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Salting-out gradients in centrifugal partition chromatography for the isolation of chlorogenic acids from green coffee beans

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ABSTRACT

In addition to sample solubility constraints, the use of polarity gradients in normal-phase centrifugal partition chromatography (CPC) for the purification of complex mixtures is also limited by the instability of biphasic systems as a consequence of dramatic changes in the settling times along the gradient, leading in many cases to column bleeding when working under maximum efficiency conditions. In this paper an electrostriction approach is proposed as a strategy in reversed-phase CPC to fractionate intermediate polarity extracts in a single run by bringing its components into the "sweet spot" in a controlled fashion through a stepwise reduction of salt concentration in the aqueous mobile phase. The salting-out gradient method was successfully tested with the separation of the major chlorogenic acids (CGAs, hydroxycinnamoylquinic acids) present in green coffee beans (5-caffeoylquinic acid (5-CQA), 5-feruloylquinic acid (5-FQA) and 3,5-dicaffeoylquinic acid (3,5-diCQA)) using ethyl acetate-hexane as the stationary phase and an ionic gradient of LiCl (5.0, 2.5 and 0.1 M) as the mobile phase in one case and $(NH_4)_2SO_4/KNO_3$ (3.0 and 1.5 M/1.5 M) in another. Regioisomers of each chlorogenic acid obtained by base-catalyzed isomerisation were also separated by CPC using isocratic elution. The best resolution for both FQAs and diCQAs was achieved with a chloroform–*n*-butanol–0.01 M pH 2.5 phosphate buffer (84:16:100; v/v) system, while CQAs were best isolated using chloroform–*n*-butanol–0.01 M pH 2.5 phosphate buffer/5.0 M LiCl (82:18:100; v/v).

1. Introduction

The liquid nature of the stationary phase in counter-current separations (CS) confers an indisputably greater versatility to this technique as compared to HPLC. This unique feature has led to a fascinating new spectrum of instrumentation setups, column designs, separation methods and polarities which have been successfully applied and there is still the potential for further developments. From the preparative point of view this characteristic makes CS even more powerful as it enables the usage of the stationary phase to increase the solubility of analytes, thus allowing the loading of highly concentrated complex samples, e.g. plant extracts, into the separation system for fractionation or isolation purposes. However, in contrast to adsorption chromatography, polarity elution gradients used for sample fractionation are not very common in CS due to the significant changes in the physical properties of the mobile phase associated to the change in solvent composition, which can lead to the instability of the biphasic system producing inconvenient column bleeding. Several ingenious strategies have been developed, however, to solve this practical downside that limits the competitiveness of CS. The most common is perhaps the elution-extrusion mode [1–3], in which the stationary phase containing retained analytes is entirely pumped out of the system preserving its separation pattern. The alternation of the mobile/stationary roles between the upper and lower phases of the solvent system is another widespread elution method, first introduced as the dual-mode in high-speed counter-current chromatography (HSCCC) [4] and later on as a multiple dual-mode in centrifugal partition chromatography (CPC) [5]. Nevertheless both polarity range and chromatographic resolution of CS could be increased with the implementation of elution gradients. As an alternative to a polarity gradient an electrostriction or salting-out gradient is used in this case to control the distribution constant (D) of analytes and hence their relative elution times or resolution.

Salting-out is a very common but not simple physical phenomenon extensively exploited by biopolymer science and ion-exchange chromatography. It has also been applied in CS separations, specifically by Ito [6], using a gradient elution CPC method for the selective precipitation of proteins called centrifugal precipitation chromatography, and at single salt concentrations to either stabilize the solvent system or to adjust D values.

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In the method described in this paper the electrostriction effect is achieved by a stepwise reduction of the ionic strength of the mobile phase to separate the different types of chlorogenic acids (CGAs) present in green coffee beans in a reversed-phase mode.

Although the term chlorogenic acid originally referred exclusively to 5-*O*-caffeoylquinic acid (5-CQA, Fig. 1), nowadays it is often used to refer to the whole family of *trans* cinnamoylquinic esters, one of the most important groups of phenolics in the plant kingdom particularly abundant in many foods and medicinal herbs. These catechol-like compounds have a broad range of proven and purported biological activities such as anti-bacterial [7] anti-fungal [8], anti-insect [9], anxiolytic [10], hepatoprotective [11], anti-thrombotic [12] and anti-viral [13], including HIV inhibition [14]. Their extensively documented antioxidant properties render them beneficial against several oxidative stress-related conditions such as atherosclerosis [15], cancer [16] and Alzheimer's disease [17]. Ubiquitous compounds such as these always have very crucial functions in Nature. Surprisingly, however, in spite of the vast amount of research conducted on them some of their roles are not yet fully understood. The dietary importance of catechol-like polyphenolics (flavonoids and chlorogenic acids) for instance is still quite controversial. Some recent studies claim that their healthy effects on humans do not result from their antioxidant properties but instead from their toxicity [18] which triggers the physiological production of the endogenous actual antioxidant uric acid [19].

Chlorogenic acid, 5-CQA, has been previously isolated from *Flos lonicerae* by CS, using multiple step isocratic HSCCC (high-speed counter-current chromatography) [20] and from *Lonicera japonica* with a simple pH-gradient CCC (counter-current chromatography) method [21]. However, no attempt has been made to neither isolate the compounds belonging to the different CGA subfamilies, which are concomitantly present in most sources, nor separate their regioisomers. Apart from 5-CQA and cynarine (1,3-dicaffeoylquinic acid) these conjugates and their isomers are not commercially available as reference standards and whenever required, for structure-activity relationship studies for instance, they have to be either isolated by tedious conventional column chromatographic procedures [22,23] or obtained in limited amounts through preparative HPLC. Since coffee is the richest and most readily available source of chlorogenic acids a good alternatively method which uses preparative CPC for their purification from green coffee beans was developed.

2. Experimental

2.1. Reagents

Chlorogenic acid (97%) and all inorganic salts were purchased from Sigma (St. Louis, MO, USA). Solvents (AR for extraction/CPC and HPLC grade) including formic and acetic acid were purchased from Merk Biosolve (Valkenswaard, The Netherlands), those used for extractions and CPC were of analytical grade but methanol and acetonitrile used for HPLC were of chromatographic grade. Ultra-pure deionised water was used. Green coffee beans of Ethiopian *Coffea arabica* tipica var. were purchased locally (Leiden, The Netherlands).

2.2. Apparatuses

Centrifugal partition chromatography experiments were performed with a hydrostatic counter-current chromatograph model HPCPC LLB-M (Sanki Engineering, Kyoto, Japan) equipped with a 4-way ascending-descending-mode switching valve and a rotor of 110 ± 3 mL as total capacity operating at a maximum speed of 2000 rpm. This CPC system was connected to a Knauer 10 mL (Knauer, Berlin, Germany) pump and a Rheodyne (Cotati, CA, USA) manual injector with a 5 or 30 mL loop. The eluate was monitored at 330 nm with a CPC UVIS 200 (Sanki Engineering) UV-Vis detector and a Kipp&Zonen BD40 (Kipp&Zonen, Delft, The Netherlands) recorder. HPLC analyses were performed on an Agilent 1200 series system comprising an auto sampler, low-pressure mixing pump and a diode array detection (DAD) system.

2.3. Green coffee bean extract

The major CGA in green coffee beans is by far 5-CQA, with a relative concentration of around 72% on dry basis. This is followed by diCQAs (dicaffeoylquinic acids) with 17% and 5% FQAs (feruloylquinic acids). Diverse CFQAs (caffeoylferuloylquinic acids) and triCQAs (tricaffeoylquinic acids) account for the remaining 6%. The extraction method described below was developed with the aim of obtaining an extract enriched in the two minor subfamilies of compounds, i.e. the FQAs and the diCQAs, ideal for the successive chromatographic steps. The best protocol worked out for this purpose was the following: green beans of Ethiopian *Coffea arabica* tipica var. (250 g) were soaked overnight in acidic water (300 mL, pH 4,0) and then ground and extracted with 80% EtOH (3×500 mL). The resulting extract was taken to dryness under vacuum and the residue was redissolved in 50% MeOH (200 mL) and defatted with hexane (3×200 mL). Methanol was removed by evaporation under vacuum. The remaining water extract was diluted to a volume of 250 mL with sufficient amount of 0.1 M pH 2.0 phosphate buffer and 2.0 M LiCl. This extract was partitioned against EtOAc (2×250 mL) and the organic phases were combined and taken to dryness, yielding 10 g of the crude CGAs blend. This residue was subsequently redissolved in hot water (200 mL), washed twice with $CHCl_3$ to remove caffeine and extracted twice with $CHCl_3$/18% BuOH. The resulting extract was taken to dryness yielding a residue (3.4 g) composed of about 60% CGAs, of which, CQAs represented around 41%, FQAs 21% and diCQAs 38%.

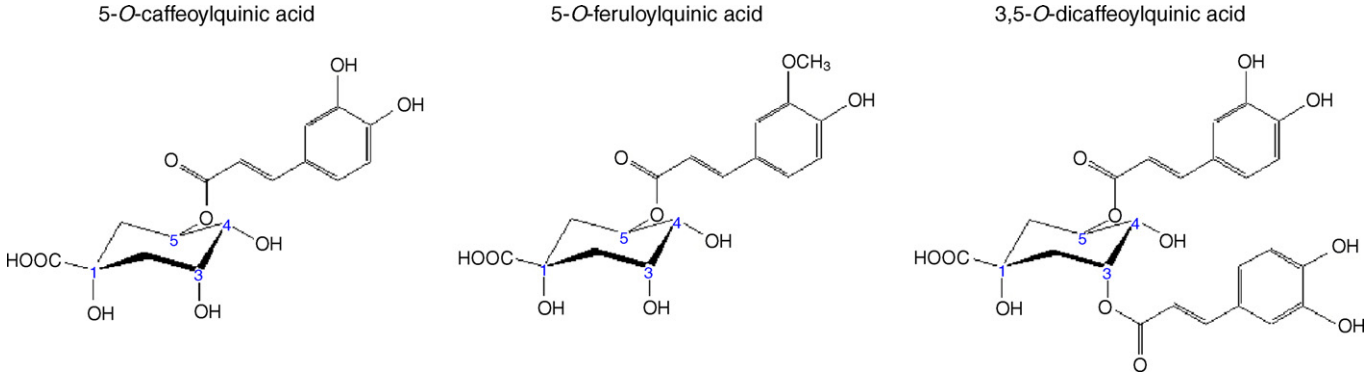

Fig. 1. Molecular structure of major representative chlorogenic acids.