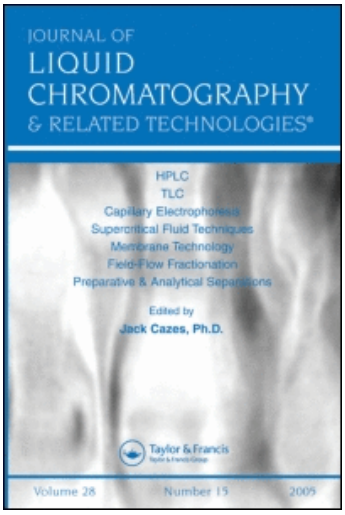

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Separation of Free and Glycosylated Flavonoids from *Siparuna guianensis* by Gradient and Isocratic CCC

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Abstract: The separation of free and glycosylated flavonoids from the ethyl acetate extract of the leaves of *Siparuna guianensis* by step-gradient and isocratic mode HSCCC is described. The initial fractionation of the extract by a two-step gradient composed of Hexane : EtOAc : MeOH : H_2O 0.6 : 4.0 : 0.05 : 1.0 v/v/v/v (A) and 0.6 : 4.0 : 0.7 : 1.0 (B), reverse phase HSCCC, yielded a sequence of a mixture of diglycosyl flavonoids, followed by a mono-glycosyl flavonoid and by quercetin, a free flavonoid. The final separation of the more polar and very closely related (structurally and chemically) glycosylated flavonoids, not easily separable by HPLC, was achieved by isocratic normal phase elution, with the solvent system Hexane : EtOAc : BuOH : MeOH : H_2O, 0.6 : 4.0 : 1.0 : 0.05 : 1.0. The optimization of the solvent ratios for the gradient and for the isocratic elution is discussed.

Keywords: *Siparuna guianensis*, Siparunaceae, Gradient elution, Countercurrent chromatography, Flavonoids

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INTRODUCTION

Flavonoids are phenolic compounds from plants that can be found in free and glycosylated forms. Plant extracts containing these kinds of compounds have great importance, since this class of secondary metabolites displays a large set of biological and pharmacological properties.[1] The separation of bioactive secondary metabolites from crude plant extracts has always been a challenge to natural products researchers, and CCC offers many advantages compared to traditional phytochemical techniques of purification, especially upon those where chromatography with solid supports is used. The main advantage of CCC is that it is a form of liquid-liquid chromatography, which does not use a solid support; there can be no loss of compounds or bioactivity due to interactions between the solid phase and the target compounds. Thus the integrity of the compounds is better maintained, which is an important factor when screening bioactive extracts.[2]

Relatively little attention has been given to gradient elution in CCC; the majority of the work on CCC deals with isocratic analysis.[3–11] Gradient elution in CCC, as in HPLC, offers a broader range of polarity to be covered in one run than in isocratic mode when slight changes of the stationary phase composition do not matter.[10] Extracts that contain free and glycosylated flavonoids have a wide range of polarity and are thus, better suited to gradient elution CCC. Previous investigation on the chemistry of the genus *Siparuna*[13,14] (Siparunaceae) showed that these plants are rich in free and glycosylated flavonoids. This work describes a practical application of how to customize a CCC gradient strategy—the initial separation of free and glycosylated flavonoids from the ethyl acetate extract from leaves of *S. guianensis* by a two-step gradient, composed by four solvents in a reverse phase mode CCC, and the final separation of a narrow polarity band fraction of glycosylated flavonoids by isocratic, five solvent normal phase CCC. The addition of a fifth solvent to improve the separation of two diglycosylated flavonoids, structurally very close, is discussed.

EXPERIMENTAL

Plant Material and Extraction

Siparuna guianensis Aubl. was collected at Crato, Ceará, Brazil, in 1997, by Prof. Dr. Ariane Luna Peixoto (Universidade Federal Rural do Rio de Janeiro), who also identified it. A voucher specimen is deposited at the Herbarium of UFRRJ. The dried and ground leaves of *S. guianensis* were submitted to extraction (maceration) with hexane and ethanol 95°GL, in this order. The ethanolic extract was evaporated under reduced pressure. The crude ethanolic extract (*ca.* 35 g) was dissolved in MeOH : H_2O 1 : 9 v/v and extracted with hexane, dichloromethane, ethyl acetate, and butanol. The ethyl acetate extract yielded 11.7 g of solid material.

Design of the Separations

A small amount of the crude ethyl acetate extract from leaves of *Siparuna guianensis* was dissolved in several different test tubes with the solvent systems described in Table 1. Fractions 8-12, originated from the first fractionation of the ethyl acetate extract (two-step gradient elution CCC), were tested with solvent systems described in Table 2. The test tubes were shaken and the compounds allowed to partition between the two phases. An aliquot of each phase was spotted beside each other, separately, onto a TLC plate. An appropriate solvent system to develop the spots for Table 1 was used (EtOAc : Acetone : H_2O 25 : 10 : 5, organic phase). For Table 2, the development solvent chosen was BuOH : AcOH : H_2O 4 : 1 : 5. The results were visualized after spraying with Folin-Ciocalteu's reagent (Merck, Darmstadt, Germany) for phenolics.

CCC Separation — Gradient and Isocratic Elutions

Two-step Reverse Phase Gradient Elution

The ethyl acetate extract from leaves of *S. guianensis* was fractionated on a CCC apparatus (P.C. Inc) in a two-step gradient (reverse phase) composed

Table 1. Solvent system optimization* for the separation of free and glycosylated flavonoids from the ethyl acetate extract of leaves of *S. guianensis* by gradient elution. A and B are the solvent systems chosen for the two-step, non-linear gradient elution

	Hexane	Ethyl Acetate	Methanol	Water
Optimization of the amount of *Ethyl acetate* in the solvent system				
	1.4	1.0	0.5	1.0
	1.4	2.0	0.5	1.0
	1.4	3.0	0.5	1.0
	1.4	**4.0**	**0.5**	**1.0**
Optimization of the amount of *Hexane* in the solvent system				
	1.0	4.0	0.5	1.0
	0.6	**4.0**	**0.5**	**1.0**
	0.4	4.0	0.5	1.0
Optimization of the amount of *Methanol* in the solvent system				
	0.6	4.0	1.0	1.0
B →	**0.6**	**4.0**	**0.7**	**1.0**
	0.6	4.0	0.4	1.0
	0.6	4.0	0.2	1.0
A →	**0.6**	**4.0**	**0.05**	**1.0**

*Chosen ratios in each optimization appear in bold.

Table 2. Optimization of the amount of Butanol in the solvent system Hexane : Ethyl Acetate : Butanol : Methanol : Water for isocratic elution*

Hexane	Ethyl acetate	Butanol	Methanol	Water
0.6	4.0	0.05	0.05	1.0
0.6	4.0	0.1	0.05	1.0
0.6	4.0	0.2	0.05	1.0
0.6	4.0	0.4	0.05	1.0
0.6	**4.0**	**1.0**	**0.05**	**1.0**
0.6	4.0	2.0	0.05	1.0
0.6	4.0	2.5	0.05	1.0

*Chosen ratios appear in bold.

of A: hexane : ethyl acetate : methanol : water 0.6 : 4.0 : 0.05 : 1.0 v/v/v/v and B: 0.6 : 4.0 : 0.7 : 1.0 as solvent systems, 2 mL/min., lower aqueous phase as mobile phase, at 850 rpm. Solvent A organic phase was used as the stationary phase with the aqueous phase of Solvent A as the initial start mobile phase. The aqueous phase of Solvent B was used as the mobile phase for the second step of the gradient. The 80 mL (PTFE coil with a bore i.d. of 1.6 mm) coil of a triple coil was initially filled with the upper organic phase of solvent system A in a head to tail mode (H → T) and then, after rotation was started, the mobile lower aqueous phase was pumped into the coil until equilibrium was obtained (observed when no further stationary phase elutes and only mobile phase comes out from the coil). The retention of stationary phase for this solvent system was 83% at 850 rpm ($V_S = 66$ mL). At this point, about 200 mg of the ethyl acetate extract from leaves of *Siparuna guianensis* were dissolved in 2.5 mL of lower phase and 2.5 mL of upper phase of the liquid system. The 5 mL were injected at the column head. Fractions of 4 mL were collected. Solvent A was pumped for 20 min (tubes 1 to 10 or 40 mL). Solvent B was used to fill tubes 11 to 26 (60 mL or 30 min). Rotation was then stopped and the column was voided to recover the retained stationary and mobile biphasic solvents and the remaining compounds. The column content extrusion was done pumping 80 mL (one coil volume) of the mobile phase. These fractions were also collected in 4 mL fractions called "wash-off".

Isocratic Normal Phase Elution of the Glycosylated Flavonoids from Fractions 8–12

Fractions 8–12 gave similar TLC plates. So, they were pooled together and the solvents were evaporated. These fractions were further purified in an isocratic CCC normal phase mode. A five solvent system composed of hexane : ethyl acetate : butanol : methanol : water 0.6 : 4.0 : 1.0 : 0.05 : 1.0 v/v/v/v/

v was used. The 80 mL coil was filled with the polar aqueous lower phase, then, with rotation started at 850 rpm, the mobile upper organic phase was pumped at a 2 mL/min flow rate in the tail to head (T → H) direction, until no more excess of stationary phase came out of the coil ($V_m = 11$ mL, $V_S = 69$ mL corresponding to 86% retention of stationary phase). Fractions 8-12 were combined, dried, and re-dissolved in 2.5 mL of the two phases (total volume injected = 5 mL). Twenty-six fractions of 4 mL were collected (104 mL or 52 min). Next, the rotor rotation was stopped and the machine content was extruded to collect the remaining compounds.

HPLC of the Crude Ethyl Acetate Extract from Leaves of *S. guianensis*

The HPLC profile of the ethyl acetate extract of leaves of *S. guianensis* was first performed in a gradient-elution mode (acetonitrile : water, with a linear gradient from 0% → 100% Acetonitrile, 1 mL/min., in 60 min.) on a LaChrom HPLC System (Merck, Darmstadt, Germany) equipped with an interface D7000, pump L-7100, diode array detector (DAD) L-7450[a], and solvent degasser L-7612. The injections were done manually with an injector valve (Rheodyne) equipped with a 20 μL sample loop. A Lichrosorb RP-18 column (Merck, Darmstadt, Germany, 5 μm particle size, 250 × 4.6 mm i.d.) was coupled to a guard column Lichrocart 250-4 HPLC cartridge (Merck). The UV detection was performed with a DAD in an integration range from 240 nm to 260 nm. Further analysis of this extract was performed with the same equipment, flow-rate, and column, but with methanol : water (pH 3.00 with TFA) as eluent, using a linear gradient from a start mixture of 20 : 80 to an intermediate of 80 : 20 over 30 min, and then to 100% methanol over 1 min, which then held isocratic for 5 min (Figure 2). An ultrasonic bath, Thornton model T28220, was used to degas the HPLC solvents (5 min.) and to dissolve the samples.

RESULTS AND DISCUSSION

HPLC Profile of the Crude Ethyl Acetate Extract of Leaves of *S. guianensis*

The ethyl acetate extract of leaves of *S. guianensis* was initially analyzed by HPLC using a gradient of acetonitrile : water, 0 to 100% acetonitrile and a C_{18} column in order to evaluate the polarity range of the compounds in the extract.[12] Figure 1 shows that the position of the sample peaks gives an idea of the sample polarity. Once this polarity is known, the CCC biphasic liquid system can be selected. The HPLC profile obtained in this procedure with the ethyl acetate extract showed two major peaks, at 18.19 min. and

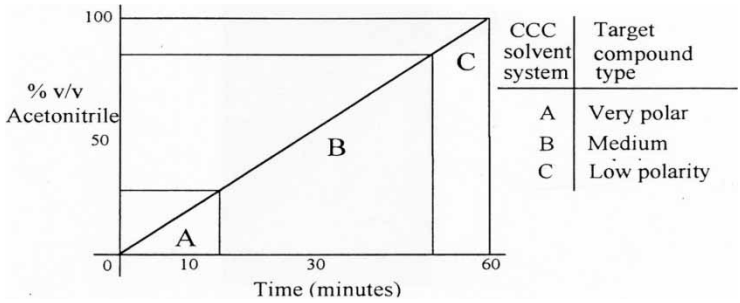


Figure 1. Proposed CCC generic strategy: schematic acetonitrile : water HPLC gradient to show the polarity region for which each CCC solvent system is suitable.

19.68 min (data not shown), with both UV spectra corresponding to quercetin derivatives.[15] The retention times for these two peaks indicated that the compounds in this extract were in a region of medium to high polarity range[12,16,17], suggesting the use of the quaternary solvent system hexane : ethyl acetate : methanol : water for performing CCC separations. The same extract was further analyzed by HPLC with the optimized system of a linear gradient of methanol : water 20 : 80 to methanol : water 80 : 20 over 30 min, and then to 100% methanol (Figure 2), in order to obtain an HPLC profile, which is more like a fingerprint and better defines the composition of the extract.

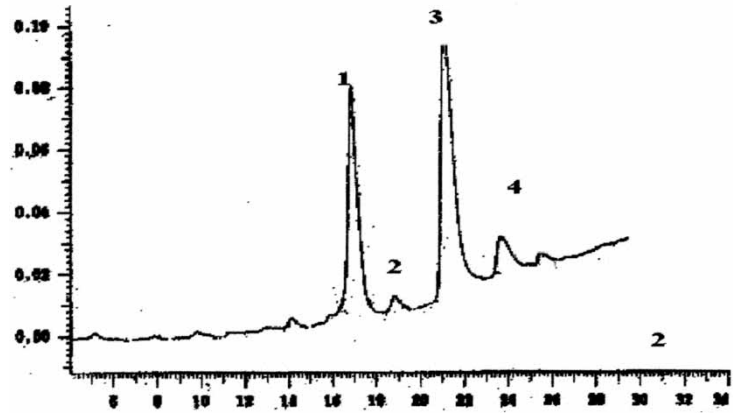
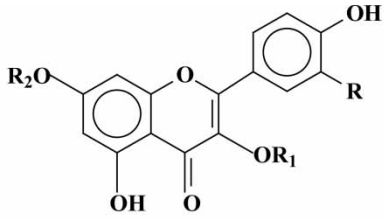


Figure 2. HPLC profile of the crude ethyl acetate extract of leaves of *Siparuna guianensis* (linear gradient of methanol : water (pH 3.00 with TFA) 20 : 80 to 80 : 20 over 30 min, and then to 100% methanol for 5 min on a Lichrosorb RP-18 column, 5 μ, 250 × 4.6 mm i.d., 1 mL/min.). Peak 1 = 16.85 min., mixture of the diglycosylated flavonoids, peak 2 = 18.83 min., rhamnosyl Kaempferol, peak 3 = 21.09 min., Quercetin, peak 4 = 25.65 min., Kaempferol.

Design of the Gradient and Isocratic Solvent Systems

The compounds isolated from this extract were quercetin, 1, a free flavonol, one of the major compounds in the extract (corresponding to the peak at 21.09 min. in Figure 2), and two derivatives of quercetin, 3–*O*-β-D–glucopyranosyl (6 → 1) –rhamnoside, 2, and 7–*O*-β-D–glucopyranosyl (6 → 1)–rhamnoside, 3, (corresponding to the peak at 16.85 min. in the chromatogram of Figure 2). Free Kaempferol, 4, and a Kaempferol monoglycoside (rhamnoside) were also isolated as minor constituents. All compounds were identified by comparison of their ^{1}H and ^{13}C NMR data with those from the literature,[18] and their purity verified by HPLC.



1. Quercetin, R = OH, $R_1 = R_2 = H$
2. Rutin (quercetin 3 – *O* – rutinoside), R = OH, R_1 = β – D – glucopyranosyl (6→1)–rhamnoside, R_2 = H
3. Quercetin – 7 – *O* – rutinoside, R = OH, R_1 = H, R_2 = β – D – glucopyranosyl (6→1)–rhamnoside
4. Kaempferol, $R = R_1 = R_2 = H$

Table 1 shows the steps for the optimization of the solvent systems used in the gradient elution of flavonoids from the ethyl acetate extract of leaves of *S. guianensis.* Chosen ratios in each optimization appear in bold. Test tube experiments from 1 to 3 (Table 1) were done to define both the optimal start ratios and the appropriate reverse phase steps required to cover the sample's polarity range necessary to semi-fractionate the crude plant extract. The aim of these experiments is to set two extreme situations: one in which we have all (or at least the major part of) compounds in the upper phase and the other where we have the opposite situation (all compounds in the lower phase). Table 2 shows the experiments designed to optimize the ratio of the fifth solvent, butanol, in the normal phase solvent system for the isocratic final purification of fractions 8–12 from the gradient separation.

In order to establish the ratios of individual solvents in the quaternary solvent system, so as to define the reverse phase gradient, a set of different solvent ratios was tested. The first set of experiments in Table 1 show the ratios of 1.4 : 1.0 : 0.5 : 1.0 for the basic solvent system Hexane : EtOAc : MeOH : H_2O, chosen for the purification of the extract.

The TLC results of this first test tube experiment showed that, except for quercetin, all compounds remained at the aqueous phase. The amount of ethyl acetate on the solvent system was next raised from 1.0 to 4.0 so that the polar compounds begin to go from the aqueous to the organic phase as the polarity of the organic phase sequentially increased. At the ratio of Hex : EtOAc : MeOH : H_2O of 1.4 : 4.0 : 0.5 : 1.0, this situation began to be improved but the amount of hexane in the solvent system is still too high, pushing the more polar compounds into the aqueous phase. So, in the next set of experiments, the amount of hexane in the solvent system is gradually lowered from 1.4 to 0.4 (Table 1). The ratio of 0.6 of hexane was optimal as all quercetin is in the upper organic phase while the glycosylated derivatives start to partition between the two phases. With 0.4 of hexane, quercetin is again partitioned between the two phases. This mixture is near the extreme of polarity of the organic layer for this particular solvent system, as further increase of ethyl acetate or decrease of hexane will result in the solvent ceasing to be biphasic and thus, becoming unsuitable for CCC. To further change the relative polarities of the organic and aqueous layers, the composition of the aqueous layer is optimized. The amount of methanol was next optimized in order to extract, selectively, the compounds from the upper organic phase (Table 1). This last set of experiments for the gradient elution showed that, with a ratio of 1.0 of MeOH, all compounds were in the lower aqueous phase. As we lowered this ratio, quercetin starts to go into the upper organic phase (practically all in the organic with 0.2 MeOH), along with the more polar derivatives (glycosyl derivatives), which start to be partitioned in the two phases with 0.4 MeOH. At this point, we set the gradient as hexane : ethyl acetate : methanol : water 0.6 : 4.0 : 0.05 : 1.0 → 0.6 : 4.0 : 0.7 : 1.0.

Figure 3 (A and B) shows the TLC results of the reverse gradient and normal isocratic elutions of the fractionation of the ethyl acetate extract of leaves of *Siparuna guianensis.* It can be seen at Figure 3A that, at the first step of the experiment, the flavonoid-rich extract was separated according to the flavonoids' polarity into 3 fractions, one composed by the mixture of more polar derivatives (diglycosylated flavonoids, tubes 8–12), the next composed by the mono-glycosyl derivative, e.g., rhamnosyl-quercetin (fractions 14–22), and the last one composed mainly by quercetin (tubes 32–50). Analysis of the last fractions (tubes 44–50) by NMR revealed a small amount of free kaempferol, a minor constituent of this extract, along with quercetin. The mixture of polar glycosylated flavonoids (Fractions 8–12) was purified on a normal phase isocratic mode, when butanol was introduced in the previous solvent system. The test tube experiments for the optimization of the solvent system can be seen in Table 2. Brown and coworkers[12,16,17] discussed the differences in selectivities of reverse and normal phase in CCC, and the benefits of adding a fifth solvent modifier with similar functionality to the target compounds to aid resolution in CCC. So, butanol was added to the system to give a better partition of the

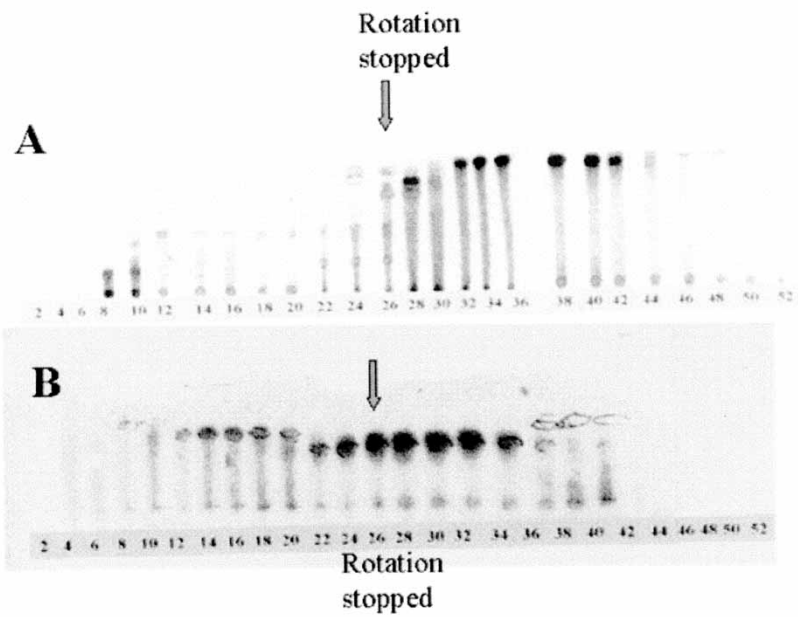


Figure 3. A — TLC results of the gradient elution — two step, four solvent, reverse-phase, non-linear gradient elution HSCCC of the ethyl acetate extract of leaves of *Siparuna guianensis.* Solvent system hexane : EtOAc : MeOH : H_2O 0.6 : 4 : 0.05 : 1 → 0.6 : 4 : 0.7 : 1. First step gradient was pumped from tubes 1–10 (Diglycosylated flavonoids, tubes 8–12) and second step from tubes 11–26 (rhamnosyl-quercetine, tubes 14–22 and quercetin, tubes 32–50). Developing solvent for TLC : EtOAc : acetone : H_2O 25 : 10 : 5. B – TLC result of the isocratic elution — five solvent, normal phase, isocratic separation of fractions 8–12 from Step 1. Solvent system: hexane : EtOAc : BuOH : MeOH : H_2O 0.6 : 4.0 : 1.0 : 0.05 : 1.0. Rhamnosyl-kaempferol, tubes 12–21; quercetin-7-*O*-rutinoside, tubes 22–25 and rutin, (quercetin–3-*O*–rutinoside), tubes 26–35. Developing solvent for TLC: BuOH : AcOH : H_2O 4 : 1 : 5. Both plates were sprayed with Folin Ciocalteu's Reagent for phenolics. Dark spots indicate positive results.

glycosylated derivatives in the two phases. With the ratios of 0.05 and 0.1 of BuOH, all compounds remain in the lower aqueous phase. With increasing amounts of BuOH, compounds start to partition between the two phases, the optimal situation being 1.0. Noteworthy, are the ratios 2.0 and 2.5 mL of BuOH: the compounds go again into the lower aqueous phase. As the purpose of the first step gradient was to heart-cut compounds of very similar polarity, there is rarely a need for anything other than an isocratic normal phase CCC, in the second stage, to obtain high purity compounds. The introduction of a second alcohol (butanol) in the solvent system yielded a better partition of the glycosylated derivatives in this system, affording the isolation, in a pure form, of the di-glycosylated derivatives quercetin-7-

O-rutinoside (tubes 22–25), with a distribution ratio K of approximately 1.3, and rutin, (quercetin–3-*O*–rutinoside, tubes 26–35), as well as rhamnosyl-kaempferol (tubes 12–21), with a K value near 1. The results demonstrate the enhanced selectivity given by butanol on this solvent system, allowing the separation of the two di–*O*-glycosylated flavonols, which were not easily separated by HPLC (Figure 2).

CONCLUSIONS

The CCC technique described here is shown to be able to initially screen a wide polarity crude plant extract into narrow polarity band by means of a four solvent two-step gradient elution. The separation of very similar compounds from a narrow polarity band of interest can then be made by logical addition of a fifth solvent to the pre-defined four solvents system, and a change from reverse to normal phase CCC operation may yield high purity compounds. Optimization of the solvent system composition using TLC separations was possible, but the authors caution that for certain CCC solvent systems, TLC may not be suitable for pre-determination of CCC gradients. Trial low volume HSCCC runs on a small (5 to 15 mL) coil of the same i.d. bore on a similarly configured HSCCC instrument will need to be carried out to obtain a good idea of the sample separation with the tested biphasic liquid system.

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