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"FLIP-FLOP" CHROMATOGRAPHY*

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SUMMARY

To separate mixtures containing substances of widely differing polarities, a technique called "flip-flop chromatography" has been introduced. The sample is deposited in the pores of a solid (*e.g.*, silica) with an average pore diameter of about 100 Å to increase its surface area and hence its rate of extraction. Depending on the pore volume of the silica, up to 40% (w/w) of sample may be loaded. The essence of flip-flop chromatography is that the sample column is extracted by a combination of four or more polar and non-polar solvents which are applied in order of alternating polarity beginning with the most extreme so that the tail ends of the polarity distribution of the sample are successively extracted, leaving behind material with a more restricted polarity range, increasing the selectivity of later extractions. All the polar solvents are washed through the sample column in one direction and the non-polar eluents in the opposite direction. The sequence of solvents can be 1, water; 2, heptane; 3, methanol; and 4, methylene chloride. At either end of the sample column stripping columns (packed with silica or a reversed phase) prevent the removal of substances whose polarity is very different from that of the solvent.

The mechanism of the separation is a combination of extraction, frontal analysis and displacement chromatography, but definitely not the conventional adsorption chromatography. However, the influence of the support on the separation is not negligible.

The advantages of flip-flop chromatography are: (1) improved quality of separation compared to conventional extraction, (2) high speed of separation, (3) economy in the use of solvents, (4) high sample concentrations (up to 5%) in the fractions, (5) the simplicity of the apparatus.

As an illustration of this method, the heart glycoside uscharine, was recovered from freeze-dried *Calotropis gigantea* sap. Both the yield and the degree of purification achieved are better with this method than with stepwise extraction.

* The term "flip-flop" is used in the sense of "sudden reversal of direction".

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INTRODUCTION

The extraction and separation of components of widely differing polarities in a mixture usually presents no difficulties. However, the separation becomes difficult if the polarity scale of the compounds spreads from non-polar (*e.g.*, squalane) to polar (*e.g.*, an amino acid) and if the mixture contains a wide range of substances with intermediate polarity. Such systems are in fact very familiar in the tars left after the isolation, particularly by crystallization, of extracts from living systems, or in the tars produced by unsuccessful attempts at synthetic chemistry, or in some hydrolyses of natural products.

It is characteristic of such tarry mixtures that one may know them to contain a particular substance, perhaps because of a biological activity, and yet have great difficulty in isolating it. Of course, by diluting the mixture with suitable solvents to the degree required for good analytical chromatography, followed by chromatography, good separation is possible. However, this involves such large volumes of solvents that it is often economically unjustified. Furthermore, the recovery of substances present at the parts per million level can become poor.

One of us (A.J.P.M.) suggested that a better approach would be to remove the tail ends of the wide polarity range distribution of the sample by extracting alternately and successively, with solvents from the extreme end of the polarity range, leaving behind material in the middle polarity range with a progressively smaller polarity distribution. For example the mixture is first extracted with water and then with heptane. The following solvents can be methanol and methylene chloride, respectively.

It was postulated that this procedure of cleanly removing the tail ends of the polarity distribution and approaching a medium polarity should greatly increase the solubility of the material left behind and greatly increase the selectivity of the later extractions, thus reducing markedly both the analysis time and the consumption of solvents.

An efficient extraction is only possible if the total sample is in close contact with the extraction solvent. The speed and efficiency of the extraction procedure decreases with increasing thickness of the sample layer. With increasing sample thickness, the probability also increases that polar substances will be trapped within a non-polar matrix so that they cannot be extracted by the polar solvent. The extraction procedure always becomes more efficient if the thickness of the sample layer is decreased, *i.e.*, if its surface area is increased.

The method of "extrography" has been described[1] for the separation of the vacuum residue of crude oil, in which the sample was dissolved or dispersed in a volatile eluent (*e.g.*, methylene chloride). This solution or dispersion was mixed with a silica having a pore volume of *ca.* 1 ml/g and with an average pore size of about 100 Å. After the solvent had been evaporated, the packing material (*i.e.*, silica coated with the sample to be separated) was a free-flowing powder, if the sample amount did not surpass the pore volume of the silica. Up to 67 g of vacuum residue was deposited into the pores of 100 g of silica as described above (*i.e.*, 40% w/w). Assuming that the depths of the pores are of the same order of magnitude as their diameters, the thickness of the layer to be extracted will then also be of the order of 100 Å. Of course, it is also possible to prepare the sample for elution by simply mixing the support with a finely powdered sample, providing this deposits the sample within the pores and

yields a free-flowing material. A particle size of 100–200 μm is suitable. The free-flowing material can be loaded into the sample column. This form of column preparation is reminiscent of the preparation of heavily loaded columns[2]. In the method of extrography the sample column was extracted with solvents of increasing polarity, *e.g.*, heptane, trichloromethane, methylene chloride, etc., and the fractions were collected. The efficiency of the separation could be increased with this method, if a "separation column" (packed for example with alumina) was connected after the sample column. If a sample column, 50 cm × 25 mm I.D. was used, up to 40 g of vacuum residue of crude oil could be separated in one batch. It was possible to scale this system up without difficulty and to separate 160 g or more of the oil residue. Because of the thin layer of the sample in the pores, the separation was speedy and efficient. Samples of the size mentioned above were separated into 10 or more fractions in 4 h or less.

Because of the high concentration of the mixture in the sample column, the concentration of the sample in the eluent can be several orders of magnitude higher (*i.e.*, more than 5% w/w) than is usual in chromatography. Therefore, the mechanism of elution will be a complicated combination of extraction, frontal analysis, displacement chromatography and solid–liquid chromatography, but definitely not, because of the high sample concentration in the eluents, a conventional adsorption chromatography. Evidently the mechanism is —among others— a function of the sample/support ratio in the sample column. This ratio decreases with the volume of the eluent pushed through this sample column.

The support should have a relatively small pore size (100 Å) in order to limit the sample thickness and therefore to encourage speedy mass transfer. On the other hand, because of its loadability, the pore volume of the support has to be large. Consequently, the specific surface area of the support must be high and therefore it will be more or less active. The activity of the support is a further parameter co-determining the separation of such systems. If the sample has been loaded as a solution or dispersion, it is possible that it may deposit itself inhomogeneously, with the sample layer becoming more polar the closer it is to the polar surface of the support.

The technique proposed in this paper combines the approach of removing the extreme ends of the polarity range of the sample to be separated with the technique applying the sample as a thin layer to a support. The support coated with sample is loaded into a sample column and flushed first with water, then with heptane, etc. This method is speedier and more efficient than normal extraction. This, however, introduces a new difficulty.

The solubility of the non-polar components in water is not negligible. Therefore the eluent (*e.g.*, water) from the sample column is passed through a "stripping column" packed with reversed phase. It is essential that the stripping column has a large enough capacity so that elution can be continued until no more polar material is eluted with water from the sample column without the stripping column being overloaded.

As the next process, a very non-polar solvent (*e.g.*, *n*-heptane) is pumped through the reversed-phase stripping column into the sample column. Thus any non-polar compounds arrested by this stripping column during the previous step are dissolved and pushed back into the sample column. This non-polar solvent (*e.g.*, heptane) will dissolve most of the non-polar material together with a small amount of the more

polar material which was not soluble in water. In order to remove this more polar material from the heptane fraction, the eluent from the sample column is passed through a polar (*e.g.*, packed with silica) stripping column. The polar stripping column must also be of an adequate size.

At this stage, the original mixture has been divided into three fractions: a polar fraction dissolved in water, a non-polar fraction dissolved in heptane, and a fraction of intermediate polarity which remains in the sample column. For some purposes this amount of fractionation may be sufficient, in which case the intermediate polarity fraction can be washed out of the column. If a further fractionation of this intermediate polarity fraction is required, then methanol is pumped into the sample column through the silica stripping column. After it leaves the sample column, it passes through the reversed-phase stripping column. After this, methylene chloride is pumped through the column train in the opposite direction.

The sample is now divided into five different fractions, four of which were dissolved in the four different solvents, and the fifth remaining in the sample column. Usually this separation is sufficient and the polarity range of this undissolved fraction will be as narrow as is required. This fraction may then be washed out of the column.

If the separation achieved by this solvent program yields fractions whose polarity ranges are too large, then either further solvents can be used or a different solvent program involving more solvents with smaller polarity differences can be used.

A further advantage of the stripping columns is that they will remove any particles of sample carried along mechanically by the solvent from the eluent stream.

We propose that the method of fractionation described shall be called "flip-flop chromatography".

In the Experimental section of this article, the mixture to be separated was a freeze-dried powder, with a large surface area, deposited as a solid powder into the pores of a silica support. The preparation of sample columns where the mixture to be separated is deposited into the pores of a support from a solution or dispersion, and the influence of the activity of the support on the separation, will be discussed in a future paper.

Flip-flop chromatography has been evaluated and compared with conventional stepwise extraction chromatography by studying the purification of heart glycosides from the lyophilized sap of *Calotropis gigantea*[3–9]. The main glycosides contained in this material are uscharine and calotropine. Their structure was described by Reichstein and co-workers[8,9]. The conventional extraction procedure is unsatisfactory. When the lyophylizate was extracted with a range of solvents (benzene, trichlorethylene and chloroform), all the different glycosides were present in each solvent. The extracts were then purified by normal low-pressure preparative chromatography on silica or alumina. In a typical experiment 10–20 g of extract were chromatographed on a 1-m long column packed with 500–700 g of alumina or silica.

As the column was eluted, the polarity of the eluent was increased by changing its composition stepwise from benzene to ethyl acetate, to ethanol and to water. A typical chromatogram of the chloroform extract is shown in Fig. 1, where more than 10 l of eluent and about 10 days were required for the chromatographic separation[6].

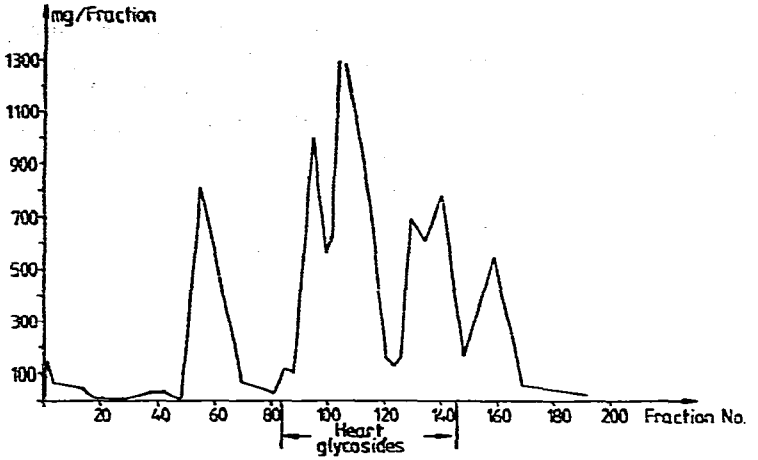

Fig. 1. Chromatographic separation of a chloroform extract of freeze-dried *Calotropis gigantea* sap (ref. 6, p. 60). Sample size, 10 g; column, 1 m × 4.5 cm I.D.; packing, silica, 715 g; eluents, benzene, chloroform, ethyl acetate, ethanol, ethanol-water (1:1), water; fraction volume, *ca.* 80 ml; time of analysis, *ca.* 10 days.

EXPERIMENTAL

The apparatus is shown in Fig. 2. The stainless-steel sample column (length 10 cm, I.D. 0.8 cm) was packed with silica (LiChrosorb SI 60, 15–25 μm; Merck, Darmstadt, G.F.R.). The polar stripping column was packed with silica (LiChrosorb SI 100, $d_p = 10\,\mu$m; Merck). The non-polar stripping column was packed with a reversed-phase support [SI 100, C_{18}, 17.5% (w/w) C].

The two pumps were two separate heads of a three-headed membrane pump (Orlita, Giessen, G.F.R.; Model MS4/3/3/3). Pump No. 1 was arranged to pump liquid from reservoir 1 through the column train in the direction non-polar stripping column–sample column–polar stripping column. Pump No. 2 was arranged to pump liquid from reservoir 2 through the column train in the reverse direction. The flow was

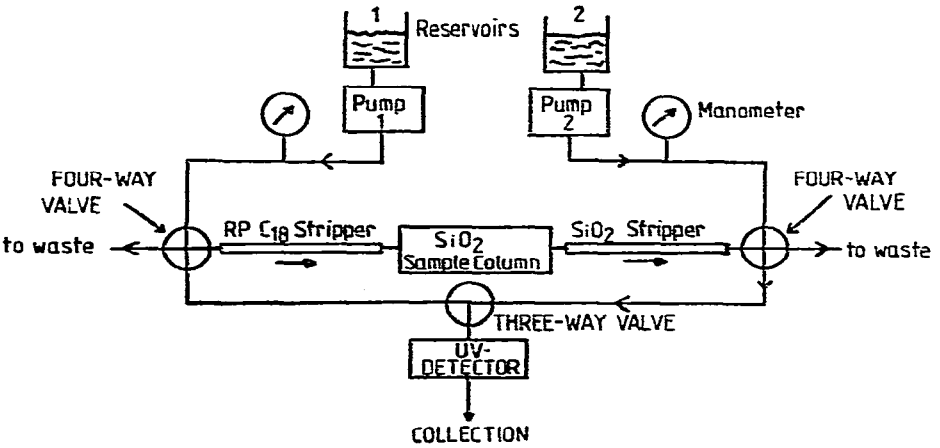

Fig. 2. General arrangements of the apparatus.

directed by three valves, two of them four-way and one three-way (Whitey, Oakland, Calif., U.S.A.), whose dead volumes were decreased by means of PTFE inserts. These valves enabled the flow from either of the pumps to be directed through the columns. The flows could also be directed to waste so that it was possible to rinse the pumps and reservoirs when changing solvents.

The detector was either a home-made UV detector (254 nm) or a differential refractometer (Waters Assoc., Milford, Mass., U.S.A.; Model R 401). Downstream from the three-way valve the stainless-steel connecting tubes were of 0.5 mm I.D., while between the columns and the three-way valve the tubes were of 1.0 mm I.D. and upstream from the columns 1.5 mm I.D. At first 0.25 mm I.D. tubing was used to connect the columns to the detector and to the collector, but such tubing was susceptible to blockage.

Procedure

A known weight (about 2.2 g) of freeze-dried *Calotropis gigantea* sap is mixed thoroughly with 80% (w/w) of silica and packed whilst dry into the sample column. This column is then connected to the column train, and eluted with the chosen solvents, elution being alternately from either end. The first solvent is supplied from reservoir 1 by pump No. 1, the second from reservoir 2 by pump No. 2, and the third also from reservoir 1 by pump No. 1.

Most of the extractions were performed with the solvent system *n*-heptane–water–methylene chloride–methanol. The use of water as an eluent introduces a complication, as it would completely deactivate the silica in the polar stripping column. However, this difficulty can be circumvented in various ways. Water can be used as the first eluent, in which case the polar stripping column need not be attached to the column train until after the water has been used. If water is not the first solvent used then the polar stripping column can be replaced when the silica has been deactivated. However, we have chosen simply to remove the polar stripping column from the column train whilst eluting with water so that the contents of this column remain to be eluted by the next polar solvent.

During the elution the concentration of the eluate was high enough to saturate the detector system completely, and the eluate was divided arbitrarily into four or five fractions. When the detector was not completely saturated then the detector response was used as a guide for cutting the fractions.

At the end of the experiment the packing of the sample column was dried and weighed.

The various fractions were analysed at first by thin-layer chromatography (TLC) on Merck silica gel 60 F_{254} plates (20 × 20 cm) using *n*-heptane–ethyl acetate–isopropanol (25:65:10) as the mobile phase. The chromatograms were rendered visible by spraying with acetic anhydride–sulphuric acid–methanol (5:5:50) followed by heating to 120° for 15–20 min. Pure uscharine and calotropine were run as standards.

The presence in the fractions of glycosides was also tested qualitatively using Kedde's[10] specific reagent: 1% (w/w) 3,5-dinitrobenzoic acid dissolved in 50% methanol which was 0.5 *N* with respect to sodium hydroxide.

If necessary, the fractions were further analysed by high-performance liquid chromatography (HPLC) on a 10-μm RP C_{18} column (length 30 cm, I.D. 0.4 cm)

using 40% methanol as the mobile phase. The pressure drop was 130 atm, which gave a flow-rate of about 1.8 ml/min, and uscharine had a retention time of 10 min ($k' = 5.0$).

RESULTS

When the solvents *n*-heptane, water, methylene chloride and methanol were used then the uscharine was usually found in the methylene chloride and the more polar glycosides in the water and in small amounts in the methanol. As was to be expected, no glycosides could be detected in the *n*-heptane fraction.

Functions of the stripping columns

The major intended function of the stripping columns is to prevent the elution of polar sample components by non-polar solvents and *vice versa*. When extractions were carried out with and without stripping columns, TLC analysis of the various fractions showed that the stripping columns caused the various components to be concentrated into fewer fractions and caused there to be less overlap of components between the fractions. Tests with Kedde's reagent showed that with the stripping columns there was little glycoside in the methanol extract but without them there was a relatively large amount.

A second possible function of the stripping columns is that they might perform some separation during the extraction, even though they are overloaded and although they are operating under non-equilibrium conditions during the change from one solvent to another. This possibility was confirmed by studying the composition of successive fractions collected during the elution with one solvent. Fig. 3 shows the analytical chromatograms of three successive methylene chloride fractions.

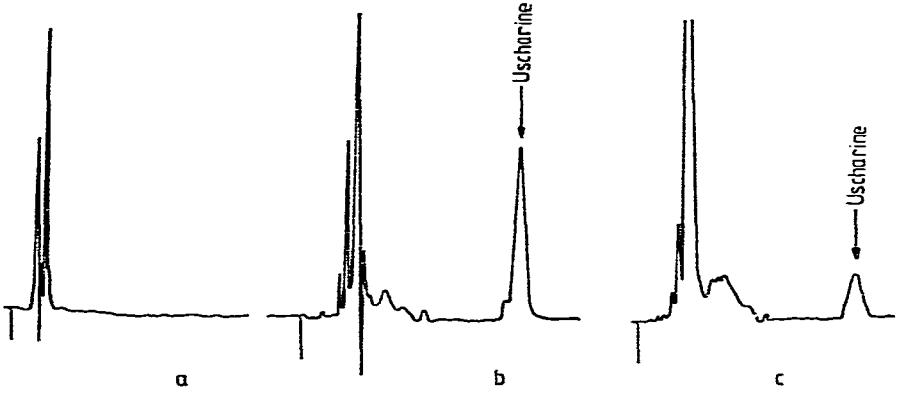

Fig. 3. Analytical chromatograms of three successive fractions of the methylene chloride extract. Stainless-steel column (30 cm × 0.42 cm I.D.); stationary phase, reversed-phase RP C_{18}; particle size, 10 μm; eluent, 40% methanol; flow-rate, 1.8 ml/min; pressure drop over column, 130 atm; capacity ratio (k') for uscharine, 5.0.

Optimal volume of eluent

The volume of liquid in the columns (the column volume), taken as 80% of the volume of the empty columns, was approximately 8 ml.

Two column volumes of each eluent were not sufficient and there was considerable overlap of components in the different eluents. After extraction with three column volumes of all the solvents except water, the extraction was complete. With water the extraction was incomplete even after the passage of six column volumes, probably because the sample was deposited inhomogeneously in the pores of the silica, as discussed above.

An extraction with three column volumes of each solvent, including water, was decided upon, because increasing the volume of water did not appear to improve the quality of the separation.

Comparison of flip-flop and stepwise elution

If the sample was extracted stepwise with the solvent system *n*-heptane–methylene chloride–methanol–water, and using a silica stripping column, then the result was very similar to flip-flop extraction with the important differences that whereas in flip-flop extraction the uscharine was found solely in the methylene chloride, in the stepwise extraction a small amount was also found in the methanol extract.

When the sample was extracted stepwise in the order, water–methanol–methylene chloride–*n*-heptane, and using a reversed-phase stripping column, then there was no separation. Almost everything, including the uscharine, was extracted by the methanol.

In Fig. 4 the chromatograms of the flip-flop and stepwise procedures using water–methanol–methylene chloride–*n*-heptane are compared. Fig. 4a shows a methylene chloride fraction obtained using the flip-flop procedure. In Fig. 4b the corresponding fraction obtained using the stepwise elution system is shown. It can be seen that in this instance uscharine is absent from the methylene chloride fraction. However, as shown in Fig. 4c, the uscharine is extracted together with more polar heart glycosides in the methanol fraction, which is uscharine-free in the flip-flop procedure. In all these instances, equal amounts were collected and injected. The area under the uscharine peak can therefore be directly correlated with the efficiency of the extraction and purification procedure.

Fig. 4. Analytical chromatograms of fractions from flip-flop and stepwise elutions. Analytical conditions as in Fig. 3. (a) Methylene chloride fraction of the flip-flop extraction; (b) methylene chloride fraction of the stepwise extraction; (c) methanol fraction of the stepwise extraction.

The extraction by the flip-flop technique was more complete than for either of the two stepwise methods; 96% (w/w) of the sample could be extracted by the flip-flop method, 76% (w/w) by stepwise elution with solvents of increasing polarity and 88% (w/w) by stepwise elution with solvents of decreasing polarity. In each instance the volume of solvent was three column volumes.

Comparison of different solvent systems

The following four-component solvent systems were compared:

(1) *n*-heptane–water–methylene chloride–methanol;

(2) *n*-propyl chloride–water–methylene chloride–methanol. The only difference is the substitution of *n*-propyl chloride in system 2 for *n*-heptane in system 1.

Two six-component solvent systems were also studied:

(3) *n*-heptane–water–*n*-propyl chloride–acetonitrile–methylene chloride–methyl acetate;

(4) *n*-heptane–water–*n*-propyl chloride–methanol–methylene chloride–methyl acetate. The only difference is the substitution of methanol in system 4 for acetonitrile in system 3.

The best results were obtained using system 1. The *n*-heptane extract contained no glycosides. The water fraction (Fig. 5a) contained large amounts of the more polar glycosides, including possibly calotropine and calotropagenin, but it contained no uscharine. The methylene chloride extract contained all of the uscharine together with smaller amounts of slightly more polar glycosides (Fig. 5b). The methanol extract also contained heart glycosides, according to Kedde's reagent, but was markedly different from the other extracts (Fig. 5c).

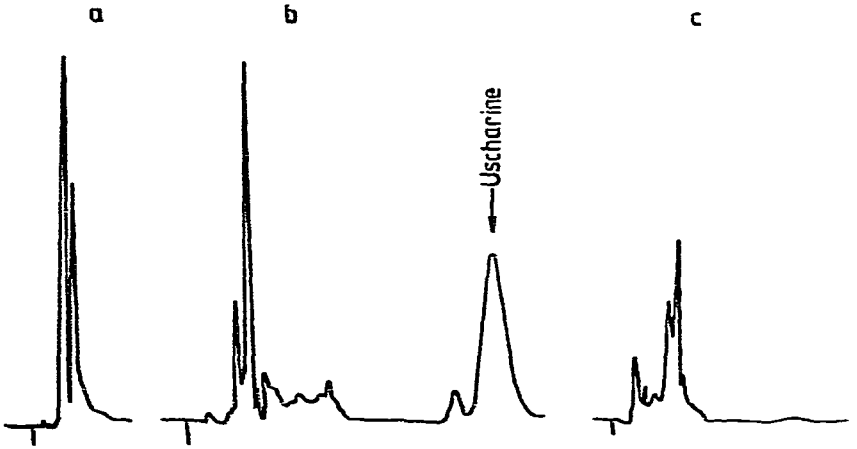

Fig. 5. Analytical chromatograms of the last three fractions obtained with the system *n*-heptane–water–methylene chloride–methanol. Analytical conditions as in Fig. 3. (a) Water fraction; (b) methylene chloride fraction; (c) methanol fraction.

The results obtained using solvent system 2 were very similar to those obtained using system 1, the only difference being that the *n*-propyl chloride extract contained trace amounts of what seemed, by TLC analysis, to be uscharine. This finding, however, could not be confirmed by column chromatography.

Both of the six component systems gave very similar results. In system 3, although TLC indicated the possible presence of uscharine in the *n*-propyl chloride extract as well as large amounts of it in the acetonitrile extract, column chromatography only confirmed its presence together with large amounts of the more polar glycosides in the acetonitrile extract. Solvent system 4 yielded the uscharine together with polar glycosides in the methanol extract.

Strangely, the six-component solvent systems were less efficient at extracting the sample than the four-component systems: system 1 eluted 96% (w/w), system 3, 73% (w/w) and system 4, 87% (w/w). In each instance the sample was eluted with three column volumes of each solvent so that a total of 50% more solvent (by volume) was used in the six-component systems.

Thus, the use of a six-component system gave no better separations than the use of a four-component system. Of the four-component systems, system 1 (*n*-heptane–water–methylene chloride–methanol) is marginally the best.

CONCLUSIONS

Flip-flop extraction of freeze-dried *Calotropis gigantea* sap is better than stepwise extraction using the same solvents. It consistently has a higher extraction efficiency and uscharine is confined to one solvent from which it could, if desired, be easily purified to give pure crystalline uscharine.

The use of stripping columns improves the separation, with the various components being concentrated in fewer fractions in comparison with extraction without the stripping columns.

In this particular separation the use of more than four solvents gives no advantages.

The amount of solvent required is much smaller than is necessary in more conventional extraction procedures.

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