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Short communication

Cation-exchange micropreparative separation of galloylated and non-galloylated sulphur conjugated catechins

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Abstract

Catechin conjugates bearing an amino function can be separated from underivatized monomers by cation-exchange chromatography. Here, chromatographic conditions for the separation of epicatechin gallate-containing conjugates from the non-galloylated conjugates at micropreparative scale are described. The separation was achieved by exploiting either the hydrophobic or hydrophilic interactions of the conjugates with the core polymer. The retention was modulated by changing the amount of organic co-solvents (MeOH, EtOH, CH_3CH, THF) in the elution buffers. The best resolution compatible with small peak widths was obtained at 20–30% EtOH. The experiments were reproducible.

Keywords: Catechins; Antioxidants; Polyphenols; Flavan-3-ols

1. Introduction

Plant polyphenols are attracting much attention as chemopreventive agents with beneficial influence on human health [1,2]. In particular, flavanols from tea, grape or pine bark are potent antioxidants of interest as potential preventative agents against cancer and cardiovascular diseases [3–7].

We have recently described the preparation and properties of a new kind of antioxidants based on naturally occurring flavanols [8,9]. The new compounds are conjugates between thiols such as cysteamine or cysteine and flavanols such as (−)-epicatechin **1**, (+)-catechin **2** or (−)-epicatechin 3-*O*-gallate **3** (Fig. 1). The conjugates are more efficient than their underivatised counterparts as free radical scavengers and inhibitors of the proliferation of colon carcinoma cells [9]. They are obtained from polymeric flavanols by acid cleavage, separated from the crude cleavage mixture by cation-exchange chromatography and purified by reversed-phase high-performance liquid chromatography (RP-HPLC). By taking advantage of the hydrophobic non-specific retention with the cation-exchange resin, the epicatechin and catechin derivatives may be resolved from the epicatechin gallate-containing conjugate, by means of an organic co-solvent, as reported for compounds **4–6** (Fig. 1) [8]. Since gallate esters are more potent free radical scavengers than non esterified flavan-3-ols [9–11], the method seemed

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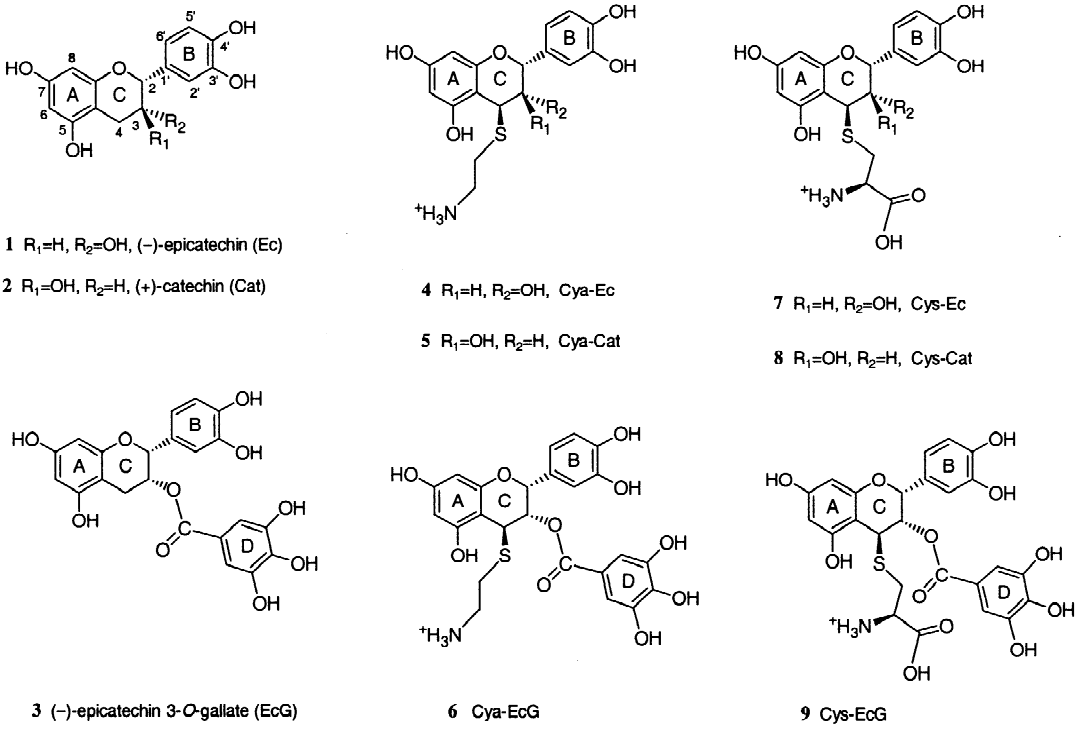

Fig. 1. Structures of monomeric catechins and their cysteamine and cysteine conjugates. Cya-Ec: 4β-(2-aminoethylthio)epicatechin; Cya-Cat: 4β-(2-aminoethylthio)catechin; Cya-EcG: 4β-(2-aminoethylthio)epicatechin 3-*O*-gallate; Cys-Ec: 4β-(*S*-cysteinyl)epicatechin; Cys-Cat: 4β-(*S*-cysteinyl)catechin; Cys-EcG: 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate.

worth optimising. To this end and to explore more extensively the potential of cation-exchange chromatography for the partial purification of the efficient antioxidant amine containing flavanol conjugates, we have tested the influence of the nature and amount of the organic co-solvent on the resolution of cysteamine and cysteine conjugates with flavan-3-ols. We have used a strong cation-exchange resin with a matrix polymer able to establish either hydrophobic or hydrophilic interactions with the solutes. Conditions have been found that discriminate between epicatechin gallate sulphur conjugates and non-galloylated flavanol derivatives by either hydrophobic retention or hydrophilic interaction–cation-exchange chromatography.

2. Experimental

2.1. Materials

The crude mixtures were obtained by cleavage of polymeric procyanidins in the presence of either cysteamine or cysteine as described [8,9]. The main components of the crudes were: **1**–**3**, **4**–**6** (mixture I, cysteamine conjugates) and **1**–**3**, **7**–**9** (mixture II, cysteine conjugates) (Fig. 1). Water and co-solvents: Milli-Q® water; HPLC grade methanol, CH_3CN, THF (E. Merck, Darmstadt, Germany) and absolute ethanol (E. Merck) filtered through a 0.5-μm pore size filter. Acetic acid, phosphoric acid (E. Merck), NaOH and sodium chloride (Carlo Erba, Milano, Italy) were of analytical grade.

2.2. Chromatography

Micropreparative cation-exchange chromatography was performed on a Smart® System, equipped with a μ-Peak monitor (Amersham-Pharmacia Biotech, Uppsala, Sweden) and fitted with a Mono-S® PC 1.6/5 (3 μm particle size, 50×1.6 mm I.D., 0.1 ml bed volume) column (Amersham-Pharmacia Biotech). The crudes were eluted with binary systems: [A] sodium acetate 20 m*M*, pH 4.75 buffer–

organic solvent or sodium phosphate 20 m*M*, pH 2.27 buffer–organic solvent; [B] sodium acetate 20 m*M*, pH 4.75 buffer–organic solvent 1 *M* NaCl or sodium phosphate 20 m*M*, pH 2.27 buffer–organic solvent 1 *M* NaCl. The column was equilibrated with eluent A, loaded with the depolymerised mixture (20 μl, 2 μg) and washed with A (10 bed volumes, 1 ml). Then, the retained flavan-3-ol derivatives were released with a salt gradient (0–100% B over 20 bed volumes, 2 ml). At high amount of co-solvent (>70% MeOH, EtOH, >40% CH_3CN, >30% THF), 1 *M* NaCl solutions resulted in two layers. We then lowered the concentration of salt to 0.5 *M* NaCl and run a shorter gradient (0–0.5 *M* NaCl over 10 bed volumes, 1 ml) with the same slope. The nature and amount of the organic co-solvent depended on the experiment and are stated in Section 3. Flow rate was 100 μl/min and detection was at 214, 280 and 320 nm. At 214 and 280 nm all flavanols are detected while only the gallate-containing flavanols will absorb at 320 nm. For every run the elution volume of two peaks, corresponding to catechin epicatechin conjugates (compounds **4**, **5** or **7**, **8**) and epicatechin gallate conjugate (compound **6** or **9**), respectively, were recorded and the following parameters were calculated: retention factor R_x = (retention volume V − dead volume V_0)/dead volume V_0, where V_0 was 100 μl; selectivity factor $\alpha = V_2/V_1$; resolution $R_s = 2 \times (V_2 - V_1)/(w_1 + w_2)$, where w is the peak width at the base.

3. Results and discussion

In cation-exchange chromatography, if no interaction other than electrostatic were to take place and the charged groups were equally accessible to the sulphonic groups on the resin, the separation would depend exclusively on the net electric charge of the solutes. In most instances, hydrophobic interaction with the core polymer causes an additional retention of the solutes, which can be eliminated totally or partially with organic co-solvents. The aim of this work was to find conditions to combine, in a single run, the separation of amine containing flavanol derivatives from uncharged species and the discrimination between charged compounds with different polar character. To this end, the interactions between the solutes and the resin polymer were modified conveniently with water-miscible organic co-solvents added to the mobile phases. Four water-miscible co-solvents covering a variety of polarities and hydrogen bond forming capabilities were selected, namely the most commonly used strong dipole forming solvent CH_3CN, two hydrogen bond accepting–donating solvents (MeOH and EtOH) and one hydrogen bond accepting solvent (THF). The amount of co-solvent ranged between 10 and 70% for CH_3CN, between 10 and 80% for MeOH and EtOH and between 10 and 50% for THF. Two buffers were selected with pH values of 4.75 and 2.27, respectively. The amino group of all derivatives was supposed to be protonated at both pH values whereas at pH 4.75 the carboxyl group of **7**–**9** was likely to exist as a mixture of protonated and unprotonated populations. Thus, at low co-solvent amount (10–20% EtOH, 10% MeOH, 10% CH_3CN) and pH 4.75, derivatives **7–9** (mixture II) were mostly retained by hydrophobic interactions, whereas derivatives **4–6** presented a positive net charge and were retained by ionic and hydrophobic interactions. At pH 2.27 all derivatives were positively charged. THF was only used with one of the buffers (sodium acetate, pH 4.75) since this solvent damaged the pumping system's O-ring seals and we decided not to proceed further with it.

3.1. Hydrophobic retention–cation-exchange chromatography

In our hands, the uncharged compounds **1** and **2** were excluded from the column already at 10% of co-solvent whereas the elimination of the hydrophobic retention established by compound **3** needed amounts of co-solvent as high as ≈35% MeOH, 30% EtOH and 20% CH_3CN in both buffers. The stronger retention of (−)-epicatechin 3-*O*-gallate **3,** already observed by RP-HPLC [8,12,13] was due to the extra aromatic ring on position 3. On the contrary, (−)-epicatechin **1** and (+)-catechin **2**, which are separated by RP-HPLC, were not differentially retained by the cation-exchange core polymer.

The same effect was observed for the salt mediated elution of the amine containing derivatives. The galloylated compounds **6** and **9** gave the highest elution volumes (Fig. 2). Since galloylation appears to be significant for the chemopreventative activity of flavan-3-ols and their thio-derivatives [9–11], we

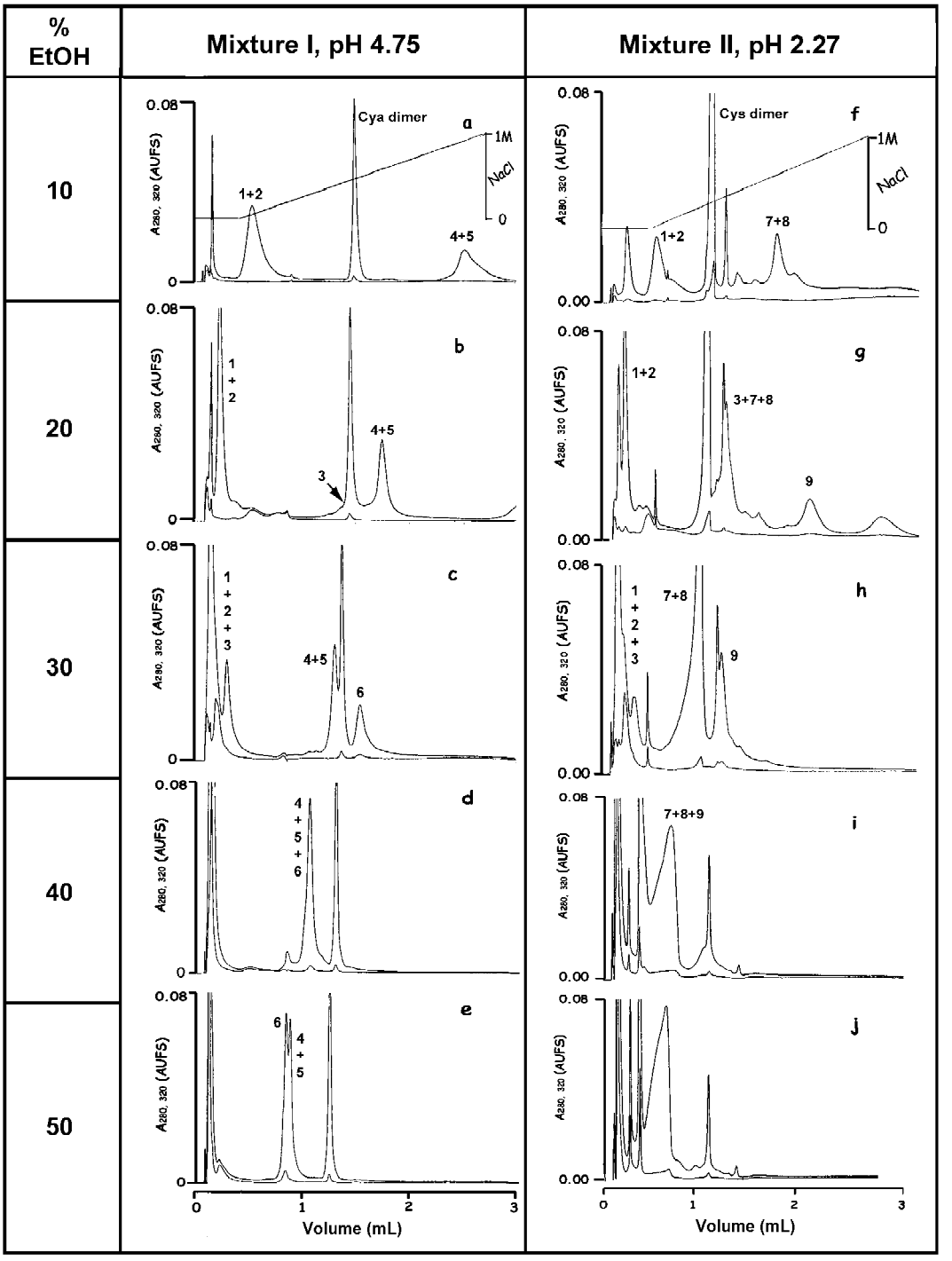

Fig. 2. Cation-exchange chromatograms corresponding to the elution of depolymerised mixtures I (compounds **1**–**6**) and II (compounds **1**–**3**, **7**–**9**). Load: 20 μl, 2 μg catechins. Column: Mono-S® PC 1.6/5. Eluent A: sodium acetate 20 m*M*, pH 4.75 buffer–EtOH or sodium phosphate 20 m*M*, pH 2.27 buffer–EtOH; eluent B: eluent A, 1 *M* NaCl. Elution: 1 ml (10 bed volumes) of eluent A followed by a 0–100% B gradient over 2 ml (20 bed volumes). Upper absorbance curve: 280 nm; lower absorbance curve: 320 nm. Cya, cysteamine; Cys, cysteine.

focused on the ability of the cation-exchange resin to retain the gallate-containing derivatives by hydrophobic interactions. The best separation conditions are those that combine a good resolution ($R_s \geq 1.5$) with small peak widths (eventually related to the fraction collected volume). For the separation of galloylated species from non-galloylated ones, the best results were obtained with 20–30% EtOH at pH 2.27 or 4.75 (cysteamine conjugates, Fig. 2b,c) and pH 2.27 (cysteine conjugates, Fig. 2g,h). Similar results were obtained with 20% CH_3CN. Lower amounts of EtOH resulted in a too strong retention of **6** or **9**. Amounts of co-solvent higher than or equal to 30% EtOH or higher than 20% CH_3CN resulted in poor resolution ($R_s < 1$) of the pair of peaks. As far as MeOH is concerned, higher amounts of co-solvent were needed to obtain similar results ($R_s \geq 1.5$ at 40% MeOH, $R_s < 1$ with at least 60% MeOH). Considering also its toxicity, MeOH appeared to be less appropriate than EtOH or CH_3CN. THF is less toxic than MeOH but gave the poorest results ($R_s = 0$ at 15% THF and higher, gallates too retained at 10% THF). In conclusion, both CH_3CN and EtOH were suitable co-solvents under hydrophobic retention conditions.

At amounts of co-solvent higher than those described so far, two different effects were observed, namely loss of retention or hydrophilic interaction with the polymer.

3.2. Loss of retention with the solvent

First, raising the amount of co-solvent resulted in early elution of the conjugates. Already at 30% EtOH, the *S*-cysteinyl derivatives **7**, **8** were slowly released from the column at low concentration of salt (Fig. 2h). At 50% EtOH, the same species were excluded from the column with the washing buffer, that is to say, in the presence of only 20 m*M* sodium acetate. The reason for the early liberation of flavanol derivatives at high amount of co-solvent might be the formation of ion-pairs with anions from the eluting buffer that would not interact with the sulphonic groups of the resin. The peak widths under these eluting conditions would be explained by equilibrium between both kinds of ion-pairs. This effect has been described for the ion-exchange purification of other chemicals such as biocompatible cationic surfactants [14].

3.3. Hydrophilic interaction–cation-exchange chromatography

A second effect was observed when working with mixture I (cysteamine conjugates). The hydrophobic retention was eliminated at 40% EtOH ($R_s = 0$, Fig. 2d), and the three compounds (**4**, **5**, **6**) eluted together when the charged amino group was exchanged. At higher amounts of co-solvent the main contribution of the polymeric matrix was hydrophilic and the elution order was inverted. The gallate-containing conjugate **6** eluted first due to its lower hydrophilic character (Fig. 2e). This has been called hydrophilic interaction–cation-exchange chromatography, described for peptidic biomolecules [15,16]. Although the R_s obtained were low (-0.33 to -0.36) our results suggest that the galloylated cysteamine conjugate may be purified from the other derivatives in a fast and low buffer consuming way by taking advantage of its lower hydrophilic character, provided that the right matrix is found. Stationary phases based on silica [15,16] may lead to better resolutions.

3.4. Reproducibility of elution volumes and R_s values with EtOH as co-solvent

In view of the results presented so far EtOH appears to be the co-solvent of choice for the purification of flavanol thio-derivatives by cation-exchange chromatography. It shows lower toxic potential (class 2 solvent according to the published FDA guidance, ‘‘Q3C Impurities. Residual solvents’’ [17]) and it is more reasonably priced than CH_3CN which is a class 3 solvent (solvent to be limited [17]). We have studied the reproducibility of the separation between galloylated and non-galloylated conjugates for EtOH under hydrophobic retention conditions (25% EtOH) and hydrophilic interaction conditions (50% EtOH). Reproducibility in 1 day was measured from five replicate determinations. The day-to-day reproducibility was ascertained over a period of 4 consecutive days. The hydrophobic

retention–cation-exchange chromatography of both mixtures I and II gave reproducible elution volumes (SEM = 0.02 ml) with a good ($R_s > 2.50$) and reproducible (SEM = 0.09) resolution between the two peaks. Under hydrophilic interaction conditions the results were also reproducible (SEM elution volume = 0.02 ml, SEM $R_s = 0.01$).

4. Conclusion

Hydrophobic retention–cation-exchange chromatography has proven to be suitable for the separation of amine containing flavanol derivatives from uncharged species and for the separation of galloylated conjugates from non-galloylated conjugates. EtOH appears to be the solvent of choice because it provided good resolution, reproducibility, low toxic potential and low cost. The same solvent might be used for hydrophilic interaction–cation-exchange chromatography.

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