CHROMATOGRAPHIC STUDIES ON CANADA BALSAM AND SOME RESIN ACIDS

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INTRODUCTION

The constituents of Canada Balsam have interested several workers for many years[1] but the development of chromatographic techniques has increased the opportunity of resolving the complex mixture of acids, neutral compounds and other constituents, particularly as strong locating reagents can be used in thin-layer chromatography[2]. JORK[3] described thin-layer chromatographic methods for the differentiation of genuine and artificial Canada Balsam, including a quantitative densitometric estimation, and other workers[4–6] have separated methylated resin acids on silver nitrate impregnated Silica Gel G plates. A critical review of methods used for the analysis of oleoresins was published by STAHL[7], who was interested, however, only in the oleoresins used as flavouring agents. Several workers[4,8–10] have studied the separation of methylated resin acids by gas–liquid chromatography. The recommended column packings are either non-polar Apiezon N, the use of which can result in isomerisation of some acids, or polar polyesters, for example, diethylene glycol succinate. BURCHFIELD AND STORRS[11] dealt with the problems of separation of structurally similar resin acids. Some of the resin acids have been studied by ultra-violet spectroscopy[12,13], the purified compounds in some cases giving characteristic spectra.

EXPERIMENTAL

Silica Gel G (Merck) layers, 0.25 mm thick, were oven dried at 100° and stored in a desiccator until used. The plates were spotted with the test solutions and developed with ethyl acetate–*n*-hexane, 3:7 v/v (solvent system I). This solvent system was used for one-dimensional[1] separations. For two-dimensional separations, solvent system I was used for the first dimension and chloroform–acetone, 9:1 v/v (solvent system II) for the second.

Detection

General test

Vanillin in sulphuric acid (1 % w/v) was used for the general detection of Canada Balsam constituents on TLC. The reagent was freshly prepared every three days[14].

Unsaturated compounds

Bromine vapour. The plates were placed in a chromatography tank containing

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bromine vapour and the spots visualised, after removal from the tank, by heating gently with hot air.

Diiodofluorescein. The plates were sprayed with alcoholic diiodofluorescein solution (0.2 % w/w) before exposure to bromine vapour.

Potassium permanganate. The plates were sprayed with a very dilute solution of potassium permanganate (0.2 % w/v) in aqueous sulphuric acid (0.04 % w/v).

Acidity

Compounds giving an acidic reaction were detected by spraying with Universal Indicator solution.

Phenols

Phenolic compounds were detected by spraying the plates with 2 % ferric chloride solution in acetone.

Copper resin acid reaction

After separation of the constituents, the plates were sprayed with a saturated solution of copper acetate in acetone.

A few drops of the reagent were applied to the starting point on the plate, followed by the solution under test. The mixture was then resolved by development in the required solvent system.

Multiple detection (Figs. 1 and 2)

After development, the plate was sprayed first with Universal Indicator, secondly with ferric chloride solution and finally treated with bromine vapour and heated. The spots produced with each locating reagent were traced and the pH noted of the compounds detected by Universal Indicator.

Methods of separation

Sodium carbonate solution. A solution of Canada Balsam in ether (5 %) was shaken three times with 5 % aqueous sodium carbonate solution ($^1/_4$ of the ether volume). The aqueous portion was washed with ether, acidified by the addition of dilute acetic acid and the precipitated acids re-extracted into ether. The ethereal solution of acids was washed three times with water (1/4 of the ether volume), then dried with anhydrous sodium sulphate and filtered.

TLC. To the origin of a Silica Gel G plate, saturated ethanolic sodium hydroxide solution was added, followed by the application of Canada Balsam or coniferous material under study. After drying, the plates were double developed using solvent system I, and the band containing the sodium salts of the resin acids was removed from the plate. The material scraped from the plate was first macerated with ether, then suspended in water that had been acidified by the addition of dilute acetic acid. The liberated acids were extracted with ether, the solution being washed with water, dried with anhydrous sodium sulphate and filtered.

Ammonia precipitation. Ammonia was passed through a 2 % ethereal solution of Canada Balsam for 30 min, and the material precipitated in this time was separated by centrifuging the mixture. The precipitate was washed with ether and then dissolved in water. Dilute acetic acid was added until the solution gave a slightly acid reaction, when the free acids were extracted with ether. The ethereal solution was washed and dried as described above.

Separation of resin acids by ion-exchange resins. Canada Balsam, or other test material, 0.5 g, was extracted with 30 ml petroleum ether (b.p. 40–60°). The insoluble

portion was separated and washed twice with 10 ml petroleum ether. The petroleum ether extract and washings were bulked and shaken three times with 5 ml of 5% sodium carbonate solution, to extract the resin acids. The aqueous solution was washed twice with 5 ml ether before being passed through a column containing 10 g Amberlite 1 R 120 (H^+). The free resin acids were eluted with two bed volumes of ether and the eluate washed with two 20 ml portions of water, dried with anhydrous sodium sulphate, filtered and evaporated to dryness.

Isolation of resin acids as amine salts. The free resin acid residue was dissolved in 20 ml acetone and then 0.5 ml of an amine–acetone solution* was added to precipitate the amine salts of abietic, neoabietic, L-piramic and palustric acids. The mixture was stored for 15 hours at 5–10° before the precipitated material was separated washed with acetone and dissolved in methanol. Both the methanol solution and the acetone solution, which contained the unprecipitated amine salts, were passed separately through columns of Amberlite 1R 120 (H^+) to deaminate the acids[16].

Fractionation of Canada Balsam. Canada Balsam was extracted with petroleum ether and the insoluble fraction (1) separated (Fig. 4). The extract (2) was divided into two portions, one portion being shaken with sodium carbonate solution, and the acids liberated from the salts using the ion exchanger 1R 120 (H^+) (3). The non-acid-containing part of the eluate was dried with anhydrous sodium sulphate (4). The second portion of the petroleum ether extract was evaporated to dryness, the residue dissolved in acetone and 0.5 ml of amine–acetone (1:1) solution added. After storage in a refrigerator for 15 h, the precipitate was separated, dissolved in methanol and the acids deaminated by passage through an ion exchange column (5). The amines remaining in the acetone solution (6) were also deaminated, the solvent evaporated, the residue dissolved in ether, and the solution shaken three times with 5 ml of 5% sodium carbonate solution, to separate the remaining acids as salts, which were freed by passage through a column of Amberlite IR 120 (H^+) (7). The remaining solution is numbered (8) in Fig. 4.

The fraction insoluble in petroleum ether (1) was dissolved in 30 ml ether and shaken three times with 5 ml sodium carbonate solution (5%). The aqueous phase was separated and passed through an ion-exchange column (9) and the organic phase was evaporated, after washing with water (10).

GLC of resin acids. To an ethereal solution of resin acids, a solution of diazomethane urea in ether was added, until a stable colour was obtained, when the solution was evaporated to dryness on a waterbath. The methylated resin acids were examined by GLC using a Perkin Elmer F 11 Gas Chromatograph, with a column 2 m long, diameter 2.2 mm, packing 10% diethylene glycol succinate (LAC-3-R-728) on Chromosorb W, mesh 100–120, oven temperature 200°, injection temperature dial setting 5, carrier gas nitrogen 30 lb/sq. in. hydrogen 15 lb/sq. in., oxygen 25 lb/sq. in. and chart speed 15 in. per hour.

RESULTS

Detection and separation

TLC gives the opportunity of using stronger locating reagents than can be used

* 2-Amino-2-methylpropan-1-ol–acetone (1:1).

with paper chromatography. Antimony trichloride[2,17] and antimony pentachloride solutions, phosphomolybdic acid, and anisaldehyde reagents[3] have been used, but with these reagents, the plates have to be heated at 100–110° for 5–10 minutes to locate the compounds. It was decided, therefore, to use 1 % vanillin in sulphuric acid, which eliminated the need of heat, although when used, deeper colours were obtained. This was important for preparative TLC in the preparation of samples for GLC studies, when the detection of only a narrow strip was possible. The observation of the colours produced was started shortly after spraying and was continued at certain intervals, because of changes and late development of colours. Vanillin in sulphuric acid reacts with iso- and D-pimaric acids to give a violet colour, with neo-

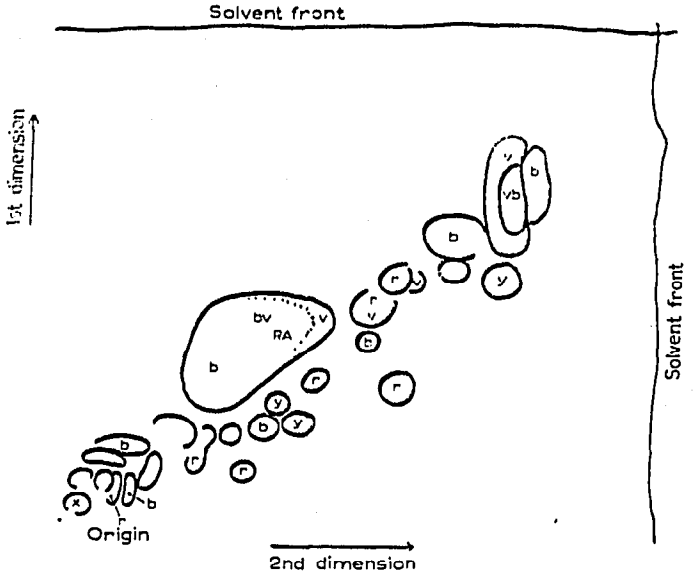

Fig. 1. Two-dimensional thin-layer chromatogram of Canada Balsam. Detection: vanillin in sulfuric acid. b = Blue, r = red, v = violet, y = yellow, RA = resin acids.

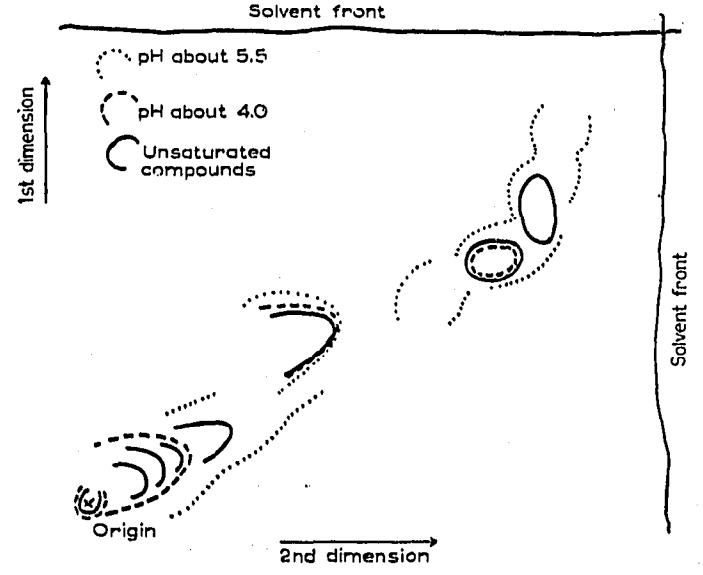

Fig. 2. The same two-dimensional thin-layer chromatogram of Canada Balsam as given in Fig. 1. Multiple detection.

abietic and palustric acids to give a blue colour and abietic acid to give a green-blue to blue colour. Plates sprayed with concentrated sulphuric acid attract moisture from the atmosphere, which can result in the production of spots of different transparency, which can be characteristic. In this way it is possible to identify the position of dehydroabietic acid, which does not give a colour reaction with vanillin in sulphuric acid.

The detection of unsaturated compounds has been important in this study of the resin acid complex of Canada Balsam. By exposure to bromine vapour, followed by gentle heating, yellow-brown spots were produced by the unsaturated compounds. In addition, when L-pimaric acid was present in sufficiently high quantities, a blue colour was produced after heating. Diiodofluorescein reagent was used also for the determination of unsaturated compounds. After spraying the plates with the reagent any fluorescence produced was noted, before the plates were exposured to bromine vapour, which located the unsaturated compounds as pale spots on a pink background. Very sensitive detection was achieved using a dilute solution of potassium permanganate[15]. The unsaturated compounds appeared as yellow spots on a pink-violet background, but when exposed to bromine vapour and gently heated, additional new white spots appeared on the yellow ones. The use of the diiodofluorescein and potassium permanganate reagents was more convenient than using bromine vapour, which was less sensitive than the other two locating reagents.

Dehydroabietic acid does not react with bromine vapour or potassium permanganate solution. Under ultra-violet light, the plate treated with diiodofluorescein and bromine vapour showed dehydroabietic acid as a light-absorbent violet spot, and with the copper reagent it produced, if sufficient quantities were present, an emerald green spot similar to that produced by the other acids.

Phenolic compounds are present in Canada Balsam in low quantities. Similarly the chloroform extracts of different coniferous materials did not contain large quantities of phenols, contrary to their alcoholic extracts.

For the determination of the acidity of the phenols and unsaturated compounds, a multiple detection method was used on one plate (Table I). When used in conjunction with a duplicate plate sprayed with the vanillin in sulphuric acid reagent, it was possible to obtain further characteristics of each spot. There are two acid reacting groups of compounds in Canada Balsam, one detected near the origin of the chromatogram and a second near the centre, which is the major spot. The so-called "neutral substance" spot, formed probably by abienol and/or esters of resin acids, also gives positive reactions for acidity and unsaturation. The compounds producing this spot are not, however, fixed at the origin like the sodium salts of the resin acids.

TABLE I

COMPOUNDS TRACED BY MULTIPLE DETECTION

Universal Indicator	*$FeCl_3$*	*Br_2*	*Results*
+	—	—	Acid reacting compounds
+	+	—	Acid reacting compounds + phenols
+	+	+	Acid reacting compounds + phenols + unsaturated compounds
+	—	+	Acid reacting compounds + unsaturated compounds
—	+	+	Phenols + unsaturated compounds

Copper has been used for the detection of the complex of resin acids in Colophony, for the detection of Colophony as an adulterant in vegetable drugs and for quantitative colorimetric evaluation[18]. For quick preliminary examinations we used spot tests by pressing freshly cut surfaces of conifer branches against paper impregnated with copper acetate. Distinct emerald green traces were obtained. Copper was of less use for the detection of resin acids on TLC except in cases when large quantities of acids were present. After treatment with bromine vapour, a light violet colour appeared, corresponding to the visible spot, but after being heated, part of the spot became violet or bluish. No violet colouration was observed when dehydroabietic acid was applied.

As mentioned earlier, the separation of methylated resin acids, using silver nitrate impregnated Silica Gel G plates, has been studied by several workers. The preparation of reliable plates, sometimes containing large quantities of silver nitrate[5,6], is not easy, and to effect good separations, care has to be taken in the preservation and storage of the plates. Moreover, detection of the separated components is difficult. For a complex material like Canada Balsam, this system is not the most convenient, particularly for the isolation of the resin acids prior to GLC studies, when separations on untreated layers are desirable.

The solvent system ethyl acetate–*n*-hexane (3:7 v/v) was found to be the best for the resin acids being studied. Using this system, the acids moved to about the middle of the plate with some separation. For two-way separations, chloroform–acetone (9:1 v/v) was used for development in the second direction. The reference samples of resin acids, even after purification by preparative TLC, always produced several spots when examined by TLC, indicating possible decomposition. Partial

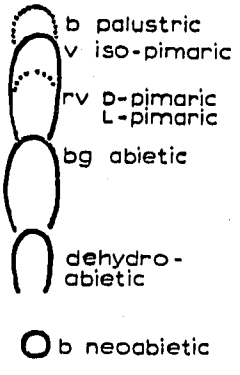

Fig. 3. Separation of resin acids (4 × running). Solvent system: ethyl acetate–*n*-hexane (3:7). b = blue, g = green, r = red, v = violet.

TABLE II

COLOURS AND R_F VALUES OF SOME RESIN ACIDS

Acid	*Colour with vanillin–H_2SO_4*	*R_F (× 100) on Silica Gel G*	
		**Ethyl acetate–n-hexane*	*Chloroform–acetone*
D-Pimaric	Violet	58	66
Iso-pimaric	Pink-violet	64	66
Abietic	Blue-greenish	39	63
Dehydroabietic	None	52	66
Neoabietic	Blue	23	44
L-Pimaric	Blue	35	60
Palustric	Blue	65	

* Run four times.

separations of the relatively pure acids available were obtained on untreated plates, after four-fold development (Fig. 3 and Table II). The R_F values obtained depended on the quantities of the resin acids applied. In mixtures it was possible to identify the spots according to their colour reactions with the vanillin and sulphuric acid reagent and the other locating reagents.

The use of Alumina G layers instead of Silica Gel G was of no benefit, as the alumina reacted with the resin acids to form salts, which remained at, or close to, the origin. This simple fixation of resin acids and their subsequent separation from other Candada Balsam constituents was utilised for their isolation in other experiments. Originally the Silica Gel G layers were made alkaline by the addition of 5 % sodium carbonate to the Silica Gel G, but a subsequent modification was the application to the bottom of the adsorbent layer of a saturated ethanolic solution of sodium hydroxide, to make alkaline only the starting line of the plate, before the application of the test material. Two chromatograms of the same material were run parallel on one silica gel plate, one half being sodium hydroxide treated and one half being untreated. Good results were obtained, which were useful for comparative chemo-taxonomic studies. In the spectra of the sodium hydroxide treated material, the resin acids were eliminated. This method cannot be considered as specific for resin acids, as phenolic and alcoholic compounds will behave in a similar manner. The phenols can be recognised, however, by using a convenient reagent.

Fractionation of Canada Balsam

Canada Balsam was fractionated to obtain information about the distribution of the compounds in each fraction. Study of the various fractions by TLC produced a total number of spots in excess of the 37 achieved from the original sample by two-dimensional TLC.

The Canada Balsam fraction insoluble in petroleum ether (1) constituted from 27 to 44 % of the total weight (Fig. 4). This portion is supposed to contain mainly resins and neutral substances and is characterised on TLC by spots of low R_F value. This fraction was dissolved in ether and shaken with sodium carbonate solution, which extracted up to 50 % of the dissolved material (9). The petroleum ether insoluble

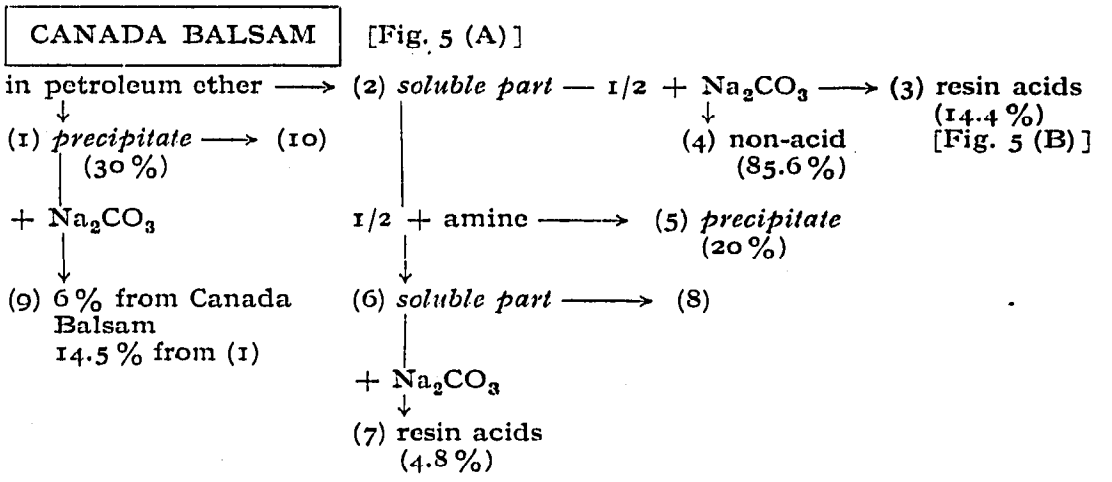

Fig. 4. Petroleum ether precipitation and further fractionation of Canada Balsam.

fraction was free from acids, but the soluble part, treated with sodium carbonate solution, showed substances in addition to acids, and required further purification. The use of ammonia for the isolation of the acid portion gave more satisfactory results.

GLC of the total acid fraction (3) showed neoabietate, L-pimarate/dehydroabietate, abietate and D-pimarate/palustrate peaks. The soluble amine salts portion of the original total acid fraction (3) was characterised by the dehydroabietate, D-pimarate and isopimarate peaks. The precipitable part (5) showed large peaks of

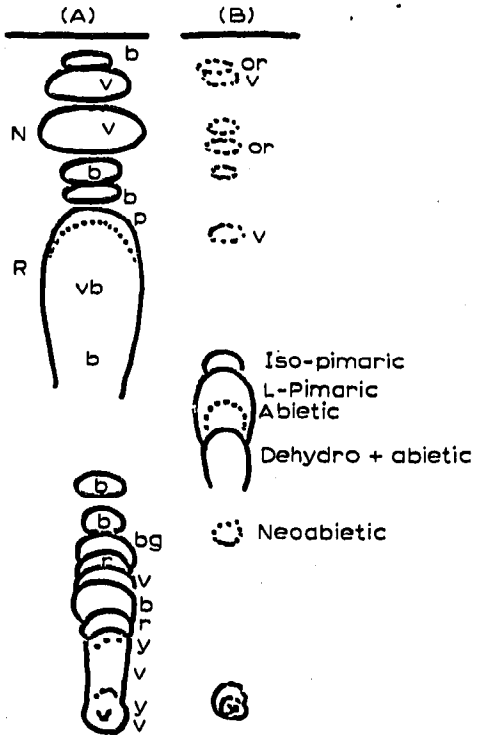

Fig. 5. (A) Natural Canada Balsam. Solvent system: ethyl acetate–*n*-hexane (3:7). (B) Acid fraction separated by ion-exchange resin. A,B: N = neutral compounds (?), R = resin acids mixed with other components of Canada Balsam; b = blue, g = green, or = orange, p = pink, r = red, v = violet, y = yellow. B: double running.

L-pimarate, abietate and neoabietate, and a double one produced by unknown compounds.

The separated and methylated acid fractions (3), (5) and (6) were estimated by GLC. The "pair" acids, showing one peak, were separated by the amine salt method. The main compound was the abietate, but the palustrate was present in traces only (Table III, Fig. 4). Quantities of some resin acids (methyl esters) were found in Canada Balsam which were not very different from those found in resins of some coniferous species[19–22].

TABLE III

QUANTITIES OF RESIN ACIDS (%) IN THE ACID FRACTION OF CANADA BALSAM

Neoabietate	*Dehydro-abietate*	L-*Pimarate*	*Abietate*	*Iso-pimarate*	D-*Pimarate*	*Palus-trate*	
		20					
10			23	11			Total
11		13	21				Insoluble amines
	5			12	1.2		Soluble amines

DISCUSSION

A chemotaxonomic study of the Coniferae requires a simple method for the separation of resin acids from relatively small quantities of starting material. This is possible in different ways, using the ability of the acids to form salts, not only with sodium, but also with copper, ammonium and even with amines. In this study TLC and GLC were used. The use of benzene as a solvent system[3a] should leave the resin acids at the origin. Comparison of the GLC results of material purified with benzene with that prepared using sodium hydroxide to fix the acids at the origin, showed small differences, particularly with compounds having the higher retention times, this being the result, probably, of migration of the acids in the solvent.

Gaseous ammonia treatment yielded a precipitate, which on examination by GLC, gave results similar to the material isolated from the sodium hydroxide treated plate. The application of this method was, however, more complicated. It required a sufficient source of ammonia and, even when the precipitate had coagulated to a sticky solid, there was still a large proportion of resin acid salts dispersed in the ether, which caused an opalescence on the addition of more ether. It is likely that some of the ammonium salts were dissolved in the strongly alkaline solvents. They were removable by washing the ether solution with water, acidifying the aqueous phase with acetic acid, and extracting the acids with ether or by passage through an ion exchange column. In this way a yield of about 28 % was obtained, which made the figure of 40 % obtained by LOMBARD *et al.*[12] appear a little high. Using the sodium hydroxide treated plates, 19 % of a crude acid complex was obtained from Canada Balsam. All acid fractions obtained by these methods needed further purification by repeated formation of salts, washing with ether and regeneration to free acids.

Preliminary treatment of the balsams, resins or extracts with petroleum ether or acetone was always helpful.

Comparison by GLC of the fractions obtained by benzene separation, ammonia precipitation, the use of sodium hydroxide treated plates and sodium carbonate extraction indicated that the use of sodium hydroxide treated plates was the most convenient method for use in chemotaxonomic studies, because of the possibility of easier separation of the non-acid part of the Coniferous material. The separation of the amine salts of the acids[16] was less convenient for large numbers of specimens. Precipitation with the amine can be applied to a previously separated resin acid complex. The amine method is very useful for the separation of the "pair" acids, L-pimaric/dehydroabietic and D-pimaric/palustric. The soluble and insoluble partners can be detected separately by GLC (Table III).

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SUMMARY

The separation of Canada Balsam was studied, with special reference to the resin acids, for their further study by gas–liquid chromatography (GLC).

Using ethyl acetate–*n*-hexane (3:7 v/v) as solvent system (I) on Silica Gel G layers, partial separation of the Canada Balsam constituents was achieved. For two-dimensional separation, solvent system I was used in conjunction with chloroform–acetone (9:1 v/v). The use of Silica Gel G alone was found preferable to silver nitrate impregnated plates.

A satisfactory separation of some resin acids was found possible after four-fold development. The R_F values of some resin acids were studied, as well as their colour reactions with 1 % vanillin in sulphuric acid and their detection with diiodofluorescein, bromine and potassium permanganate. The blue colour of the resin acid spot of Canada Balsam, which appeared on the chromatogram after bromine treatment and heating, was caused by L-pimaric acid.

For the determination of Colophony, the much used copper reaction was found insensitive, unspecific and useless for thin layer chromatograms.

For chemotaxonomic studies, the isolation of the resin acid portion of Canada Balsam or coniferous material was studied and, as the most convenient method, sodium hydroxide treated plates are recommended.

A packing of 10 % diethylene glycol succinate (LAC-3-R-728) on Chromosorb W and a temperature of 200° gave satisfactory results for GLC. Five distinct peaks of the neoabietate, L-pimarate/dehydroabietate, isopimarate, and D-pimarate/palustrate were obtained. The quantities of some resin acids in Canada Balsam were evaluated.

The pair resin acids, giving one peak on GLC, could be separated by the different solubilities of their amine salts in acetone.

Good isolation of the resin acid complex was achieved using ion-exchange resin columns.

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