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RECOVERY OF PLANT AMINES WITH CARBOXYLIC ION-EXCHANGE SUBSTRATES AND THEIR SUBSEQUENT ANALYSIS BY THIN-LAYER CHROMATOGRAPHY

LAURA SOPHIA ROSE ALBERT, STEPHEN DENNIS MITCHELL and DAVID OCTAVIUS GRAY*

Department of Botany and Biochemistry, Westfield College, Kidderpore Avenue, Hampstead, London NW3 7ST (U.K.)

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SUMMARY

Two grades of carboxymethylcellulose (CMC) and five ion-exchange resins were compared for their effectiveness in recovering endogenous amines from plant extracts. If amine loss was to be limited to 10%, the H^+ and Na^+ forms of CMC could not be loaded with the solutes from more than 0.25 and 1.5 gram fresh weight (gfw) tissue per ml bed volume respectively. The corresponding ionic forms of a typical resin, Amberlite CG-50, would tolerate loadings *ca.* 5× higher than this. However, it was then necessary to use very slow flow-rates (13 ml cm^{-2} h^{-1}) for both applying and eluting the amines and, even so, they could not be quantitatively displaced from any resin tested, with the possible exception of Duolite C433. If the extract was acidic, maximum permissible loadings were reduced by a factor of 3 to 20, depending on substrate and ionic form. The composition of the amine fraction was essentially the same whatever substrate was used for its recovery and whatever percentage of it had been lost.

INTRODUCTION

Amines can be provisionally defined as substances lacking COOH groups but containing a primary, secondary, tertiary or quaternary nitrogen atom that does not form part of a heterocyclic ring. Theoretically there is no sharp dividing line between amines and alkaloids but in practice techniques revealing primary and secondary amines are relatively specific for the simple aliphatic monoamines, aliphatic di- and polyamines, the phenylethylamines, tryptamines and histamines.

When working up the amine fraction, the greatest need is to eliminate amino acids: these make analysis of the amines much more difficult because they are almost always present at much higher concentrations and react with the same reagents. Most amines, accompanied by up to 5 amino acids, can be effectively precipitated as their tetraphenylborates[1,2] but the method has been seldom used. The most popular approach has been to absorb the amines with a cation-exchange resin and subsequently displace them with acid. Resins bearing SO_3H groups retain amino acids as well, and

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though these can be selectively removed by washing the bed with, say, sodium acetate[3–5] such methods have never been used to isolate the amine fraction as a whole. This has only been done using carboxylic resins, which, in principle, can be washed free of all but the basic amino acids with water. Even so, most of their applications have been specific ones, as for the purification of aliphatic amines[6], metanephrines[7,8], catecholamines[7,9,15], polyamines[16], serotonin[14] and histamine[9,17,30]. There have, however, been four more general studies[18–21].

These carboxylic substrates have been used in either the Na^+ (refs. 7, 9–14, 17), H^+ (refs. 6, 16, 18–21) or NH_4^+ (ref. 8) forms and bound amines have been eluted from them with hydrochloric acid[6,8,9,11,14,16,17,19,21], boric acid[7,10–13,15], acetic acid[18,20] or sulphuric acid[9]. The most popular substrates have been Amberlite IRC-50[9,13–17,30] and Amberlite CG-50[6,8,11,13,18,20,21] which are different grades of the same resin, followed by Bio-Rex 70[7,10–12], Zeo Karb 226[19,21] and carboxymethyl cellulose[21]. Some authors who have used these resins in the H^+ form have sequentially or simultaneously treated their samples with an anion-exchange resin in the OH^- form[6,16,19]. This arrangement certainly increases the effective capacity of the cation-exchange resin but generates high pH values at least transiently, so encouraging loss of volatile amines and oxidation of phenolic ones. In addition, decomposition of the anion-exchange resin releases trimethylamine or its equivalent. The only other modification to the basic procedure has been to add EDTA and/or a reducing agent to the sample to minimise amine oxidation[6,10–13,20].

Very little is known about how efficiently these carboxylic substrates absorb amines from the complex mixture of bases and competing ions found in extracts and physiological fluids. Measurements based on the retrival of individual compounds from pure aqueous solutions[18,21] are clearly inadequate, as it is already known that salts depress amine recovery[7,8,17]. The recovery of aliphatic amines from plasma[6] has been measured as has that of the metanephrines from urine[7] and plasma[8], histamine from urine[17,30] and the catecholamines from plasma[12], urine[7,10,13] and tissue extracts[9,11]. However, the literature is limited to this type of specific investigation. No-one has recorded how efficiently a large proportion of the amine fraction is absorbed or how its loss increases with column loading.

We describe our attempts to make such measurements here. We have used plant extracts as our samples, partly because these have been completely neglected and partly because we are interested in the unusual amines they contain[22]. In general we have followed the type of technique most popular with previous investigators. Thus our substrates have been used in either the Na^+ or H^+ form and have been eluted with hydrochloric acid: the Na^+ procedure was slightly modified to avoid exposing the amines to alkaline conditions. We have compared a number of common resins with carboxymethylcellulose, because this has a considerably more hydrophobic matrix and a smaller tendency to bind aromatic amino acids[21]. There is no simple and universal method for measuring amines so we have used several general reagents sensitive to amino and imino groups for the purpose. We have been working with complex mixtures of incompletely-characterised compounds, so it has not been practical to use standards: the work has been organised as a series of comparative and, where necessary, overlapping experiments. Since the material bound by these carboxylic substrates always includes basic amino acids, it is often described as the "basic nitrogen fraction" (BNF) here.

EXPERIMENTAL

Preparation of plant extracts

Table I gives details of the major plant materials used, which were measured by fresh weight throughout.

Diced fresh plant material (1 kg) was homogenised with 3 l methanol (analytical reagent grade) and the residue was re-extracted with 750 ml 70% (v/v) methanol. Smaller tissue samples were extracted with relatively larger volumes, adjusted so that the final methanol concentration was always 70%.

After passing through muslin, the methanolic extract was evaporated *in vacuo* at 50°C. The residue was dissolved in water, normally at a concentration equivalent to 0.3 g tissue ml^{-1}, and was clarified by centrifugation and filtration.

TABLE I

PLANT MATERIALS

Common name	*Botanical name*	*Tissues extracted*
Leek	*Allium porrum* L. var Musselburgh	Leaves + stem
Cabbage	*Brassica capitata* L. var January King.	Leaves + stem
Squirting Cucumber	*Ecballium elaterium* L. A. Rich.	Stem, leaves and fruit
	Equisetum telmatiea	Cones
Ivy	*Hedera helix* L.	Leaves + petioles
Apple	*Malus pumila* Mill var normally Cox's Orange.	Fruit
Rhubarb	*Rheum rhaponticum* L.	Petiole
Potato	*Solanum tuberosum* L var Maris piper	Tubers

Ion-exchange materials and their pretreatment

Carboxymethylcellulose. A pre-swollen microgranular form of carboxymethylcellulose (CM52) was supplied by Whatman Biochemicals, Springfield Mill, Maidstone, U.K.

When required in the H^+ form, 40 g of the original CM52 was stirred for a total of 60 min with 2 × 500 ml 0.5 *M* hydrochloric acid and washed by precipitation from 6 × 500 ml water. The Na^+ form used in this work was actually a mixed Na^+/H^+ form in equilibrium with a supernatant of pH 7.0. This was obtained by stirring 40 g of the CM52 as supplied in 500 ml 0.2 *M* sodium dihydrogenphosphate and continually adjusting the suspension to pH 7.0 with 0.2 *M* sodium hydroxide for 1½ h. The product was washed as before.

Resins. Details of the commercial ion-exchange resins used are shown in Table II. Zeo Karb 226 is no longer available: the Duolite resins were supplied by Duolite International, Hounslow, U.K. and Amberlite CG-50 by Rohm and Haas, Philadelphia, PA, U.S.A.

These resins were normally supplied in the H^+ form. Before use, this was

TABLE II

SPECIFICATIONS OF ION-EXCHANGE SUBSTRATES

Product and abbreviation	*Mesh size*	*Polymer type*	*Cross linkage (% age DVB)**	*Small ion capacity (mequiv. ml^{-1})*	
				H^+ form	*Na^+ form*
CM52	80–200	Carboxymethyl-cellulose	–	0.29	0.25
Zeo Karb 226 (ZK226)	100–200	Acrylic–Methacrylic	4.5	4.3	
Zerolit 236	14–52	Acrylic–Methacrylic	4	4.3	2.1
Duolite C436 (C436)	14–52 and 60–120**	Acrylic–Methacrylic	4	4.3	2.1
Duolite C433 (C433)	14–52	Acrylic	3.25	4.3	3.6
Amberlite CG-50 (CG-50)	100–200	Methacrylic	5	3.5	2.5

* The cross linkage is the percentage of commercial divinylbenzene (DVB) added to the original monomer mixture. This is normally the molar percentage but is the percentage by weight for C433 and probably for CG-50.

** Normally used in the 14–52 mesh form.

stirred with 10 bed volumes of 2 *M* hydrochloric acid for 10 min and washed free of chloride in the same way as the CM52. When the Na^+ form was required, the H^+ form was stirred with 10 bed volumes of 0.2 *M* sodium dihydrogenphosphate and adjusted to pH 7 with 2 *M* sodium hydroxide before washing as usual.

Recovery of the BNF from plant extracts

Deamination of wash water. The distilled water used to wash the ion exchange substrates was sometimes a significant source of amino compounds. If so, these interfering substances were removed at the outset by passing the water through a 250 × 25 mm I.D. column of the strong cation exchange resin, Dowex 50 (X8, 20–50 mesh) in the H^+ form at a flow-rate of *ca.* 3.3 ml min^{-1}. Such a column would deaminate at least 5 l water and was as effective at reducing the reagent blanks as was the Na^+ form of ZK226.

Recovery of BNF with carboxymethylcellulose. A column of CM52 was packed to give a bed nominally 50 × 10 mm I.D., having a volume of exactly 4.0 ml. This column was loaded with plant extract, washed with 10 + 100 ml water, and eluted with 50 ml 0.5 *M* hydrochloric acid, all at a flow-rate of 40 ± 7 ml h^{-1} and a temperature of 25 ± 5°C.

When an 8.0-ml, 100-mm long column was employed, this was eluted with 100 ml acid but was otherwise treated as before.

Recovery of BNF with resins. The standard bed volume of the column was again 4.0 ml with nominal dimensions of 50 × 10 mm. After loading with plant extract at a flow-rate of 10.6 ml h^{-1}, the column was washed with 10 + 200 ml water at 40 ml h^{-1}. Elution was with 50 ml 2 *M* hydrochloric acid at 10.6 ml h^{-1} and the temperature was maintained at 25 ± 5°C throughout.

8.0-ml columns, 100-mm long, were treated similarly but eluted with 100 ml acid.

Quantitative analysis of the BNF

The eluates from the ion exchange columns were taken to dryness *in vacuo* at 50°C in a rotary film evaporator. Hydrochloric acid was removed from the residue by redissolving this twice in 10 ml water and re-evaporating. This procedure did not cause detectible loss of methylamine, the most volatile of all the amines. For example, when 14 μmoles methylamine hydrochloride was dissolved in 50 ml 2 *M* hydrochloric acid and taken to dryness 4×, the recovery of the amine in the residue was still quantitative within experimental error (±3%).

After removal of the hydrochloric acid, the BNF was dissolved in 5 ml water, diluted as appropriate, and determined by reaction with one of the following reagents.

1,2-Naphthoquinone-4-sulphonic acid (NSA). The method used was a modified form of that recommended by Blau and Robson[23]. The diluted sample (5 ml) was heated with 2 ml 0.025 *M* borax buffer (pH 9.8) and 0.5 ml 0.5% (w/v) sodium salt of NSA for 10 min at 45°C. The mixture was cooled and excess reagent was bleached with 0.5 ml acetate buffer (pH 3.0) and 1.0 ml 8% (w/v) sodium thiosulphate. The absorbance at 476 nm was recorded 2 min later. The acetate buffer was prepared by mixing 5% (w/v) sodium acetate with 50% (v/v) acetic acid.

All determinations were carried out in triplicate and corrected for reagent blanks or, where appropriate, for the readings given by the hydrochloric acid eluates of blank columns which had been loaded with distilled water in place of plant extract.

2,4,6-Trinitrobenzene sulphonic acid (TNBS). The manual procedure used was based on that of Snyder and Sobocinski[24]. The sample (2 ml) was warmed with 2 ml 0.1 *M* borax buffer (pH 9.7) and 2 ml aqueous TNBS (0.13 g l^{-1}) for 3 h at 30°C. The resulting absorbance was measured at 420 nm. Samples were normally analysed in triplicate and the results were corrected for reagent blanks or column blanks. The TNBS solution was replaced every 14 days to minimise the reagent blanks.

Fluorescamine (FS). A form of Imac's procedure[25] was used. The amine sample (1 ml) was mixed with 1.5 ml 0.2 *M* sodium phosphate buffer (pH 8.0) and 0.5 ml FS [0.02% (w/v) in acetone (analytical-reagent grade)] was added as the sample tube was oscillated with a vortex mixer. The resulting fluorescence was measured within 1 h at 475 nm, excitation being at 390 nm. Sample concentration was adjusted to ensure that the calibration graph was linear over the whole range employed. Samples were normally analysed in duplicate and the results were corrected for reagent blanks or column blanks and expressed in arbitary emission units (EUs).

Chromatography of the BNF

After removing the excess hydrochloric acid, the BNF was washed out of the evaporator flask in 5 × 0.5 ml water and taken to dryness in a vacuum dessicator over sulphuric acid and solid sodium hydroxide.

Thin-layer chromatography (TLC). The BNF was first dansylated by a modification of the method described by Pataki and Niederwiesser[26]. The sample amines, normally those recovered from 1 gfw plant tissue, dissolved in 1 ml water, were incubated for 15 h at 22 ± 3°C in darkness with 1 ml Dns chloride (5 mg ml^{-1} in acetone) in the presence of excess solid $NaHCO_3$. Proline [1 ml of a 15% (w/v) aqueous solution] was added and the reaction mixture allowed to stand for another 2 h. The derivatised products were then extracted with 2 × 2.5 ml ethylacetate, which was evaporated to dryness in a stream of air at 50°C.

The residue was redissolved in 0.2 ml ethyl acetate and half this solution was normally spotted onto 20-cm square, 0.25-mm thick layers of Kieselgel 60G, pre-activated at 110°C for 1½ h. The layers were routinely chromatographed in the first direction with cyclohexane–ethyl acetate (2:3, v/v) and in the second with benzene–triethylamine (5:1, v/v). The fluorescent dansylated amines were located under 350-nm light. When comparing isolates with standards the two solvents already mentioned were used, together with chloroform–butyl acetate (8:3, v/v) and cyclohexane–benzene–methanol (2:7:1, v/v/v).

Liquid chromatography (LC). The column was composed of a polymer of methacrylic acid, crosslinked with 11% 1,4-butanedioldiacrylate, pre-treated with 50% (v/v) aqueous acetone and having a mean particle diameter of 29 μm[27].

Samples (typically 200 μl) containing amine hydrochlorides in 1 *M* sucrose, were layered onto the surface of the resin bed, 300 × 6 mm I.D., which had been packed in 30-mm sections and was maintained at 55 ± 1°C. This column was equilibrated and eluted with 0.02 *M* borax containing 0.4 *M* sodium nitrate, adjusted to pH 8.5 with nitric acid. The flow-rate was 6.9 ± 0.5 ml h^{-1} and amines were detected in the column effluent with an automated form of the TNBS reaction[27].

The recovery of amines from plant extracts: preliminary experiments

First it was necessary to decide how carefully variables such as column length, flow-rate and temperature must be controlled to obtain consistent results. In all these preliminary experiments, for each set of conditions, duplicate 4-ml columns were loaded with the extract from 4 g leek. This caused all ion-exchange substrates in the H^+ form to be considerably overloaded, while those in the Na^+ form were not. Amine recovery was monitored with TNBS or FS: the average relative standard error of these determinations was ±2%.

Effect of column length. The columns available had mean internal diameters ranging from 9.0 to 12.0 mm, so that 4-ml beds varied in length from 35 to 64 mm. An experiment with CM52 (H^+) showed that this variation had no significant effect on amine recovery (< 1%). Despite this, the columns were selected so the lengths of 4-ml beds were kept within the range 40–60 mm.

Effect of temperature. Loading, washing and elution were all carried out at the same temperature. Judging by results obtained with CM52 (H^+), temperature had no effect in the range 20–35°C (± 1.6%) but amine recovery declined by 12% at 10°C.

Effect of flow-rate when loading with plant extract. In the case of CM52 (H^+), the extract could be applied at flow-rates from 22 to 75 ml h^{-1} without affecting the recovery of fluorescamine reactors (variation ±2.5%). However, Table III shows that this was not true for the H^+ forms of all the carboxylic resins tested. Here optimum recovery of the BNF only occurs at inconveniently low flow-rates (< 10 ml h^{-1}). Amberlite CG-50 was the best of the resins studied: an increase in flow-rate from 10.6 to 26.6 ml h^{-1} only caused recovery to decline by 4.6% and this may not even be significant. The corresponding decreases for C436 (14–52 mesh), C436 (60–120 mesh) and C433 were 24, 29 and 19%, respectively.

Some components of the BNF might have been held preferentially by the resins. To test this, the usual volume of leek extract was passed through a 4-ml column of C436 (H^+) at a high flow-rate (266 ml h^{-1}) and then through an identical column at a much lower rate (15 ml h^{-1}). TLC showed that the hydrochloric acid

TABLE III

CARBOXYLIC RESINS (H^+): EFFECT OF RATE AT WHICH COLUMN WAS LOADED WITH EXTRACT ON AMINE RECOVERY

The BNFs were recovered from 4 g samples of leek as already described except for the variation in flow-rate as the extract was applied. Aliquots of each BNF were reacted with FS and the results have been corrected for column blanks and variations in fluorimeter sensitivity. Each result is the average given by two columns and four analyses.

Flow-rates (ml h^{-1})	*Amine recovered (EU with FS)*			
	C436 (14–52 mesh)	*C436 (60–120 mesh)*	*C433*	*CG-50*
10.6	38.3	38.9	45.2	39.9
26.6	29.1	27.6	36.8	38.1
133	–	18.8	–	36.5
200	10.2	–	23.9	–

eluates of both columns contained the same amines in apparently the same proportions.

Effect of flow-rate when eluting the BNF. Fig. 1a and b show that when tested at the same moderate flow-rate (40 ml h^{-1}), CM52 (H^+) elutes very cleanly, whereas CG-50 (H^+), like all the other carboxylic resins examined, does not. In order to give a quantitative measure of the efficiency of elution, the total amine recovered in the first 30 ml of hydrochloric acid has been divided by that recovered in the next 20 ml. The higher this "elution ratio" the more cleanly the BNF will have been displaced from the column. Table IV shows how this "elution ratio" varies from one substrate to another at different flow-rates. The ionic form of the substrate makes little difference to the results and the most satisfactory carboxylic resin was found to be CG-50, used at the lowest standard flow-rate (10.6 ml h^{-1}). The elution pattern given by CG-50 under these conditions (Fig. 1c) suggested near quantitative displacement of the BNF with 50 ml hydrochloric acid, though later evidence showed this to be untrue.

When elution was inefficient, the amines present in the early fractions were apparently identical to those found in the later ones. This was shown by eluting leek amines from a 4-ml column of C436 (H^+) at 120 ml h^{-1} and analysing the 0–30 ml and 30–100 ml fractions separately by TLC.

Long term stability of ion-exchange substrates. Unused damp CM52 as supplied in the Na^+ form has a shelf life of 3–4 years according to its manufacturers.

Aqueous suspensions of CM52 (H^+) can be stored for 14 days at 4°C without noticeable loss of capacity towards leek amines (<4.5%). However, when the same substrate is frozen at −20°C for 3 days, 10% of its capacity is lost, possibly due to a partial collapse of the matrix through hydrogen bonding.

Commercial carboxylic resins are usually considered to have an indefinite shelf life. An unused sample of CG-50 (H^+) which had been stored for at least 15 years at room temperature was used to illustrate this. When compared with a freshly purchased preparation, the aged sample only recovered 6% less amine from 4 g leek. Thus this resin is not only chemically stable but must be a consistent product.

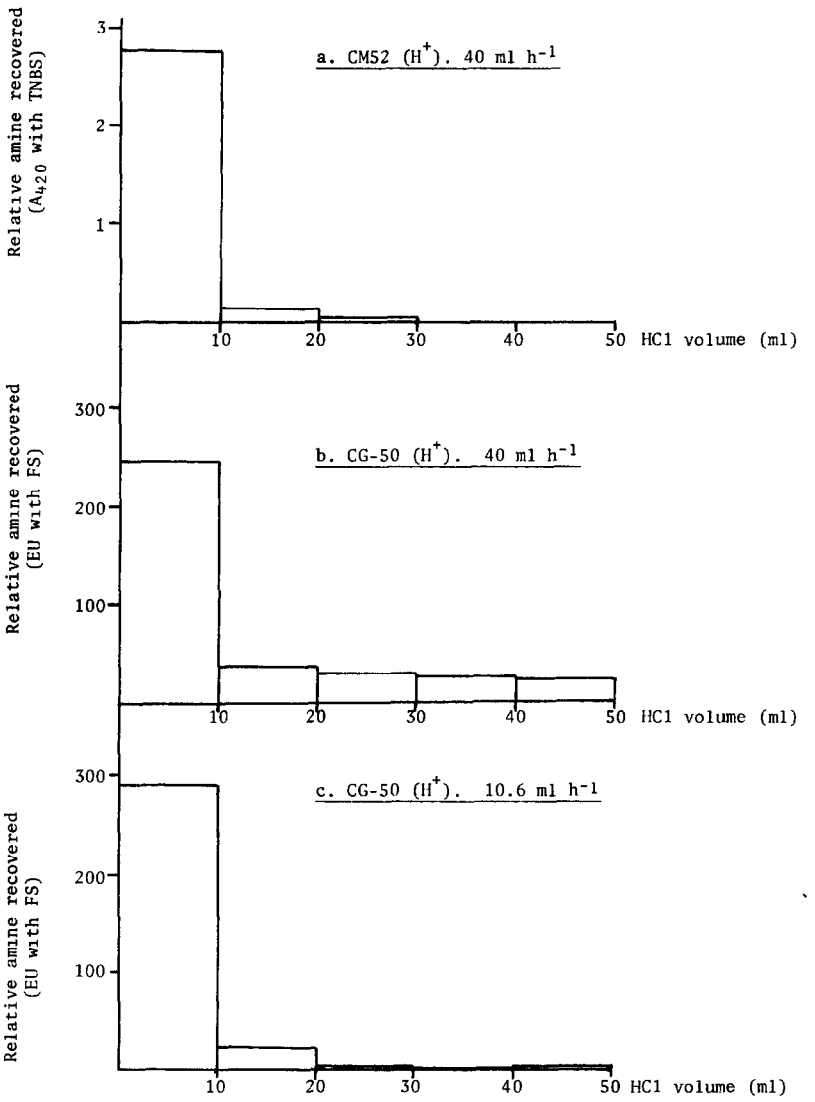


Fig. 1. Elution profiles of leek amines. The substrates were loaded with the amines from 4-g samples of leek, as already described. However, when the columns were eluted at the stated flow-rates, the HCl was collected in 10-ml fractions. Each fraction was separately evaporated, diluted and reacted with either TNBS (a) or FS (b and c). Blank columns, loaded with water instead of plant extract, were treated identically. The mean reading given by each fraction from the blank column was subtracted from the corresponding experimental reading and the remainder was multiplied by the dilution factor to give the relative amine recovered. Each set of results is the average of duplicate columns and was obtained on a separate day.

Re-use of ion-exchange substrates. When CM52 was used a second time for the recovery of leek amines, the apparent loss of capacity, 1.8% for the H^+ form and 1.1% for the Na^+ form, was insignificant. However, this substrate was never used a third time because it did not then give free flowing columns: the recycling had

TABLE IV

EFFECT OF SUBSTRATE AND FLOW-RATE ON ELUTION EFFICIENCY

The elution ratios were calculated from elution profiles obtained as described under Fig. 1.

Ion-exchange substrate	*Ionic form*	*Mesh size*	*Flow-rate (ml h^{-1})*	*Elution ratio*
CM52	H^+	80–200	40	∞
CM52	Na^+	80–200	40	∞
C436	H^+	14–52	10.6	16.8
C436	H^+	14–52	20	11.3
C436	H^+	14–52	400	4.0
C436	Na^+	14–52	40	6.2
C436	H^+	60–120	40	10.1
C433	H^+	14–52	40	6.8
CG-50	H^+	100–200	10.6	63
CG-50	H^+	100–200	40	6.8
CG-50	Na^+	100–200	40	8.1

caused sufficient breakdown to degrade its mechanical properties. Recycling in this case was kept to the minimum: the once-used CM52 was in the H^+ form so it was either washed and used directly or washed, suspended in sodium phosphate and adjusted to pH 7.

More thorough recycling is probably desirable for carboxylic resins. The evidence is that C436 (H^+) and CG-50 (H^+ and Na^+) can all be used a second time for the recovery of leek amines without obvious loss of capacity (< 3%) providing they are recycled as follows before reconversion to the correct ionic form: the once-used material is treated successively with 10 bed volumes each of 2 *M* hydrochloric acid, 2 *M* sodium hydroxide and 2 *M* hydrochloric acid, being washed after each treatment. The once-used resins gave column blanks as low as those of the unused substrates, proving that this treatment had effectively removed residual amines.

On the other hand, when 8-ml columns of ZK226 (H^+) were loaded with a series of 2.5-g aliquots of *Ecballium elaterium* extract, apparent amine recovery declined by an average of 30% for each cycle. The essential difference between the two experiments is probably that the ZK226 was merely washed and did not receive an acid/alkali treatment after each elution. Flame photometer measurements showed that the resin was progressively accumulating Ca^{2+} from the extract so was probably becoming saturated with other plant cations too.

Comparative ability of different substrates to recover amines from the same extract. Table V shows the relative performance of a number of carboxylic resins. Two of these comparisons (b and c) were made under conditions when CG-50 was known to retain 90% of the BNF. In the H^+ form, C436 and CG-50 gave identical results while C433 was substantially better, especially when the columns were overloaded (Table Va). However, the apparent superiority of C433 is most marked in Table Vc where the Na^+ forms are being compared.

Table VI shows a comparison between a typical carboxylic resin and CM52 under conditions when the latter is so lightly loaded it will retain 90% of the BNF. The CG-50 was distinctly inferior as its H^+ and Na^+ forms retained 6 and 12.5%

TABLE V

CARBOXYLIC RESINS: THEIR COMPARATIVE ABILITY TO RECOVER LEEK AMINES

The BNFs were recovered from leek samples as already described except for the variation in column loading. Aliquots of each BNF were reacted with FS and the results corrected for column blanks. Each EU figure is the mean result given by two columns and four analyses. It should be assumed that the results in each sub-table were obtained at a different fluorimeter sensitivity.

		Resin	*Amine recovered (EUs with FS)*
a	H^+ forms, loading: 4 g	C436 (14–52 mesh)	38.3
		C436 (60–120 mesh)	38.9
		CG-50	39.9
		Zerolit 236	41.8
		C433	45.2
b	H^+ forms, loading: 1.7 g	C436 (14–52 mesh)	24.8
		CG-50	24.9
		C433	26.9
c	Na^+ forms, loading: 6.5 g	C436 (14–52 mesh)	12.2
		CG-50	16.6
		C433	23.1

less amines, respectively, than did the corresponding CM52 forms. These small differences seem to be real ones according to the consistency of the duplicates.

Little work has been done on the relative capacities of different preparations of carboxymethylcellulose but A. Hayman[28] has tested CM11, a now obsolete fiberous grade. When preswollen and loaded with the extracts from 1.7 g raspberry (*Rubus idaeus*) fruit, orange (*Citrus aurantium*) fruit and *Arum pictum* spadix, 8-ml columns recovered 60% less amine on average than did equivalent columns of CM52 when both substrates were in the H^+ form. The small ion capacity of CM11, 40% less than that of CM52 on a dry weight basis, correlates reasonably well with its reduced ability to bind amines.

TABLE VI

CM52 AND CG-50: THEIR COMPARATIVE ABILITY TO RECOVER LEEK AMINES

Conditions were as for Table V except that each EU figure is the mean result of two FS analyses from one column.

	Substrate	*Amine recovered (EUs with FS)*
a H^+ forms, loading: 0.6 g	CM52	23.8
		23.8
	CG-50	22.5
		22.2
b Na^+ forms, loading: 3.6 g	CM-52	15.7
		16.2
	CG-50	14.1
		13.7

Finally, the Na^+ form of CM52 used in this work, which contained *ca.* 1.5% of the H^+ form and was in equilibrium with a supernatant at pH 7, was compared with the pure Na^+ form as supplied by the manufacturers (pH 8.8). When tested in the usual way, the pH 7 material retained 6.3% more amine. This very small difference was a genuine one, judged by the consistency of the results given by 4 columns of each material.

Extract concentration. This is another factor that can affect amine recovery. Table VII shows that when columns of CM52 are massively overloaded with apple extract, dilution of that extract by a factor of 3 enhances amine recovery by 50% for the H^+ form and reduces it by 18% for the Na^+ form. Nevertheless the Na^+ form always has the higher capacity.

TABLE VII

CM52: EFFECT OF EXTRACT CONCENTRATION ON THE RECOVERY OF APPLE AMINES

Columns (8 ml) of CM52 were each loaded with the extract from 10 g apple, diluted as above, and the BNFs were recovered as usual. Aliquots of each BNF were reacted with TNBS and the mean absorbances, each corresponding to a separate column, were corrected for reagent blanks.

Extract concentration (gfw tissue ml^{-1})	*Amine recovered (A_{420} with TNBS)*	
	H^+ form	*Na^+ form*
0.33	0.08	0.44
	0.08	0.44
0.11	0.13	0.36
	0.11	0.36

Plant BNFs are probably recovered as efficiently from aqueous alcohols as from water. For example, 8-ml columns of ZK226 (H^+) recovered as much amine from extracts of *Ecballium elaterium* in 70% ethanol as from equivalent extracts that had been evaporated and taken up in water. The column loading, here equivalent to 2.5 gfw, should have allowed near quantitative recovery.

The recovery of added standards from plant extracts

Initially, a deliberately unfavourable situation was chosen in which the columns were overloaded with plant extract as judged by their ability to recover endogenous amines. Thus extracts from 5 g apple were partially deaminated with 8-ml beds of CM52 (H^+). The fraction passing directly through each column, together with the wash water, were evaporated, redissolved in 30 ml and supplemented with standards (*n*-hexylamine, 1.5 μmole; *n*-propylamine, 1.5 μmole and 2-phenylethylamine, 2.0 μmole). This mixture was applied to 8-ml CM52 (H^+) columns for the recovery experiment. Identical columns were loaded with the same mixture of standards in 30 ml water. The BNFs were recovered as usual and analysed by LC. Recovery of the standards from water averaged 93%, nearly quantitative allowing for manipulative losses, but mean recovery from the extract was only 43%.

Table VIII illustrates the opposite situation when columns were used well within their capacity. Endogenous amines were removed from aliquots of *Ecballium*

TABLE VIII

ZK226 (Na^+): THE RECOVERY OF STANDARDS ADDED TO *ECBALLIUM* EXTRACTS

For the 1-μmole experiments, deaminated extracts (25 ml), supplemented separately with the stated standards, were passed through 8-ml columns of ZK226 (Na^+) which had been precycled twice between 5 *M* HCl and 5% NaOH to reduce their blanks. The columns were washed with 10 × 10 ml water and eluted with 50 ml 2 *M* HCl + 10 ml water, all at 40 ml h^{-1}. Blank columns were loaded with 25 ml deaminated extract by itself. The eluates from the experimental and blank columns were evaporated to dryness, redissolved, mixed with 1 ml BDH universal buffer, adjusted to pH 6.0 and diluted to 25 ml before analysis with NSA. Standards, used for comparison, were added to the neutralised blanks. All distilled water was first deaminated. The main modifications in the 140-μmole experiments were that 50 ml of deaminated extract was used each time and the BNFs were eluted with 50 ml formic acid.

	Percentage recoveries	
	140 μmole samples added	*1 μmole samples added*
Methylamine	100	102
Ethanolamine	97	101
p-Tyramine	99	101
1,4-Diaminobutane	100	96
Dimethylamine	103	99

elaterium extract (equivalent to 5 gfw at 0.2 g ml^{-1}) with 8-ml columns of ZK226 (Na^+) as indicated in the previous paragraph. After adding standards, the BNF was isolated with identical columns and recovery was measured with NSA. The results show that representative amines could be recovered quantitatively from tissues even at concentrations comparable with physiological ones (0.2 μmole g^{-1}).

The recovery of endogenous amines from plant extracts

CM52 quantitatively recovers all amines so far tested from water[21]. However, plants contain unknown and untested amines. The only useful working hypothesis is that these, too, will be quantitatively retained providing the CM52 is very lightly loaded with extract. The essential problem is to decide how much extract can be applied to a standard column (in gfw equivalents) before the percentage loss becomes excessive. We have attempted to measure this in two ways.

The single column method. Here the approach is to decide the point at which amine recovery is no longer directly proportional to column loading. This is illustrated by Fig. 2 in which the graph departs sharply from linearity at column loadings greater than 3.75 g, suggesting that the maximum capacity of ZK226 (H^+) for *Ecballium* extract is *ca.* 0.5 g for each ml of bed volume. Table IX gives some further estimates of column capacity obtained in this way.

The multiple column method. A more satisfactory approach is to use another column to detect and measure amine loss from the primary one. A crude but useful form of this method is illustrated in Table X, in which a single heavy loading is applied to a series of small columns which are eluted and analysed separately.

However, capacity can only be measured precisely by taking a number of pairs of columns and varying the sample loading. In our experiments, the extracts were applied to a 4-ml column which flowed directly into an 8-ml one of the same substrate

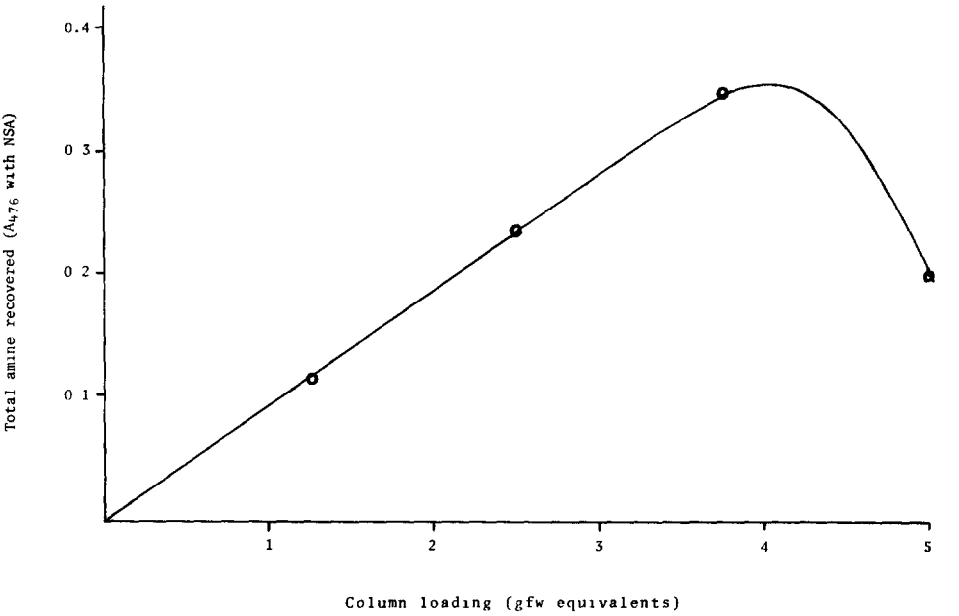


Fig. 2. ZK226 (H^+): its apparent capacity for the endogenous amines of *Ecballium. Ecballium elaterium* extract at 0.25 g ml^{-1} was loaded onto 8-ml columns of ZK226 (H^+) which were washed with 10 × 10 ml water and eluted with 50 ml 2 *M* HCl. BDH universal buffer (5 ml) was added to each elute, which was adjusted to pH 6.5 and diluted to 100 ml before analysis with NSA. Each result is the mean given by duplicate columns and has been corrected for neutralised HCl blanks. All distilled water was deaminated before use.

in the same ionic form. Conditions were the usual ones except that after column 2 had received the effluent and washings from the first bed, it was rewashed with the standard volume of water. The BNF was eluted from each column separately and measured with FS. The amine fractions from both members of a column pair were diluted by the same factor so, when the substrate was in the Na^+ form, the sodium

TABLE IX

CAPACITIES OF SUBSTRATES FOR ENDOGENOUS AMINES AS ESTIMATED BY THE SINGLE COLUMN METHOD

Extract	*Substrate*	*Capacity/unit bed volume (g ml^{-1})*
Eichornia crassipes[29] leaves	CM52 (H^+)	3.0
Spinach (*Spinacea*[29] *oleracea*) leaves	CM52 (H^+)	2.5
Cabbage[29]	CM52 (H^+)	2.7
Apple	CM52 (H^+)	0.25
Apple	CM52 (Na^+)	0.25
Ecballium elaterium	ZK226 (H^+)	0.5
Ecballium elaterium	ZK226 (Na^+)	>6.2

TABLE X

ZK226 (Na^+): ITS CAPACITY FOR ENDOGENOUS AMINES AS ESTIMATED BY A MULTIPLE COLUMN METHOD

Each extract, at a concentration equivalent to 1 g ml^{-1} was allowed to flow through two or three 4-ml columns arranged in series. Each bed received the washings from all those earlier in the series and was then washed with an additional 100 ml. Elution and evaporation were as usual except that the flow-rate was 40 ml h^{-1} throughout. The BNF was analysed with NSA after adjustment to pH 6.0.

Extract	*Loading (gfw)*	*Percentage of total amine recovered*			*Approx. capacity/ unit bed volume (g ml^{-1})*
		Column 1	*Column 2*	*Column 3*	
Ecballium elaterium	42	98.7	1.3	–	10
Apple, var. Golden Delicious	50	87.3	12.7	–	10
Rhubarb	50	15.5	83.5	1.0	6
Equisetum telmatiea	50	97.5	2.0	0.5	12
Ivy	50	95	3.4	1.5	12

chloride concentration in the column 2 eluate was 2 × that for column 1. Fortunately the increased sodium chloride concentration reduced the FS reading by less than 1% on average (n = 15, BNFs from leek, potato and cabbage). Apple contained so little amine that here the concentration of the FS in the reaction mixture was routinely doubled to increase sensitivity. An example of the results and calculations is given in Table XI. These show that the total FS reaction/g tissue was monitored so that if heavy loadings caused amine to be lost from both columns, this could be detected and allowed for: such loss from the system was very rare.

TABLE XI

CM52 (Na^+): THE EFFECT OF LOADING LEVEL ON THE RECOVERY OF ENDOGENOUS LEEK AMINES

The method is described in the text. All EU figures are the average of duplicates and have been corrected for column blanks. Though this work was done over 3 days, the EU values have been adjusted so they are all comparable.

Loading (gfw)	*Amine recovered (EU with FS)*		*Total amine recovered/unit loading (EU g^{-1})*	*Percentage amine held by column 1*
	Column 1	*Column 2*		
0.7	94	1.5	136	98.4
1.0	135	2.2	137.5	98.4
1.7	228	2 5	135.5	98.9
2.5	342	10	141	97.2
3.0	389	15	134.5	96.3
3.5	434	43	136.5	91
3.0	371	33.4	135	92
4.0	474	64.2	134.5	88
5.0	531	173	141	75.5
6.0	489	333	137	59.5

The method was used to measure, for the first time, the precise relationship between column loading and percentage recovery of endogenous amines, using extracts of both leek and cabbage. It was also used to compare the capacities of both ionic forms of CM52 with those of CG-50. The latter was chosen as a representative carboxylic resin, partly because its performance was close to average in most of the

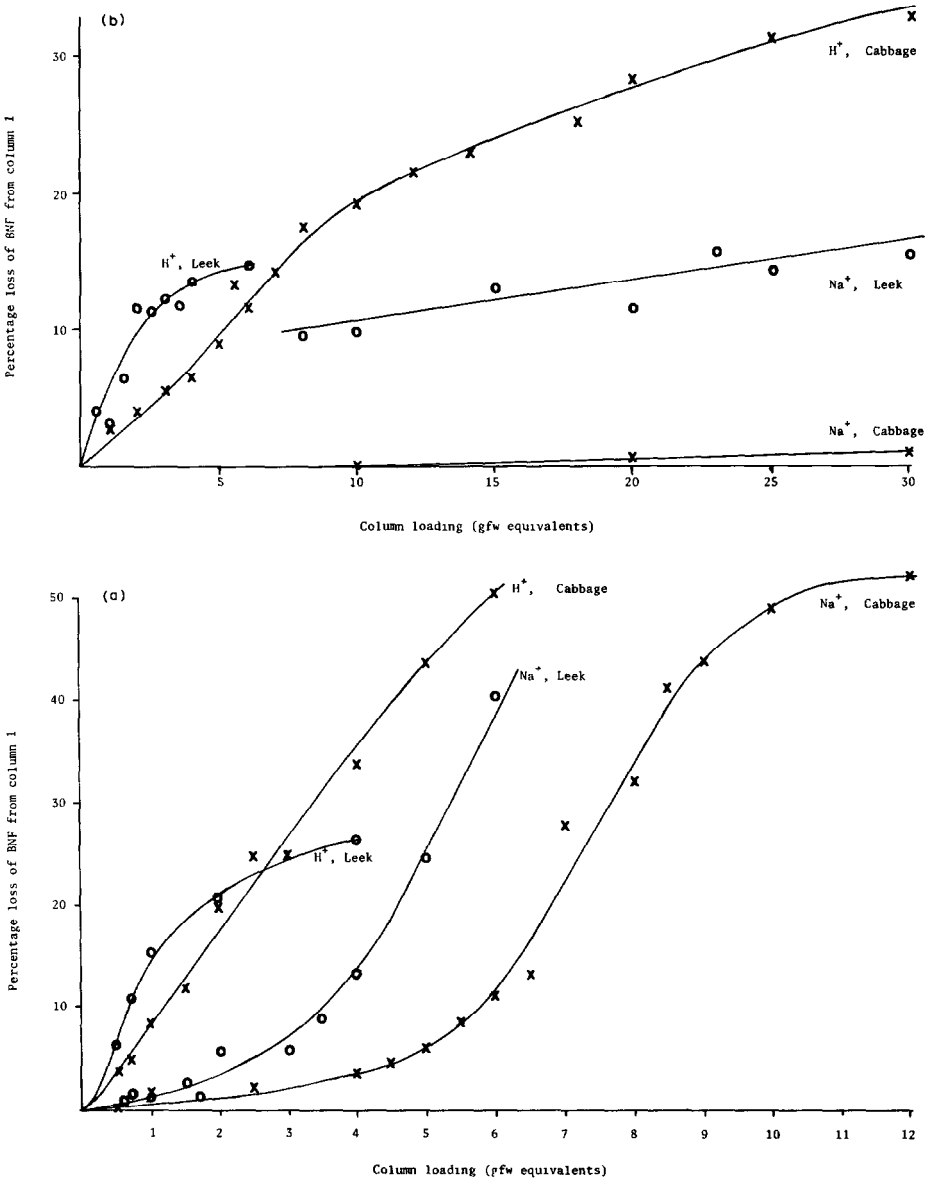


Fig. 3. The effect of loading level on the percentage loss of endogenous leek and cabbage amines. (a) CM52; (b) CG-50. The method is described in the text and specimen results are shown in Table XIV.

preliminary experiments and partly because it had been so popular with previous investigators.

The results are shown by Fig. 3a and b. Observations on the Na^+ form of CG-50 were extended beyond the range illustrated. The percentage loss of leek amines from 4 ml columns of this substrate remained constant, at *ca.* 15%, as loading was increased from 30 to 60 g. For cabbage amines, loss increased progressively with loading, being 4% at 40 g, 9% at 60 g, 17% at 80 g and 26% at 100 g.

Fig. 4a–d presents a different aspect of the same data and shows how the total amine accumulated by a 4-ml column depends on its loading.

There is clearly some loss from the H^+ substrates under all practical conditions so to obtain useful comparative figures for capacities we have calculated them at the point when a 4-ml column is retaining 90% of the BNF applied. The results for four plant extracts are shown in Table XII.

TABLE XII

CAPACITIES OF SUBSTRATES FOR ENDOGENOUS AMINES AS ESTIMATED BY THE DOUBLE COLUMN METHOD

These figures are the sample loadings at which 10% of each BNF is lost from a 4-ml column as measured from Fig. 3a and b and similar graphs.

Extract	*Capacities/unit bed volume (g ml^{-1})*			
	CM52 (H^+)	*CM52 (Na^+)*	*CG-50 (H^+)*	*CG-50 (Na^+)*
Cabbage	0.26	1.5	1.3	15.5
Leek	0.15	0.9	0.44	2 3
Potato	0.19	1.1	0.6	7.8
Apple	0.07	0.58	0.06	2.2

Table XIII lists several properties of the same four extracts that may influence amine recovery. The total cation concentrations are such that when columns are loaded as in Table XII, only a very small proportion of the exchange sites will be occupied by extract cations, 1% on average for H^+ substrates and 10% for Na^+ substrates.

Effect of recovery method on subsequent chromatography of the BNF

Analysis by TLC. The derivitisation mixture itself generates fluorescent components which must be taken into account during amine analysis. The hydrochloric acid eluates of unloaded columns of CM52 and CG-50, of both ionic forms, enhance this blank reaction but all do so to the same degree. Similarly the method used for recovering the BNF makes no noticeable difference to its tendency to streak during chromatography.

When examining a given extract, all methods gave chromatograms showing the same major spots. Most of the differences implied by Table XIV were due to very faint spots and we should not like to conclude too much from these results. Nevertheless some of the differences were certainly genuine. For example, the medium-intensity spot co-chromatographing with spermine in leek and cabbage was only

TABLE XIII

SOME PROPERTIES OF THE PLANT EXTRACTS USED IN THE COLUMN CAPACITY EXPERIMENTS

Initial pH values were measured on extracts of normal concentration (0.3 g ml^{-1}). The second column of results was obtained by diluting 1 ml of each extract to 30 ml and passing it through a 4-ml bed of CM52 (H^+). The first 10 ml emerging from the column was discarded and the pH recorded was that of the remaining 20 ml. This dilute sample was backtitrated to its original pH with 0.02 *M* NaOH to estimate exchangeable cation concentration. Amine concentration was measured by recovering the BNF from the equivalent of 2 gfw with 4-ml beds of CM52 (Na^+) as usual. After reaction with FS the results were corrected for the small losses from the columns and the EU values were made comparable to those of Fig. 4 and Table XIV.

Extract	*Initial pH*	*pH after passing through CM52 (H^+)*	*Total exchangeable cation concentration (μequiv./gfw)*	*Amine concentration (EU with FS/gfw)*
Cabbage	5.25	3.65	62	223
Leek	5.35	3.75	43.5	206
Potato	5.85	3.59	28.5	280
Apple	3.80	3.55	2	15

found in CM52 eluates, while a distinct unknown running near ethanolamine in leek was only obvious after isolation with CG-50. In these cases the differences were correlated with the substrate and not its ionic form.

The TLC work allowed the major compounds present to be provisionally identified. Co-chromatography with standards in the 3 solvents specified suggested that ammonia, putrescine/agmatine, ethanolamine, methylamine, dimethylamine, lysine and spermidine were present in all the extracts while tyramine and spermine were found in most of them. The rest of the components were too faint to be eluted and

TABLE XIV

COMPOSITIONS OF THE BNFs RECOVERED BY DIFFERENT SUBSTRATES

Columns (4 ml) were loaded with the volume of extract that would allow 90% retention of the amines present (Table XV) but were otherwise treated as usual. A sample of each BNF obtained, corresponding to 1 gfw tissue, where possible, was dansylated and the equivalent of 0.5 gfw was chromatographed. In order to remain within the column loading limitations, these quantities were halved for the apple extract. The whole experiment, including the isolation of the BNFs was duplicated. The results have been corrected by subtracting the spots given by dansylated column blanks.

Extract	*Number of components found after reaction with Dns chloride*				
	Direct dansylation of extract	*CM52 (H^+)*	*CM52 (Na^+)*	*CG-50 (H^+)*	*CG-50 (Na^+)*
Cabbage	20	18	17	12	13
Leek	16	15	17	14	17
Potato	20	18	18	18	16
Apple	14	12	12	10	13

chromatographed against standards: 4 of them ran in positions similar to hexylamine, propylamine, cadaverine and ethylamine but the remaining 9 did not have the chromatographic characteristics of any common amine.

Analysis by LC. When recovered with a carboxylic substrate in the Na^+ form, the BNF contains sufficient sodium chloride to interfere with its subsequent fractionation by LC on an ion-exchange resin. Thus Table XV shows that standard amines, added to a plant extract, can be chromatographed perfectly normally if re-isolated with the H^+ form of CM52. However, if re-isolation is with the Na^+ form, peak sharpness during LC is much reduced. This effect has nothing to do with the plant extract. It is still seen if re-isolation is from water and can be mimicked by adding the calculated quantity of sodium chloride to a mixture of pure standards. The only remedy is to take a smaller sample for LC. The maximum loading our LC system would tolerate without substantial loss of efficiency was the eluate of a 2-ml bed of CM52 (Na^+), containing 28 mg sodium chloride.

TABLE XV

EFFECT OF RECOVERY METHOD ON THE SUBSEQUENT LC OF STANDARD AMINES

Where indicated, the standards were added to a partially deaminated apple extract and re-isolated with 8-ml columns as described in the text (*The recovery of added standards*) before separation with LC. Each result is the mean of duplicate or triplicate experiments.

	Chromatographic efficiencies (plates cm^{-1})		
	Standards chromatographed directly	*Standards re-isolated with CM52 (H^+)*	*Standards re-isolated with CM52 (Na^+)*
n-Hexylamine	104	105	22
2-Phenylethylamine	113	145	28
n-Propylamine	129	143	7

Effect of overloading on the composition of the amine fraction recovered

It might be predicted that some amines would bind much more strongly than others and would come to represent a larger and larger proportion of the residual amine fraction as a column was progressively overloaded. This has not been our experience based on 16 sets of comparisons representing all the substrate–plant extract combinations listed in Table XIV. In each case the BNF eluted from a column lightly loaded as in Table XIV was compared to that from a column that had received 10× the volume of extract (5× the volume for the Na^+ form of CG-50). Amine losses were estimated and used to equalise the quantities of dansylated amines applied to the chromatograms. Altogether 240 visual comparisons of individual spots were made. In 19 cases, spots were scored as being more intense where the columns had been lightly loaded: this is probably a measure of the experimental error of the visual comparisons. In 29 cases spots appeared intensified in the eluates of the heavily loaded columns. This effect was seen on 12 out of 16 occassions when "spermidine" spots were being compared. Thus, apart from the polyamines, the composition of the

BNF seems remarkably constant whether loss is high or low during its isolation.

This is supported by some limited quantitative evidence obtained by loading both forms of CM52 to different degrees with apple extract and using LC to analyse the BNFs recovered. LC revealed four main peaks but the excess sodium chloride present in some samples degraded resolution so much, it was only possible to measure the overall concentration ratio between the two faster and the two slower components. Table XVI shows that this ratio is constant within experimental error whether the CM52 is recovering 98 or 13% of the BNF applied. It is surprising that the CM52 does not discriminate between two groups of components separated so clearly by another ion-exchanger during LC.

TABLE XVI

THE EFFECT OF OVERLOADING CM52 ON THE COMPOSITION OF THE BNF RECOVERED

Each BNF was recovered as usual, with an 8-ml column. When this was analysed by LC the peaks, given in order of increasing elution volume, were provisionally identified as *n*-propylamine, arginine, ethanolamine and methylamine. The ratio of the two faster to the two slower components were calculated from the relative peak areas. All results are the mean of duplicates.

	Column loading (gfw equivalents apple extract	*Recovery of BNF (%)*	*Ratio of faster/ slower components*
CM52 (H^+)	1	83	0.25
	2	75	0.26
	5	57	0.25
	10	34	0.27
	20	14	0.21
CM52 (Na^+)	1	98	0.26
	2	96	0.26
	5	71	0.23
	10	28	0.20
	20	13	0.24

DISCUSSION AND CONCLUSIONS

None of the statements made here necessarily apply to tertiary and quaternary amines. The NSA reagent reacts with both primary and secondary nitrogen, so it should be sensitive to all the compounds revealed by the Dns chloride–TLC method. However, the more sensitive TNBS and FS reagents, that proved necessary for later work, only react with primary amines. Of the plant amines so far isolated, 53% have been primaries and 27% secondaries but this probably underestimates the preponderance of primary amines in extracts. The BNFs of the 4 extracts that have been most carefully examined here contained on average 10 major primary amines including representatives of the aliphatic mono-, di- and polyamines as well as of the phenylethylamines and tryptamines. Thus though we cannot claim that our results apply to the whole amine fraction, they apply to a large and important part of it.

If general amino-group reagents are to usefully measure the recovery of a BNF, its composition must be constant, since reagent sensitivity will not be the same towards all amines. This condition does seem to be satisfied as judged qualitatively and

quantitatively (Table XVI), for the BNF from a given extract, regardless of percentage loss. The other requirement for valid comparisons is that the BNF must not contain neutral or acidic amino acids: almost all such compounds are quantitatively rejected by CM52, CG-50 and ZK226 when applied one at a time in water[18,21]. Moreover, when plant BNFs are separated by LC, less than 1% of the total TNBS/NSA reacting material, on average, elutes in the position of neutral/acidic amino acids.

Another major requirement is that none of the components of the BNF are generated chemically by the interaction between the ion exchange substrates and plant amino acids. If this happened, amines would be formed continuously as the same extract was passed through a series of columns. This is not what is observed (Table X); unless the column system is overloaded, the BNF recovered represents a constant proportion of the extract's total fluorescamine reaction (Table XI). This proportion is 4.3% for apple, 11.4% for leek, 11.7% for potato and 9.4% for cabbage. Table XIV provides even better evidence that the amines are not artifacts. It indicates that dansylation reveals fewer, not more, components in the BNFs than in the original extracts. Moreover, judging by chromatographic position, no new compounds are generated by either CM52 or CG-50.

These results also illustrate that our form of the dansylation procedure discriminates strongly against amino acids. Such compounds do react with Dns chloride but most of the products are left in the reaction mixture when this is extracted with ethylacetate. Thus it might seem unnecessary to preisolate the BNF here. Experience varies from extract to extract. Direct dansylation of the extracts of several tobacco-derived crown galls gives satisfactory chromatograms but spot intensity is reduced by a factor of 2.2 due to the presence of competing amino acids in the reaction mixture. Direct dansylation of cabbage, leek, potato and apple extracts gives poorer results while seed extracts of *Bidens ferulaefolia, Crepis neglecta, Crepis pulchra, Gaillardia aristata* and *Senecio elegans* yield chromatograms sufficiently distorted to make amine identification difficult. In all these cases, dansylation of the corresponding BNFs, as isolated with CM52 (H^+), gives clean, unstreaked, chromatograms.

Complete recovery of any compound naturally present in an extract cannot be proven. Therefore, it is encouraging that conditions allowing good recovery of endogenous amines, according to our results, also give quantitative recovery of added standards (Table VIII), and *visa versa*. The same results show that reisolation of amines from water is much easier than it is from an extract, where there will be a substantial concentration of competing inorganic cations.

The single column method of capacity measurement is at best approximate. The point at which the graph ceases to be linear is a matter of opinion and will be influenced by small deviations in the analytical results. Moreover, there will be gross errors if the percentage loss of the BNF stabilises at a fairly high value over a wide range of loadings, as happens quite often (Fig. 3). For example, Fig. 4a shows a graph for leek extract that would be considered linear, within experimental error, up to a loading of at least 4 g, and this corresponds to a loss of 26%. As for the results given in Table IX, the apple-CM52 (Na^+) and *Ecballium*-ZK226 (Na^+) estimates of column capacity are reasonably consistent with those obtained by the more reliable multiple column method (Tables X and XII) but the cabbage and apple figures for CM52 (H^+) are not.

Recovery of amines from an extract will depend on how far the reaction zone has extended down the column. At low loadings recovery of the BNF should be quantitative whereas at high loadings, when the whole bed tends to equilibrium with the extract, loss will stabilise at a value that depends on the concentration and effectiveness of the competing ions. These considerations not only explain the form of the graphs shown in Fig. 3 but indicate why amine recovery by a heavily loaded column depends on the volume and not the length of the bed. It is not surprising that the Na^+ forms have the higher capacity and are the only ones to give quantitative recovery of the BNFs over a reasonable range, as the Na^+ ion is easier to displace than the H^+ ion.

As loading increases, the total amine bound to a given column reaches a maximum and then begins to decline (Figs. 2, 4b and d and Table X). This corresponds to the point at which 15–60% of the exchange sites could be occupied by extract cations in the case of cabbage (Table XIII). Presumably it is the saturation of the bed with strongly bound inorganic cations that ultimately displaces the BNF.

When different substrates in the same form are compared, the one with the higher small ion capacity binds the most amine (Table XVII). The H^+ form of CG-50 has a smaller advantage than expected probably because its capacity will be suppressed more under acidic conditions: the mean pK_a of CG-50 is 6.1 and that of CM52 is 4.85. Even so, the correlation between exchange capacity and amine capacity is rather poor.

Results vary greatly from one extract to another. Amines are more difficult to recover from apple than from any other tissue tested (Table XII) probably due to its low pH (Table XIII): the Na^+ substrates have a disproportionately higher capacity for this extract because they take up the excess H^+ ions. Perhaps as important as initial pH, is the pH of the extract after it has passed through a bed of H^+ substrate: this will depend on the balance between buffering power and the concentration of total exchangeable cations. If the pH at this stage falls to 3.15, the column will take up no more amine. This factor helps explain the comparative behaviour of cabbage

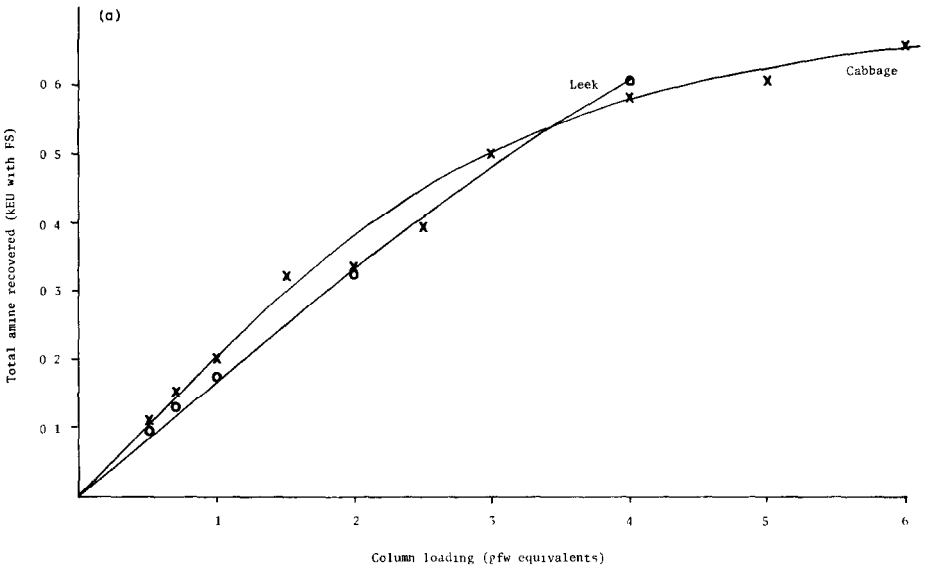
TABLE XVII

CM52 AND CG-50: THEIR RELATIVE CAPACITIES FOR SMALL IONS AND PLANT AMINES

		Small ion capacity (mequiv. ml^{-1})	*Capacity towards plant extracts (g ml^{-1})**	*Maximum cabbage amine accumulated (kEU ml^{-1})***
H^+ forms	CM52	0.29	0.17	–
	CG-50	3.5	0.60	–
	CG-50/CM52 ratio	12	3.6	–
Na^+ forms	CM52	0.25	1.02	0.3
	CG-50	2.5	6.95	4.0
	CG-50/CM52 ratio	10	6.8	13.3

* Average figures taken from Table XV.

** Calculated from Fig. 4.


(a)
Total amine recovered (kEU with FS)
0 6
0 5
0 4
0 3
0 2
0 1
Leek
Cabbage
1
2
3
4
5
6
Column loading (pfw equivalents)

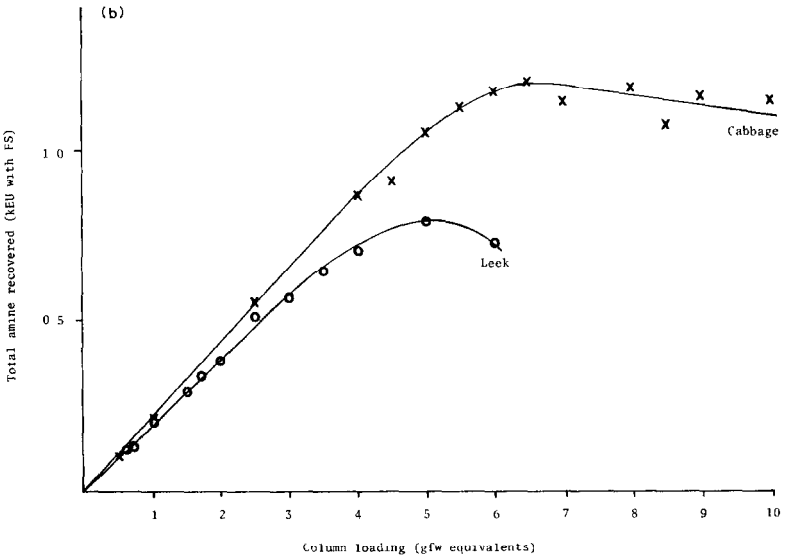

(b)
Total amine recovered (kEU with FS)
1 0
0 5
Cabbage
Leek
1
2
3
4
5
6
7
8
9
10
Column loading (gfw equivalents)

Fig. 4

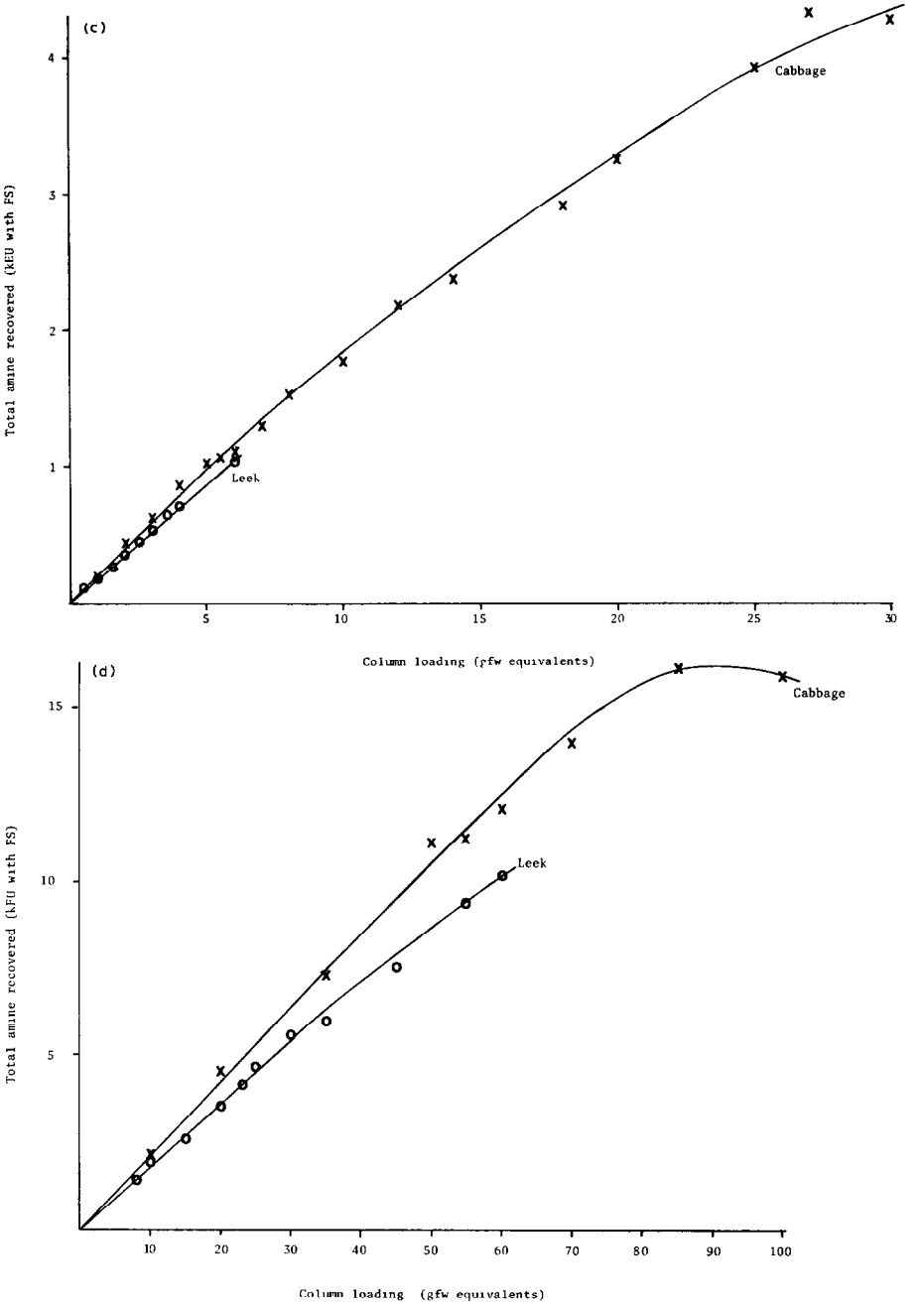


Fig. 4. The effect of loading level on the total amine recovered from leek and cabbage extracts. (a) CM52 (H^+); (b) CM52 (Na^+); (c) CG-50 (H^+). This represents the amine bound to the resin: *ca.* 11% of it is not released by acid elution. (d) CG-50 (Na^+). This represents the amine bound to the resin: *ca.* 19% of it is not released by acid elution. The method is described in the text. All the EU values are comparable with each other and with those shown in Table XIV.

and leek extracts. When tested on Na^+ substrates, leek consistently shows a higher loss so must contain the more effective competing ions (Fig. 3). The same is initially true on H^+ substrates but as loading increases the situation reverses because the cabbage extract has the lower equilibrium pH (Table XIII). Certainly the relative capacity of a substrate for these extracts will depend on the percentage loss standard adopted.

Different samples of the same tissue can give consistent results. For example, the leek extract used for the Na^+ graph of Fig. 3b was prepared from mature plants: this gave the same results, within experimental error, as an extract of much younger material prepared 0.8 years later. On the other hand, as tested with the Na^+ form of two resins, the BNF is much easier to recover from Golden Delicious (Table X) than from Cox's Orange apples (Table XII). This is not surprising: pH and cation concentration are likely to vary with growth conditions even within one variety.

Table X is useful as it gives capacities for 4 additional extracts. It was obtained using the Na^+ form of ZK226, which is equivalent to C436 (Table II). C436 has, if anything, a lower capacity for the BNF than CG-50 (Table Vc). Thus the capacity of CG-50 (Na^+) for the extracts listed in Table X would be 6–12 g ml^{-1} or greater, corresponding to the upper end of the range shown for the same substrate in Table XII. This suggests that extracts as "difficult" as apple are rather rare.

Modification of the extract itself is of limited value. Dilution would be expected to reduce amine recovery due to the law of mass action and, indeed, does so for a Na^+ substrate (Table VII). However, when CM52 was used in the H^+ form, dilution increased the equilibrium pH, after passage through the column, from 3.15 to 3.35 and the net result was an increase in recovery (Table VII). Pre-adjustment of extract pH with soluble alkalis does not improve BNF recovery by H^+ substrates: the added cations simply exchange for H^+ and make the situation worse.

The behaviour of different samples of the same substrate have been quite consistent. Four batches of CM52 were used for the work shown in Fig. 3. Whenever a graph was drawn using overlapping results from more than one batch, these agreed within experimental error. The capacities for leek amines of two samples of C436, of different mesh sizes, were identical (Table Va) while those of two preparations of CG-50 differed by 6%. Tested in the same way, Zerolit 236 recovered 8% more amine than its equivalent, C436 (Table Va).

CM52 can only be re-used once and its H^+ form has a limited storage life. The chief disadvantage of the ion exchange resins is that they take at least 6 h longer to isolate a BNF, largely due to the low flow-rates found necessary, even when the particle size was small (Table IV). The difficulty in eluting the amines could be explained if they were partially bound by adsorbtion, as at alkaline pH values[27]. However, adsorbtion cannot account for the slow uptake of the BNF and is inconsistent with its constant composition. Therefore, the sluggish equilibration between the mobile and stationary phases must be due to the low rate at which the amines diffuse into and out of the resin particles. The more open matrix of the carboxymethylcellulose allows much faster diffusion, which only seems to become restricting and cause a reduction in amine recovery at low temperatures combined with moderate flow-rates.

Most of the resins were not releasing plant amines quantitatively in our experiments, despite the low flow rates. For example, according to Fig. 3, under the

conditions of Table VIa, the H^+ form of CM52 should have been retaining 90% of the BNF. Yet CG-50 releases less, not more, fluorescamine reacting material. The discrepancy, *ca.* 11%, is presumably due to amine that remains bound to the resin. A similar calculation, based on Table VIb, suggests that the Na^+ form of CG-50 retains 19% of the BNF after elution. The problem is greater for the Na^+ form because here the matrix closes down on the amine bearing sites as the particles shrink in contact with the eluting acid. When Na^+ forms are converted to the H^+ forms, resin beds shrink by the following percentages: C433, 16; CG-50, 29; C436, 51. It is striking that in Table Vc, the resins that shrink the least, release the most amine. In fact, calculations suggest that C433 may well be giving quantitative recovery and release of the BNF in Table Vc, though not in Va or Vb.

In contrast to these results, standard amines can be retained and eluted from resins like CG-50[18,21], and ZK226[21] (Table VIII) completely quantitatively when all operations are carried out at quite high flow rates (53 ml cm^{-2} h^{-1}). However, these "model" experiments only utilised a small proportion of the total ion-exchange capacity of the beds. Thus the amines were adequately bound by superficial COOH groups and did not need to diffuse deeply into the resin particles.

The BNF isolation procedure can be modified in several ways. One evaporation stage can be avoided by passing the filtered extract directly through a column of the washed substrate, re-suspended in 70% alcohol. The bed must then be rinsed with 70% alcohol, followed by water, before it is eluted. As indicated, this modification works well for ion exchange resins but has not been tested with carboxymethylcellulose. Formic acid (Table VIII) and acetic acid will elute standard amines as efficiently as hydrochloric acid.

In conclusion, for analytical work, CM52 seems the most satisfactory substrate so far. The Na^+ form is to be preferred where subsequent contamination with Na^+ salts can be tolerated: this form will accept a substantial loading and no other alternative tested behaves as consistently towards different extracts (Table XII). For preparative purposes, the Na^+ forms of the resins have their advantages as they can accumulate much more amine/ml bed volume. More lightly cross linked resins would have a more open matrix, so would allow higher flow rates. However, of the resins actually tested, C433 was the most promising due to its more quantitative elution. C433, like other resins containing divinylbenzene, will probably have some affinity for aromatic amino acids but this remains to be demonstrated. For preparative work where salts may not be added there is at present no real alternative to the combined cation/anion-exchange systems discussed in the introduction[6,16,19].

The Dns chloride–TLC technique reveals more components in plant amine fractions than any other alternative so far tested and is certainly superior to our LC method. We therefore give the chromatographic characteristics of all the known higher-plant amines we have been able to obtain, both in our two dimensional system (Fig. 5) and in the most useful "confirmatory" solvent (Table XVIII). Choline, galactosamine, glucosamine and hordenine give exceptionally weak fluorescent spots after dansylation so the technique is insensitive to these compounds. A number of amines, especially those bearing phenolic hydroxy groups, give more than one derivatisation product. Where these are listed separately, they have been isolated and the number of Dns groups present has been determined by nuclear magnetic resonance. In all other cases the characteristics of the major product has been given. It is often

TABLE XVIII

R_F VALUES OF DANSYLATED "PLANT" AMINES IN CHLOROFORM–BUTYL ACETATE (8:3, v/v)

Dansylation and chromatography were as described in the text. All derivatives were chromatographed at the same time, as far as possible, and their mobilities were initially measured relative to that of Dns ammonia. Each R_F given is the mean of two or more measurements and the data was shown to be reliable by re-running 23 derivatives in a separate series of experiments. Agmatine is not listed as it gives the same derivatisation product as putrescine.

		R_F
1	N-ω-Acetylhistamine	0.35
2	Isoamylamine	0.91
3	6-Amino-1-hexanol	0.13
4	2-Aminoimidazole	0.11
5	5-Amino-1-pentanol	0.09
6	1-Amino-2-propanol	0.17
7	Ammonia	0.35
8	Aniline	0.11
9	Benzylamine (1-phenylmethylamine)	0.87
10	*n*-Butylamine	0.88
11	Isobutylamine	0.90
12	*sec*-Butylamine	0.78
13	Cadaverine (1,5-diamino-*n*-pentane)	0.30
14	N-Caffeoylputrescine	0.07
15	Choline [(2-hydroxyethyl)trimethylammonium]	0.33
16	Coniine (2-propylpiperidine)	0.98
17	1,6-Diamino-*n*-hexane	0.40
18	1,3-Diaminopropane	0.28
19	1,2-Diaminopropane	0.33
20	Diethylamine	0.87
21	3,4-Dimethoxyphenylethylamine*	0.64
22	Dimethylamine	0.82
23	Dopamine (3,4-dihydroxyphenylethylamine*), didansyl deriv.	0.28
24	Dopamine (3,4-dihydroxyphenylethylamine*), tridansyl deriv.	0.48
25	Ephedrine (1-phenyl-2-methylaminopropanol)	0.56
26	Epinephrine (N-methyl-3,4-dihydroxyphenylethanolamine*)	0.31
27	Epinine (N-methyl-3,4-dihydroxyphenylethylamine*)	0.50
28	Ethanolamine	0.07
29	Ethylamine	0.51
30	Galactosamine	0.30
31	Galegine (3-methyl-2-butenylguanidine)	0.15
32	Glucosamine	0.32
33	Gramine (N,N-dimethyl-3-aminomethylindole)	0.84
34	*n*-Heptylamine	0.96
35	*n*-Hexylamine	0.95
36	Histamine [2-(4-imidazoyl)ethylamine]	0.17
37	Hordenine (N,N-dimethyl-4-hydroxyphenylethylamine*)	0.86
38	4-Hydroxy-3-methoxybenzylamine	0.53
39	3-Hydroxy-4-methoxyphenylethylamine*	0.48
40	Mescaline (3,4,5-trimethoxyphenylethylamine*)	0.48
41	Metanephrine (N-methyl-4-hydroxy-3-methoxyphenylethanolamine*)	0.32
42	4-Methoxyphenylethylamine*	0.59
43	6-Methoxytryptamine	0.42
44	3-Methoxytyramine (4-hydroxy-3-methoxyphenylethylamine), monodansyl deriv	0.38
45	3-Methoxytyramine (4-hydroxy-3-methoxyphenylethylamine), didansyl deriv.	0.51

TABLE XVIII (*continued*)

		R_F
46	Methylamine	0.47
47	2-Methylbutylamine	0.82
48	N-methyl-3,4-dimethoxyphenylethylamine*	0.76
49	1-Methylhistamine [1-methyl-4-(2-aminoethyl)imidazole]	0.38
50	N-methylmescaline (N-methyl-3,4,5-trimethoxyphenylethylamine*)	0.60
51	N-Methyl-4-methoxyphenylethylamine*	0.91
52	N-Methyl-5-methoxytryptamine	0.64
53	N-Methylphenylethylamine*	0.96
54	N-Methylserotonin (N-ω-methyl-5-hydroxytryptamine)	0.45
55	O-Methylserotonin (5-methoxytryptamine)	0.45
56	N-ω-Methyltryptamine	0.75
57	Norephedrine (2-amino-1-phenyl-1-propanol)	0.40
58	Norepinephrine (3,4-dihydroxyphenylethanolamine*), monodansyl deriv.	0.07
59	Norepinephrine (3,4-dihydroxyphenylethanolamine*), didansyl deriv.	0.10
60	Norepinephrine (3,4-dihydroxyphenylethanolamine*), tridansyl deriv.	0.19
61	Normetanephrine (4-hydroxy-3-methoxyphenylethanolamine*)	0.15
62	*p*-Octopamine (4-hydroxyphenylethanolamine*), monodansyl deriv.	0.08
63	*p*-Octopamine (4-hydroxyphenylethanolamine*), didansyl deriv.	0.20
64	*n*-Octylamine	0.97
65	*p*-Phenylenediamine	0 64
66	*o*-Phenylenediamine	0.69
67	Phenylethanolamine*	0.34
68	Phenylethylamine*	0.78
69	N-Phenyl-2-naphthylamine	0.88
70	Piperidine	0 86
71	*n*-Propylamine	0.79
72	Isopropylamine	0.72
73	Putrescine (1,4-diamino-*n*-butane)	0.27
74	Serotonin (5-hydroxytryptamine)	0 29
75	Spermidine [N-(3-aminopropyl)-1,4-butanediamine]	0.23
76	Spermine [N,N'-bis(3-aminopropyl)-1,4-butanediamine]	0.18
77	Synephrine (N-methyl-4-hydroxyphenylethanolamine*)	0.36
78	*p*-Tyramine (4-hydroxyphenylethylamine*), monodansyl deriv.	0.29
79	*p*-Tyramine (4-hydroxyphenylethylamine*), didansyl deriv.	0.54
80	*m*-Tyramine (3-hydroxyphenylethylamine*)	0.53
81	Tryptamine [3-(2-aminoethyl)indole]	0.58

* In all these compounds the ethylamine moiety is substituted at the 2 (= β) position.

useful to know the chromatographic positions of all possible products. For example, when a *p*-tyramine standard is treated with the usual reaction mixture it yields mainly its didansyl derivative. Yet when amine fractions from *Nicotiana tabacum* crown galls are treated in the same way, most of the *p*-tyramine recovered is in its monodansylated form.

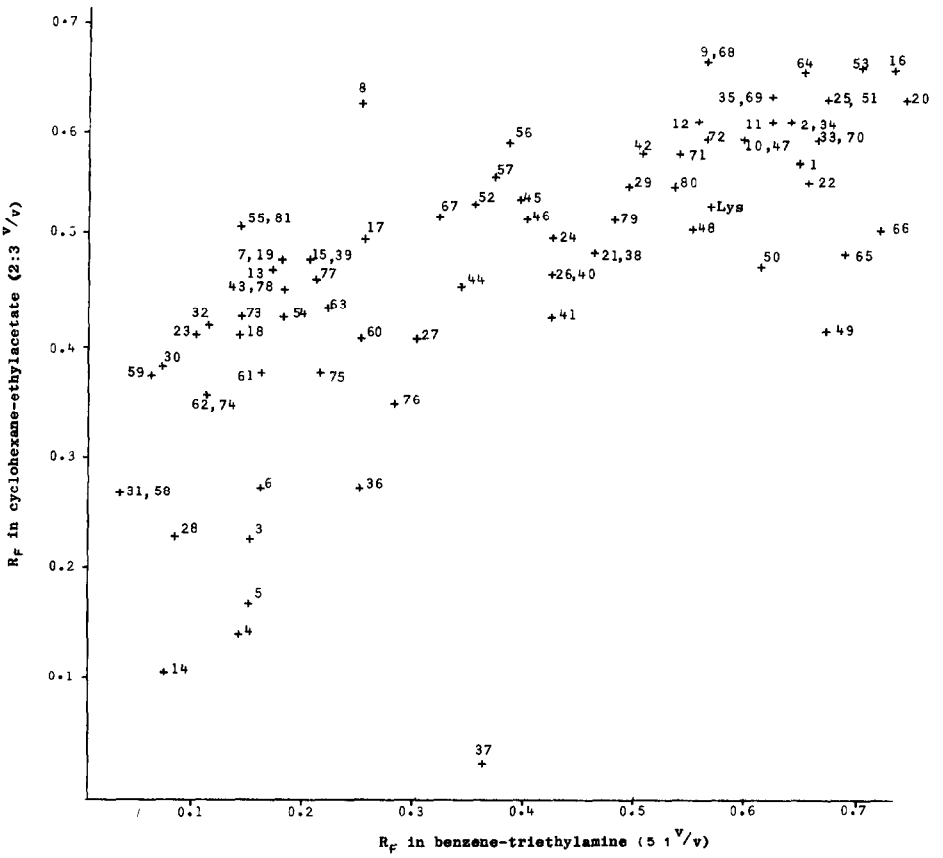


Fig. 5. Chromatographic map for dansylated "plant" amines in the standard two dimensional solvent system. Dansylation and chromatography were as described in the text. The numbers are those of the amines listed in Table XVIII. The position of lysine (Lys) is also shown as this is the only amino acid that regularly appears on chromatograms of plant amine fractions.

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