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SEPARATION OF NATURAL PRODUCTS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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SUMMARY

Centrifugal liquid–liquid partition chromatography (CPC) with a Sanki CPC apparatus has been used to perform efficient separations of various classes of natural products. Applications involving flavonoids, saponins, coumarins, anthraquinones, phenolic acids and naphthoquinones are described. Some of the examples concern isolation of pure compounds from crude plant extracts. CPC is also compared with other counter-current chromatography techniques.

INTRODUCTION

Centrifugal partition chromatography (CPC) relies on the principle of liquid–liquid partition for the separation and purification of compounds. The technique differs from that of Craig counter-current distribution and droplet counter-current chromatography (DCCC)[1] in the application of a centrifugal force in the course of separation, which results in time saving. CPC or centrifugal counter-current chromatography (CCCC), as it is also known, is a continuous process of non-equilibrium partition between two immiscible phases contained in rotating coils[2] or cartridges[3]. The cartridge system[3] is a recently introduced technique which has, until now, found relatively little application[4]. Most work has been carried out on tannins[5], but examples of separations also include the purification of labile tunichromes[6] and the isolation of retinals[7]. We report here an extension of the applications of CPC to a diverse range of natural products. Separations with the Sanki cartridge CPC system were performed, *e.g.*, on flavonoids, saponins, coumarins, anthraquinones and naphthoquinones.

Centrifugal partition chromatography does not rely on solvent systems which have to form droplets, as in DCCC. With few exceptions, most solvent mixtures which form two immiscible layers are acceptable. While the choice of solvent system cannot definitely be determined by thin-layer chromatography (TLC), as is the case with DCCC[8], preliminary results indicate that for optimum separations, the R_F of the compounds to be separated should lie in the range 0.2–0.5 when TLC is carried out with the mobile phase of the solvent system on silica gel plates. In most (but not all) cases, a mixture which can be resolved by TLC is also resolved by CPC. In some instances, CPC provides the resolution of a mixture that cannot be resolved by silica gel TLC.

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Representative solvent systems include: chloroform–methanol–water (33:40:27) [for polar substances]; ethyl acetate–94% ethanol–water (2:1:2) [for polar substances]; light petroleum (b.p. 60–95°C)–ethyl acetate–methanol–water (18:42:30:30) [for non-polar substances[9]]; hexane–acetonitrile–methanol (40:25:10) [for non-polar substances]. The choice of these solvents is based on TLC investigations of the particular sample to be separated (to get a first idea of the suitability of a two-phase system), as well as literature references. For example, the system ethyl acetate–94% ethanol–water (2:1:2) has previously proved suitable for the rotation locular counter-current chromatography (RLCC) separation of saponins[10] and is here used for the separation of flavonoid glycosides.

EXPERIMENTAL

All separations were carried out at 20°C on a CPC Model LLN (Sanki Engineering, Kyoto, Japan), connected to a 2238 Uvicord SII detector (254 nm) (LKB, Bromma, Sweden), 600 chart recorder (W + W Scientific, Basle, Switzerland) and an LKB Ultrorac II fraction collector. The continuous-flow centrifuge was fitted with six Type 250W cartridges (total volume 125 ml). Samples were injected by means of a six-way valve and a 3-ml sample loop. Monitoring of fractions was carried out either by UV at 254 nm or TLC on silica gel aluminium-backed plates (Merck, Darmstadt, F.R.G.). The apparatus was first filled with stationary phase and then the mobile phase was pumped through. When elution of the stationary phase was complete and mobile phase exited from the cartridges, the sample was introduced.

With the Sanki CPC Model LLN apparatus, the speed of the centrifuge rotor can be regulated. Higher rotation speeds generally lead to a better resolution of the sample but at the same time increase pumping pressures. Therefore, speeds of 1000–1500 rpm are desirable for carrying out efficient separations at a relatively high flow-rate of mobile phase, keeping analysis times short. The viscosity of the mobile phase is important, however, and for chloroform-containing solvents, rotation speeds and flow-rates have to be diminished in order to avoid pressure build-up. The choice of parameters is thus a rather empirical process, the aim being to increase both the rotation speed and flow-rate (via higher pump speeds) without causing overpressure problems or loss of resolution.

RESULTS

Separation of flavonoid aglycones

Fig. 1 shows the separation of the flavanone hesperetin (1) and the flavonols kaempferol (2) and quercetin (3), with the same solvent system, chloroform–methanol–water (33:40:27) (descending) for (a) RLCC[11], (b) DCCC[11] and (c) CPC. In each case, elution was according to the order of increasing polarity: hesperetin, kaempferol, then quercetin. Whereas DCCC and RLCCC required more than 30 h for complete separation, CPC took only 2.5 h. The solvent consumption for RLCCC was *ca.* 1500 ml, for DCCC *ca.* 550 ml and for CPC *ca.* 300 ml. The low solubility of the flavonoid aglycones limited the sample size in these examples.

The Sanki CPC system offers the possibility to work in the reversed-phase mode. Thus, during separation, the elution mode can be changed, while simultaneously

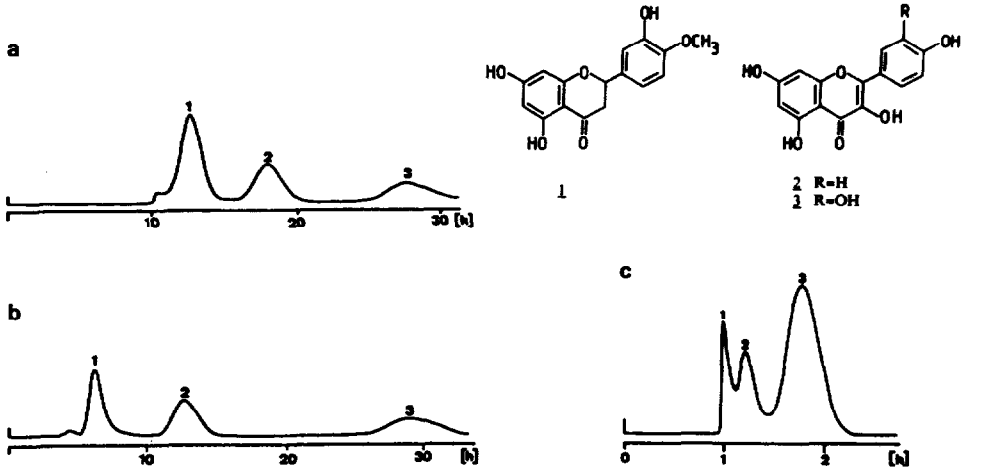

Fig. 1. Counter-current chromatography of hesperetin (1), kaempferol (2) and quercetin (3). Solvent system: chloroform–methanol–water (33:40:27), descending mode. Detection: 254 nm. (a) RLCC separation. Flow-rate, 48 ml/h. (b) DCCC separation. Flow-rate, 18 ml/h. (c) CPC separation. Flow-rate, 2 ml/min. Rotational speed: 600 rpm. Sample: 6 mg in 1 ml of upper phase and 1 ml of lower phase.

changing the phases. An example of this very useful technique is shown in Fig. 2. With chloroform–methanol–water (33:40:27) in the ascending mode, and the aqueous phase as the mobile phase (Fig. 2a), a good separation of the three flavonoid aglycones was obtained. Starting the separation in the ascending mode and then reversing the modes, *i.e.*, changing to the descending mode and eluting with the lower phase of the system, after elution of aglycone 3, produced a much more rapid separation of compounds 1 and 2 (Fig. 2b). However, in this example, aglycone 1 was eluted before aglycone 2.

Separation of flavonoid glycosides

A mixture of rutin (4), hyperoside (5) and quercitrin (6) was resolved by CPC with ethyl acetate–94% ethanol–water (2:1:2) in the descending mode (Fig. 3). The quantity of stationary phase eluted before the appearance of mobile phase (and injection of the sample) was 70 ml. Baseline separation was achieved within 2.5 h,

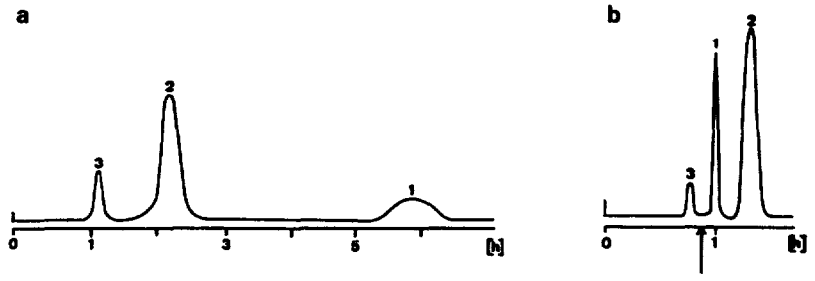

Fig. 2. Separation of hesperetin (1), kaempferol (2) and quercetin (3) by CPC. Conditions as in Fig. 1. (a) Ascending mode. Flow-rate, 1 ml/min. (b) Ascending mode to 55 min. After 55 min, descending mode with the organic phase as the mobile phase. Flow-rate, 1.6 ml/min.

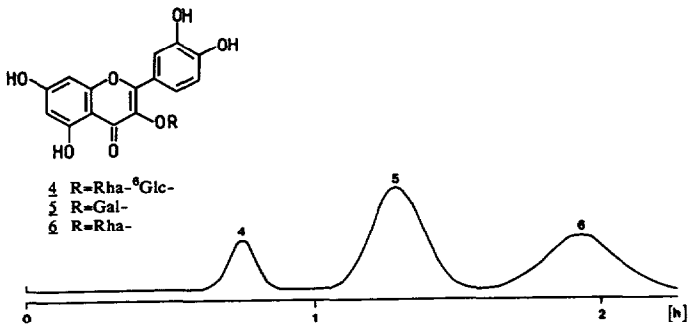

Fig. 3. Separation of rutin (4), hyperoside (5) and quercitrin (6) by CPC. Solvent system: ethyl acetate–94% ethanol–water (2:1:2); descending mode; flow-rate, 3 ml/min. Rotational speed: 1500 rpm. Detection: 254 nm. Sample: 15 mg in 1 ml of upper phase and 1 ml of lower phase. Rha = Rhamnose; Glc = glucose; Gal = galactose.

whereas DCCC separation of the mixture required 9 h[12]. However, direct comparison with the DCCC method was impossible because separation by CPC with the DCCC solvent system chloroform–*n*-butanol–methanol–water (10:1:10:6) (ascending) gave problems of overpressure.

Flavonoid glycosides were separated from sugars and less polar constituents when a methanol extract of *Tephrosia vogelii* (Leguminosae) leaves was subjected to CPC (Fig. 4). There was also partial resolution of rutin (4), isoquercitrin (7) and

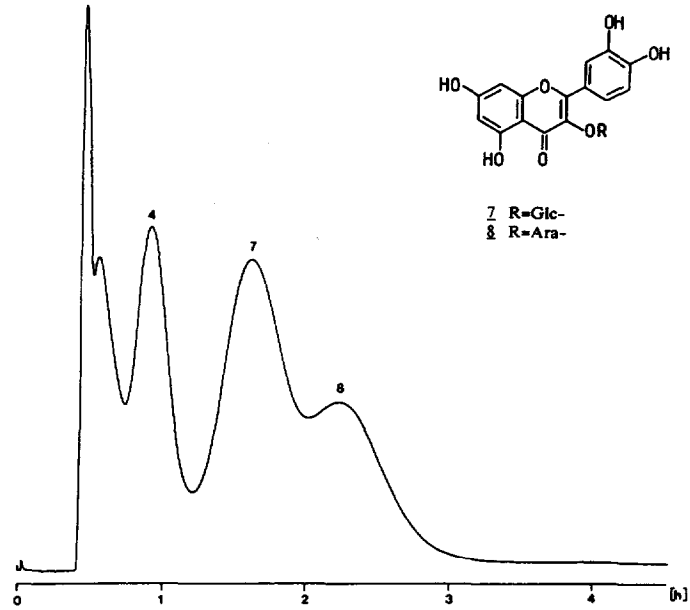

Fig. 4. CPC of a crude extract of *Teprhosia vogelii* leaves. Solvent system as in Fig. 3. Flow-rate: 2.2 ml/min. Rotational speed: 1000 rpm. Detection: 254 nm. Sample: 100 mg of extract in 1 ml of upper phase and 1 ml of lower phase. Ara = Arabinose.

quercetin 3-O-α-L-arabinopyranoside (8) with ethyl acetate–94% ethanol–water (2:1:2) (descending)[13]. It is noteworthy that in both the examples of Figs. 3 and 4, separation of glycosides differing only in the nature of their monosaccharide moieties was achieved.

Separation of triterpene glycosides

Counter-current chromatographic techniques, and especially DCCC, have proved of immense value for the separation of very polar saponins[4]. One example is the isolation of four pure triterpene glycosides from a molluscicidal methanolic extract of *Hedera helix* (Araliaceae) berries by DCCC[8]. The extract was first partitioned between *n*-butanol and water; the molluscicidal *n*-butanol layer was directly subjected to CPC, with the lower layer of a chloroform–methanol–water (7:13:8) mixture as the mobile phase (Fig. 5). The fractions were monitored by TLC. The hederagenin saponins 9–12 were separated within 2 h. Changing the elution mode to ascending after fraction 30, with the upper phase as the mobile phase, enabled two more polar saponins to be eluted in fractions 33 and 35. Fractions 31–34 also contained large quantities of polysaccharide material.

Saponins 9–12 have also been separated by DCCC using the same solvent system[14]. However, this method required 20 h to give the same result.

Separation of coumarins

Attempts to separate the coumarins herniarin (13), umbelliferone (14) and scopoletin (15) with the solvent system chloroform–methanol–water (13:7:8), pre-

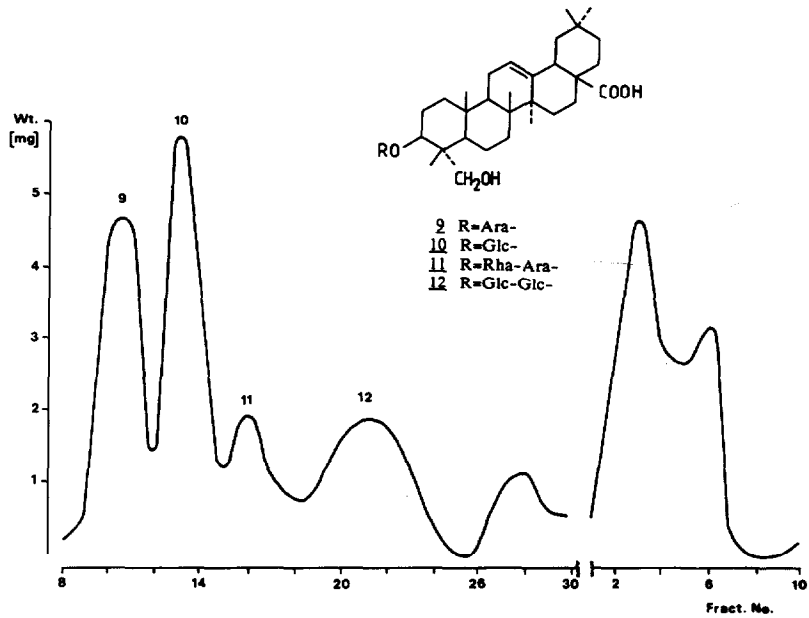

Fig. 5. CPC of a methanol extract of *Hedera helix* berries after *n*-butanol–water partition. Solvent system: chloroform–methanol–water (7:13:8); descending mode; flow-rate, 1.5 ml/min. Rotational speed: 700 rpm. Sample: 100 mg of extract in 1 ml of lower phase.

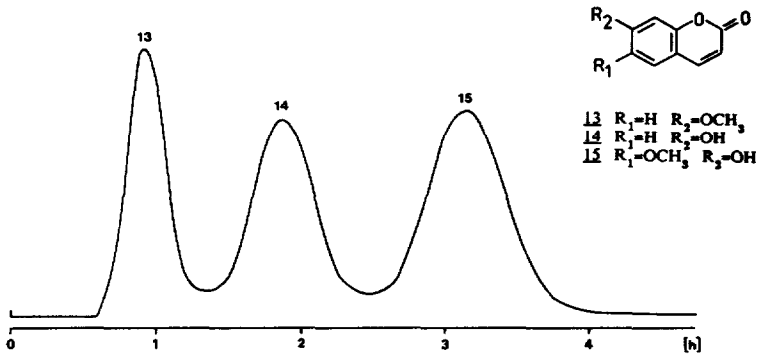

Fig. 6. CPC separation of herniarin (13), umbelliferone (14) and scopoletin (15). Solvent system; light petroleum (b.p. 60–95°C)–ethyl acetate–methanol–water (18:42:30:30); ascending mode; flow-rate, 1.7 ml/min. Rotational speed: 800 rpm. Detection: 254 nm. Sample: 9 mg in 1 ml of upper phase and 0.2 ml of lower phase.

viously used in DCCC[12], gave unsatisfactory results. In contrast, elution with light petroleum (b.p. 60–95°C)–ethyl acetate–methanol–water (18:42:30:30) in the ascending mode resulted in a baseline separation of all three components of the mixture (Fig. 6).

Separation of phenolic acids

Baseline separation of cinnamic (16), ferulic (17) and caffeic (18) acids by CPC was possible with light petroleum–ethyl acetate–methanol–water (18:42:30:30) in the ascending mode (Fig. 7). Thus, the same system was capable of separating both coumarin and phenolic acid mixtures. All three acids were eluted within 2.5 h, with the least polar acid (16) being the first to emerge from the chromatograph and the most polar acid (18) being the last to exit.

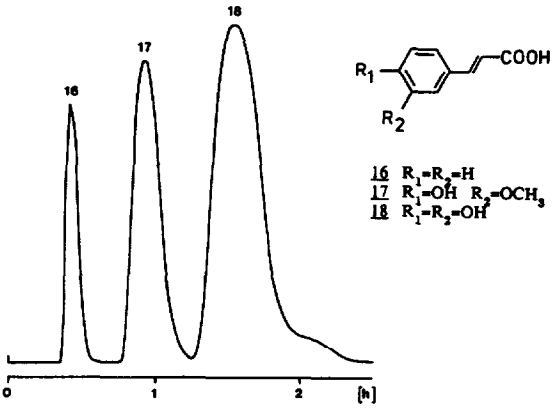

Fig. 7. CPC separation of cinnamic (16), ferulic (17) and caffeic (18) acids. Solvent system as in Fig. 6. Flow-rate: 3.2 ml/min. Rotational speed: 1000 rpm. Detection: 254 nm. Sample: 9 mg in 1 ml of upper phase and 0.2 ml of lower phase.

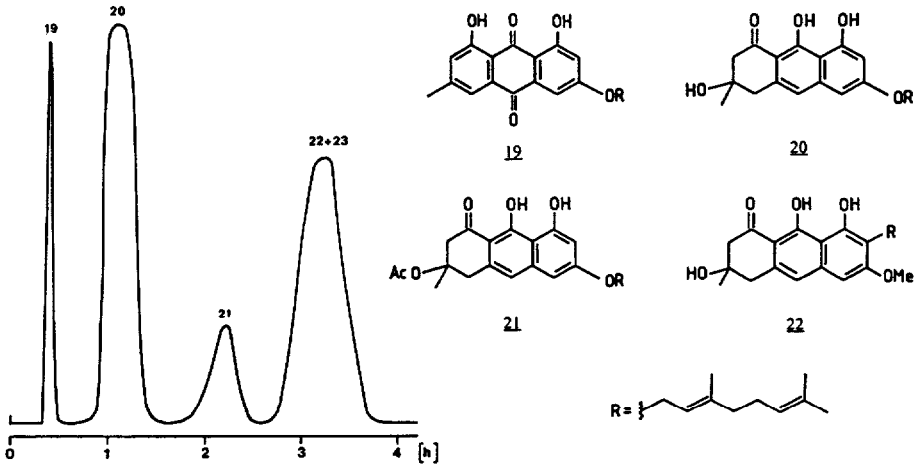

Fig. 8. CPC separation of a light petroleum extract of *Psorospermum febrifugum*. Solvent system: hexane–acetonitrile–methanol (40:25:10); ascending mode; flow-rate, 5.5 ml/min. Rotational speed: 1500 rpm. Detection: 254 nm. Sample: 100 mg in 1 ml of upper phase and 1 ml of lower phase. Ac = Acetyl; Me = methyl.

Separation of anthranoid pigments

The light petroleum extract of the root bark of *Psorospermum febrifugum* (Guttiferae) contains a mixture of anthraquinone, anthrone and tetrahydroanthracene pigments, some of which have strong cytotoxic activities[16]. Separation of these constituents by multi-step flash chromatography and low-pressure reversed-phase

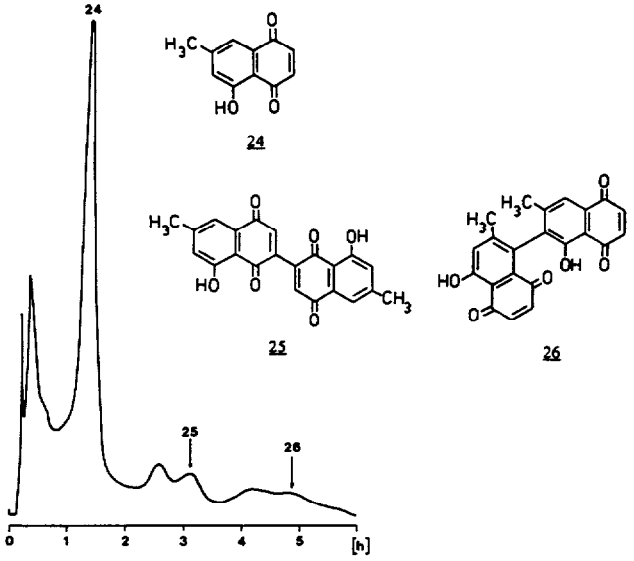

Fig. 9. CPC of a light petroleum extract of *Diospyros usambarensis*. Solvent system as in Fig. 6. Flow-rate: 5.5 ml/min. Rotational speed: 1400 rpm. Detection: 254 nm. Sample: 100 mg in 1 ml of upper phase and 1 ml of lower phase.

liquid chromatography resulted in considerable losses, due to irreversible adsorption on the sorbents. On the other hand, a single CPC step was used to obtain directly three pure compounds (19–21) and a mixture of two anthranoids (22 and a minor component, 23) (Fig. 8), *without* loss of product. With the solvent system hexane–acetonitrile–methanol (40:25:10) in the ascending mode, a 100-mg quantity of the extract was reasonably well separated within 4 h.

Naphthoquinones

A crude light petroleum extract of *Diospyros usambarensis* (Ebenaceae) root bark was injected into the CPC apparatus, and after passage of the organic phase of a light petroleum–ethyl acetate–methanol–water (18:42:30:30) system, 28 mg of pure 7-methyljuglone (24) were obtained (Fig. 9). Furthermore, a partial separation of the naphthoquinone dimers mamegakinone (25) and isodiospyrin (26) was possible.

The liquid–liquid partition one-step isolation method for 7-methyljuglone has certain advantages over chromatographic methods on solid sorbents[16]. Not least of these is the avoidance of losses arising from irreversible adsorption of the naphthoquinone on the packing material. In addition, the elution of compound 24 was complete within 1.5 h.

CONCLUSION

Centrifugal partition chromatography has proved to be a very useful addition to the existing array of liquid–liquid separation techniques. The application of centrifugal force fields to counter-current chromatography drastically shortens the time required for preparative separations of complex mixtures, when compared with DCCC, RLCC, etc. In the absence of a solid sorbent there is no sample loss and, hence, total recovery of injected mixtures. Contamination from solid sorbents is also avoided and there is only minimum decomposition or denaturation of sensitive compounds. Experience with the Sanki CPC apparatus has shown that the resolution is as high as in DCCC and that separations of the order of 100 mg are possible. Mixtures require less than 1 l of mobile phase for total elution and the method is thus inexpensive. Unlike DCCC, droplet formation is not a prerequisite for the choice of solvent systems.

The results presented here demonstrate a variety of separations of natural products, including both polar and non-polar substances: saponins, flavonoids, naphthoquinones, anthranoids and coumarins. Thus, CPC provides a convenient complement to adsorption chromatography. In addition to the features listed, the Sanki CPC instrument is capable of being operated in the reversed-phase mode. This is well illustrated in Fig. 2b by the separation of flavonoids. Changing the elution mode enables a much more rapid elution of hesperetin than would otherwise be possible (Fig. 2a). This leads not only to time saving but also reduces the volume of solvent required.

Experiments are presently underway to extend the range of substances separated by CPC and to investigate the maximum sample loads that may be injected.

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