Chapter 5

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

*J. Chrom. A. (2009), 1216: 4245-4251*

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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 (hydroxycinnamoylquinic acids, HCQAs) present in green coffee beans (5-caffeoylquinic acid, 5-CQA, 5-feruloylquinic acid, 5-FQA, and 3,5-dicafeoylquinic 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 (NH₄)₂SO₄-KNO₃ (3.0 and 1.5 M-1.5 M) in another. Regioisomers of each chlorogenic acid obtained by base-catalysed 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).

Introduction
The liquid nature of the stationary phase in counter-current separations (CCS) confers a greater versatility to this technique compared to HPLC. This unique feature has led to a fascinating new spectrum of instrumentation setups, column designs, separation methods and polarities that have been successfully applied and there is still potential for further developments. From the preparative point of view this characteristic makes CCS even more powerful as it enables the usage of the stationary phase to increase the solubility of analytes. Allowing in this way the loading of highly concentrated complex samples, e.g. plant extracts, for fractionation or isolation purposes. However, in contrast to adsorption chromatography, polarity elution gradients used for sample fractionation are not very common in CCS. The significant changes in the physical properties of the mobile phase associated with the change in solvent composition can lead to instability of the biphasic system in CCS and cause inconvenient column bleeding. Several ingenious strategies have been developed to solve this practical downside that limits the competitiveness of CCS. The most common is perhaps the elution-extrusion mode (Berthod, 2007; 2003; Lu et al., 2008), 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) (Agnely and Thiebaut, 1997) and later on as a multiple dual-mode in centrifugal partition chromatography (CPC) (Delannay et al., 2006). Nevertheless both polarity range and chromatographic resolution of CCS 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 CCS, specifically by Ito (Ito, 2002), 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 distribution constants. 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 present in green coffee beans in a reversed-phase mode.

Although the term chlorogenic acid originally referred exclusively to 5-Ο-caffeoylquinic acid (5-CQA), nowadays it is often used to refer to the whole family of trans hydroxycinnamoylquinic acids (HCQAs), 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 (Li and Steffens, 2002), anti-fungal (Shadle et al., 2003), anti-insect (Beninger et al., 2004), anxiolytic (Bouayed et al., 2007), hepatoprotective (Xiang et al., 2001), anti-thrombotic (Satake et al., 2007) and anti-viral (Chiang et al., 2002), including HIV inhibition (Robinson et al., 1996). Their extensively documented antioxidant properties render them beneficial against several oxidative stress-related conditions such as atherosclerosis (Cheng et al., 2007), cancer (Lee and Lee, 2006) and Alzheimer’s disease (Silva et al., 2005). 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 HCQAs) for instance is still quite controversial. Some recent studies claim that their health benefits in humans do not result from their antioxidant properties but instead from their toxicity (Galati and O’Brien, 2004) which triggers the physiological production of the endogenous actual antioxidant uric acid (Lotito and Frei, 2006).

Chlorogenic acid, 5-CQA, has been previously isolated from Flos lonicerae by CCS, using multiple step isocratic HSCCC (Lu et al., 2004) and from Lonicera japonica with a simple pH-gradient CCC (counter-current chromatography) method (Wang et al., 2008). However, no attempt has been made to either isolate the compounds belonging to the different CGA subfamilies, which are concomitantly present in most sources, or 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 (Nakatani et al., 2000; Parejo et
al., 2004) or obtained in limited amounts through preparative HPLC. Since coffee is the richest and most readily available source of HCQAs an alternatively method based on preparative CPC was developed for their isolation from green coffee beans.

Methods

Reagents
Chlorogenic acid (97%) and all inorganic salts were purchased from Sigma (St. Louis, MO, USA). Solvents, including formic and acetic acid, were purchased from Merk Biosolve (Valkenswaard, The Netherlands). Those used for extractions and CPC were of analytical grade while methanol and acetonitrile were of chromatographic grade. Ultra-pure deionized water was used. Green coffee beans of Ethiopian Coffea arabica cv. Tipica were purchased locally (Leiden, The Netherlands).

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 mL 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 autosampler, low-pressure mixing pump and a diode array detection (DAD) system.

Green coffee bean extract
The major hydroxycinnamoylquinic acid 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 (tr caffeoylquinic 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 (3x500 mL). The resulting extract was taken to dryness under vacuum and the residue was redissolved in 50% MeOH (200 mL) and defatted with hexane (3x200 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 (2x250 mL) and the organic phases were combined and taken to dryness, yielding 10 g of the crude blend of HCQAs. This residue was subsequently redissolved in hot water (200 mL), washed twice with CHCl₃ to remove caffeine and extracted twice with CHCl₃/18% BuOH. The resulting extract was
taken to dryness yielding a residue (3.4 g) composed of about 60% in HCQAs, of which, CQAs repre-
sented around 41%, FQAs 21% and diCQAs 38%.

**Chlorogenic acid isomerisation**

Chlorogenic acids can be easily converted into their respective regioisomers by means of a simple
base-catalysed intramolecular migration of the cinnamoyl group around the vicinal hydroxyls on the
quinic acid moiety (Clifford et al., 1989). Reaction conditions, *i.e.* base nature, base-substrate con-
centration ratio and time, were optimized using 5-CQA as a general reference for the preparation of a
total of nine HCQAs from the coffee extract: 3-CQA (neochlorogenic acid), 4-CQA (cryptochloroge-
nic acid), 5-CQA, the three analogous feruloyl conjugates and three isochlorogenic acids 3,5-diCQA,
3,4-diCQA and 4,5-diCQA. Independent isomerization of the major HCQAs present in coffee beans,
one separated by CPC in a first step, was carried out then by treating an aqueous solution of 5-CQA,
5-FQA or 3,5-diCQA (30 mL, 10 mg mL$^{-1}$) with sodium hydroxide (15 mL, 1 M) for five seconds while
stirring. The mixture was immediately acidified by adding enough 6.0 M HCl and desalted on a poly-
amide column eluted with water and acidic methanol. Evaporation under reduced pressure of the
organic solvent yielded in all cases a nearly equimolar isomeric mixture of the CQAs.

**Distribution constants and settling times**

Aliquots of 0.5 mg of either extracts or reference compounds were placed in 1.5 mL Eppendorf tubes
along with 300 µL of each partitioning phase and vortexed for 60 s. After centrifugation for 30 s at
6000 rpm 200 µL of each layer were transferred to vials, taken to 500 µL with methanol and analy-
sed by HPLC as described below. Settling times were determined by vigorously shaking 2 mL of each
phase for 10 s in a 10 mL measuring cylinder and registering the time needed for the system to sepa-
rate into two clear layers. This was done in triplicate.

**CPC procedures, solvents and samples**

Biphasic systems were prepared by thoroughly mixing convenient volumes of the different solvents
in their nominal ratios and letting them settle overnight. In all CPC runs the aqueous layer was the
mobile phase, whose pH was adjusted to 2.5 with either H$_3$PO$_4$-Na$_2$HPO$_4$ or H$_2$SO$_4$-(NH$_4$)$_2$SO$_4$
buffering systems. The stationary phase for the gradient procedures was saturated in both cases with a middle
concentrated salt solution to minimize composition changes along the gradient. The stationary phase
was always loaded first into the column at 100 rpm and then the mobile phase, in the adequate mode
(ascending or descending according to density) at 600-1000 rpm at a flow of 1-2 mL min$^{-1}$. Samples
were injected only when the dynamic equilibrium was reached. The volume composition of the bip-
hasic system for the different experiments was: (a) green coffee bean extract LiCl salting-out gradient:
ethyl acetate-hexane/0.01 M phosphate buffer-LiCl (68:32:100, v/v) with a stepwise gradient of 5.0 M
(60 mL), 2.5 M (40 mL) and 0.1 M (250 mL) LiCl; (b) green coffee bean extract (NH$_4$)$_2$SO$_4$-KNO$_3$
salting-out gradient: ethyl acetate-hexane/(NH$_4$)$_2$SO$_4$-KNO$_3$ (70:30:100, v/v) with a stepwise gradient of
3.0 M (85 mL) and 1.5 M (105 mL) (NH$_4$)$_2$SO$_4$, and a final step of 1.5 M (260 mL) KNO$_3$; (c) scale-up of
green coffee bean extract (NH$_4$)$_2$SO$_4$-KNO$_3$ salting-out gradient: ethyl acetate-hexane/(NH$_4$)$_2$SO$_4$-KNO$_3$
(70:30:100, v/v) with a stepwise gradient of 3.0 M (110 mL) and 1.5 M (170 mL) (NH$_4$)$_2$SO$_4$, and a final
step of 1.5 M (270 mL) KNO₃; (d) CQA regioisomers: chloroform- n-butanol/0.01 M phosphate buffer-5.0 M LiCl (82:18:100, v/v); (e) FQA regioisomers: chloroform-n-butanol/0.01 M phosphate buffer (84:16:100, v/v); (f) diCQA regioisomers: chloroform-n-butanol/0.01 M phosphate buffer (84:16:100, v/v). Injection volume was 5 mL in all cases except for the scale-up run (30 mL). In order to maximize solubility and yield in most cases samples were prepared by adding suitable volumes of the biphasic systems and vortexed or heated up to 60 °C, if necessary. The stationary-like phases of the resulting limpid or cloudy samples were injected firstly, followed by the aqueous ones. In all runs fractions were collected manually on a peak elution basis.

**HPLC analyses**

Fractions and standards were analysed by HPLC using a ODS-Luna 150x4.6 mm 3 µm column (Phenomenex, Torrance, CA, USA) and eluted at 0.7 mL min⁻¹ flow with a gradient of water-0.5% formic acid (solvent A) and methanol-0.5% formic acid (solvent B) according to the following gradient program (v/v): 0 min 25% B, 40 min 50% B linear, 43 min 60% B linear and 45 min 25% B linear, followed by 10 min for reequilibration. With the exception of 5-CQA all HCQAs were identified by means of their relative elution pattern and DAD spectrum analysis as consistently found in the literature (Clifford et al., 2003; Farah et al., 2005; Guerrero and Suarez, 2001), while quantification was achieved using a 5-CQA calibration curve in combination with the relative molar absorptivity coefficients reported for HCQAs (Trugo and Macrae, 1984).

**Results and discussion**

In an octanol-water system the separation factor (α) between 5-CQA and 3,5-diCQA, respectively the most and the least polar molecules in the targeted group of coffee HCQAs, was above 30. This implied that their separation evidently required either gradient, extrusion or dual-mode elution. In an effort to preserve the same column for possible multiple injections while keeping the maximum efficiency conditions and avoid the phase redistribution that takes place in dual-modes, an electrostriction gradient was chosen instead.

The main criterion for salt choice in these gradients is the salt overall electrostrictive or salting-out capacity. Even though salting effects are basic principles ubiquitous in chemistry and biology, the mechanism of their action has not been fully explained yet, relying on several different theories for their full description (Grover and Ryall, 2004), out of which the ones based on ionic hydration shells, while unsatisfactory, are the most widely accepted.

Figure 2 shows the electrostrictive power of some effective salting-out electrolytes on the distribution constant of 5-CQA in an ethyl acetate-salt(aq, pH 2.5) biphasic system. The relative salting-out power observed for the different salts is very much in agreement with the Hofmeister series for cations and anions usually reported as: F⁻ > PO₄³⁻ > SO₄²⁻ > AcO⁻ > Cl⁻ > NO₃⁻ > I⁻ > CNS⁻ and Mg²⁺ > Ca²⁺ > Ba²⁺ > Li⁺ > Na⁺ > K⁺ > NH₄⁺ > Cs⁺ (Cacace et al., 1997; Napper and Netschey, 1971; Zhang and Cremer, 2006), and directly correlated with the ion hydration enthalpies. As predicted by these series ions with the greatest charge density, e.g. HPO₄²⁻, SO₄²⁻ and Li⁺, proved to be the most electrostrictive, with KNO₃ on the lower limit in the set showing a slight salting-in effect.
Most likely due to metal complex-derived salting-in effects on the catechol group (Ghasemi and Shamsipur, 1995; Lapouge and Cornard, 2007) some tightly hydrated and hence supposedly good kosmotropic cations, such as Mg\(^{2+}\), Ca\(^{2+}\) and Al\(^{3+}\), were not effective salting-out agents for 5-CQA. Similar phenomena may explain the inverted effect observed for Li\(^+\) at extremely high salt concentrations (Fig. 2).

Lithium chloride -the lightest among the most electrostrictive salts- was chosen for the solvent system optimization and resolution studies. It is important to note that the selection of solvent systems for this group of compounds was particularly laborious due to their peculiar solubility behaviour, ranging from the affinity of CQAs with very polar organic solvents such as methanol to more lipophilic ones such as methyl tert-butyl ether for diCQAs, making it almost impossible to devise single stable biphasic systems suitable for all classes of HCQAs without compromising yield. The distribution constants for all isomers in the three most efficient candidate solvent systems found for HCQAs are summarized in Table 1. In the disk-type HPCPC rotor used in this case minimally satisfactory resolutions were obtained at optimal working conditions only for compounds with an experimental value of \(\alpha \geq 2\), which unfortunately could not be achieved by any of the biphasic combinations for all 10 phenolics, including caffeic acid. The chloroform-n-butanol system for instance provided good \(\alpha\) values between the regioisomers of all subfamilies (CQAs, FQAs and diCQAs) but not between 5-FQA and 3,5-diCQA, thus ruling out its application in the gradient experiments. A similar situation occurred in the methyl tert-butyl ether system for 5-CQA and 4-FQA. As an additional drawback in this system the polarity differences between CQAs and diCQAs are accentuated, as evidenced by an average \(\alpha > 55\), exceeding then the electrostriction capacity of LiCl to afford practical distribution constants (0.4 < \(D\) < 3) for most isomers. Surprisingly, the ethyl acetate-hexane system, while apparently the least discriminant
(no satisfactory α were obtained for the regioisomers of either subfamily), provided convenient values for the main representatives, that is 5-CQA, 5-FQA and 3,5-diCQA. At this point a two-step purification approach was taken for the HCQAs, separating 5-CQA, 5-FQA and 3,5-diCQA-4,5-diCQA from the green coffee bean extract in a gradient run with the ethyl acetate-hexane system in a first stage and secondly using independent CPC experiments to purify the regioisomers, obtained by isomerization, with suitable chloroform-butanol mixtures.

Table 1. Salting-out effect of lithium chloride on chlorogenic acids in different solvent systems.

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<th>[LiCl] (M)</th>
<th>3-CQA</th>
<th>5-CQA</th>
<th>4-CQA</th>
<th>CA</th>
<th>5-FQA</th>
<th>4-FQA</th>
<th>3,4-diCQA</th>
<th>3,5-diCQA</th>
<th>4,5-diCQA</th>
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St, settling time; CQA, caffeoylquinic acid; CA, caffeic acid; FQA, caffeoylferulic acid; diCQA, dicaffeoylquinic acid. >> = D above 100

Using the salty aqueous layer as a mobile phase in descending mode, the green coffee bean extract rich in HCQAs was successfully fractionated into the major subfamilies of HCQAs by means of a LiCl salting-out gradient (Fig. 3). The stepwise variation included three salt concentrations, 5.0, 2.5, and 0.1 M, applied in decreasing electrostrictive order, emulating thus a conventional reversed-phase gradient, aiming at the respective elution of the three major subclasses, CQAs, FQAs and diCQAs. The length of each step, as indicated by the dashed horizontal lines in the figures, was arbitrary, depending only on the elution of the major part of each peak. The maximum amount
of extract that could apparently be dissolved in a 5 mL portion of a 1:1 biphasic system was 50 mg, yielding 10.3 mg of CQAs (88% recovery of the total content in 50 mg of extract), 5.1 mg of FQAs (85% recovery), 0.7 mg of 3,4-diCQA (70% recovery) and 5.8 mg of a 3,5-diCQA-4,5-diCQA mixture (53% recovery). The low recoveries registered for the least hydrophilic compounds reveal a clear solubility preference of this solvent system towards the more hydrophilic molecules and a general loading limitation common to most gradient chromatographic techniques.

A second gradient run was designed for ammonium sulphate, (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, as a more electrostrictive, less expensive and more environmentally friendly salt. This salt however is much heavier and hence prone to bring about significant changes in the settling times of the biphasic system along the gradient. The most suitable concentrations of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} for a similar set of distribution constants compared to LiCl were 3.0, 1.5 and 0.1 M, but unfortunately the settling time net variation was around 10 s, enough to cause undesirable stationary phase bleeding. To overcome this difficulty another common application of salts in CCS was used: non electrostrictive salts such as KNO\textsubscript{3} can be introduced to stabilize the biphasic system as the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} concentration is reduced. Based on the settling times determined for several KNO\textsubscript{3} concentrations (data not presented) the last step of this (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} gradient was replaced by a KNO\textsubscript{3} 1.5 M solution. The result of this multistep and multisalt method (Fig. 4) was a separation with a resolution comparable to that obtained with LiCl (Fig. 3). With this system same amount of extract yielded 9.5 mg of CQAs (81% recovery), 3.6 mg of FQAs (60% recovery), 0.8 mg of 3,4-diCQA (80% recovery) and 6.7 mg of 3,5-4,5-diCQA (61% recovery). Observed differences in recoveries when compared to the LiCl experiment were again

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**Figure 3.** CPC separation of the major chlorogenic acids present in green coffee beans with a LiCl salting-out gradient. Experimental conditions: rotation speed: 800 rpm; solvent system: ethyl acetate-hexane/0.01 M pH 2.5 phosphate buffer-LiCl (68:32:100; v/v); salting-out gradient: 5.0 M (60 mL), 2.5 M (40 mL) and 0.1 M (250 mL) LiCl; mobile phase: lower aqueous phase; flow rate: 2.4 mL min\textsuperscript{-1}; retention of stationary phase: 76%; injection volume: 5 mL; back pressure: 50 bar (5.0 M LiCl), 40 bar (2.5 M LiCl) and 30 bar (0.1 M LiCl); sample: 50 mg CGA-enriched green coffee bean extract dissolved in 2.5 mL of mobile phase and 2.5 mL of stationary phase.
most likely a result of preferential solubilisation.

As mentioned before, CCS have the advantage, in contrast to HPLC, of being able to count on the liquid stationary phase to deal with a major solubility drawback, partly dissolving complex samples in as much stationary phase as the retained column volume allows. An attempt in this direction was made using a second (NH₄)₂SO₄-KNO₃ gradient, injecting in this case 250 mg of green coffee bean extract dissolved in 12.5 mL of mobile phase and 12.5 mL of stationary phase. In spite of the five-time scale-up, the separation obtained was as satisfactory as that obtained with the previous system as can be appreciated in the CPC chromatogram in Figure 5. It is important to note that in this case the second step ((NH₄)₂SO₄ 1.5 M) was extended until all FQAs eluted, achieving in this way a remarkable increase in the resolution between the FQAs and 3,4-diCQA peaks. The 250 mg extract sample yielded in this case 46.7 mg of CQAs (80% recovery), 17.6 mg of FQAs (59% recovery), 3.7 mg of 3,4-diCQA (74% recovery) and 28.3 mg of 3,5,4,5-diCQA (52% recovery), for an average yield increase of 4.5 times.

The best solvent system for the separation of regioisomer of all subfamilies was chloroform-n-butanol based. Unfortunately the salt-free composition polar enough to give distribution constants around 1 for CQA regioisomers (chloroform-n-butanol, 82:12; v/v) was relatively unstable (settling time: 27 s) and hence inefficient. In consequence a chloroform-n-butanol/0.01 M phosphate buffer-5.0 M LiCl system was implemented in order to achieve a good purification of CQA isomers (Fig. 6). A sample of 70 mg of isomerized mixture dissolved in 1.0 mL of mobile phase was injected into the column, yielding 19.6 mg of neochlorogenic acid (3-CQA), 20.3 mg of cryptochlorogenic acid (4-CQA) and 25.0 mg of chlorogenic acid (5-CQA), all over 95% pure. Fractions containing the pure compounds were desalted by loading them on a polyamide column, which was washed with deionized water to
Figure 5. CPC scale-up separation of the major chlorogenic acids present in green coffee beans with a \((\text{NH}_4)_2\text{SO}_4-\text{KNO}_3\) salting-out gradient. Experimental conditions: rotation speed: 600 rpm; solvent system: ethyl acetate-hexane/pH 2.5 \((\text{NH}_4)_2\text{SO}_4\) (70:30:100; v/v); salting-out gradient: 3.0 M (110 mL) and 1.5 M (170 mL) \((\text{NH}_4)_2\text{SO}_4\) and 1.5 M (270 mL) KNO_3; mobile phase: lower aqueous phase; flow rate: 2.0 mL min\(^{-1}\); retention of stationary phase: 82%; injection volume: 30 mL; back pressure: 22 bar (3.0 M \((\text{NH}_4)_2\text{SO}_4\), 18 bar (1.5 M \((\text{NH}_4)_2\text{SO}_4\)) and 16 bar (1.5 M KNO_3)); sample: 250 mg CGA-enriched green coffee bean extract dissolved in 12.5 mL of mobile phase and 12.5 mL of stationary phase.

Figure 6. CPC separation of the regioisomers of chlorogenic acid (CQA). Experimental conditions: rotation speed: 1000 rpm; solvent system: chloroform-n-butanol/0.01 M pH 2.5 phosphate buffer-5.0 M LiCl (82:18:100; v/v); mobile phase: lower aqueous phase; flow rate: 1.0 mL min\(^{-1}\); retention of stationary phase: 73%; injection volume: 5 mL; back pressure: 42 bar; sample: 70 mg of a mixture of chlorogenic acid regioisomers dissolved in 1.0 mL of mobile phase.
remove the salts after which pure compounds were eluted with acidic methanol. Regioisomers of the FQA and diCQA subfamilies were submitted to CPC using a chloroform-n-butanol/0.01 M phosphate buffer (84:16:100; v/v) solvent system. A 33 mg FQAs sample yielded 11.4 mg of 3-FQA (83% pure, with an impurity consisting mainly of CQAs) (Fig. 7), 9.3 mg of 4-FQA (92% pure, contaminated mainly with an unidentified flavonoid also present in the 3-FQA fraction) and 8.4 mg of 5-FQA (96% pure).

Separation of isochlorogenic acids (diCQAs) was less satisfactory as can be observed in the CPC chromatogram in Figure 8. Resolution between 3,4-diCQA and 3,5-diCQA was low and HPLC analysis of the 3,4-diCQA peak (10.2 mg) showed only 63% of purity, co-eluted with CQAs (resulting from the partial hydrolysis of diCQAs during the isomerization procedure) and 3,5-diCQA.

The latter was obtained with a 76% of purity (15.4 mg) and 4,5-diCQA (4.3 mg, 43% pure) contaminated with caffeic acid (6.8 mg), the main hydrolysis product. The purity of all compounds could have been increased in detriment of yield by collecting partial chromatographic regions instead of the entire eluting peaks.

With the separation of CQAs, FQAs and diCQAs from green coffee beans it has been proven that the manipulation of electrostriction in an aqueous solution by changing the concentration of one or more salts can constitute an effective method of performing a reversed-phase-like gradient elution in CCS. A major contribution of these salting-out gradients lies on the fast single-column elution of compounds largely differing on their distribution constants without experiencing significant column bleeding. Although this achievement is also possible with a conventional normal-phase gradient in CCC, very few biphasic systems meet the conditions to actually do it. In addition, notwithstanding the fact that this proposed method may suffer from some impracticability for routine application
when compared to other powerful ones like the dual (Agnely and Thiebaut, 1997; Delannay et al., 2006), the elution-extrusion (Berthod, 2007; 2003; Lu et al., 2008) and the cocurrent (Berthod and Hassoun, 2006) modes, these ionic gradients represent a proof and a reminder of how salting effects could open new possibilities in poor resolution cases limited by solvent system stability.

Figure 8. CPC separation of the regioisomers of isochlorogenic acid (dICQA). Experimental conditions: rotation speed: 900 rpm; solvent system: chloroform-n-butanol/0.01 M pH 2.5 phosphate buffer (84:16:100; v/v); mobile phase: lower aqueous phase; flow rate: 1.0 mL min⁻¹; retention of stationary phase: 80%; injection volume: 5 mL; back pressure: 42 bar; sample: 38 mg of a mixture of isochlorogenic acid regioisomers dissolved in 1.5 mL of mobile phase and 3.0 mL of stationary phase.