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# Novel separation of bioactive catechin derivatives from complex plant mixtures by anion-exchange chromatography

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**Abstract**

The paper presents the use of anion-exchange chromatography to separate bioactive cysteine containing catechin conjugates from underivatised polyphenols and oligomeric material. The conjugates were excluded from the anion-exchange resin using a low percentage of organic solvent, enough to eliminate the weak hydrophobic interactions with the matrix. The rest of the uncharged polyphenols were unexpectedly retained by the matrix and eluted by the addition of salt. Cation–π interactions between the quaternary amino group on the resin and the electron-rich faces of the flavanol's aromatic rings might be responsible for this unexpected behaviour. Because immobilized anions retain uncharged polyphenolic material, anion-exchange chromatography may be a convenient approach to separate polyphenols from other bioactive compounds in complex mixtures of plant origin.
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*Keywords:* Catechins; Antioxidants; Purification; Anion-exchange chromatography; Cation–pi

## 1. Introduction

The strong interest in plant polyphenols as possible chemopreventive agents against a variety of diseases is leading to intensive investigation on convenient separation methods at both analytical and preparative scale. The method of choice will depend on the nature of the target compounds (monomers of different polarity and charge, oligomers, polymers) and the feasibility of scaling up the process. At analytical level polyphenols have been separated by reversed-phase high performance liquid chromatography [1], normal-phase HPLC–MS [2], capillary electrophoresis [3], micellar electro kinetic chromatography [4] and ion exchange chromatography [5,6]. At preparative scale natural and semisynthetic polyphenols have been fractionated and purified by high-speed counter-current chromatography [7], membrane extraction [8], supercritical fluid extraction [9], centrifugal partition chromatography [10], size-exclusion chromatography [11], ion exchange chromatography [12,13] and solid-phase extraction with Amberlite XAD resins [14].

Biologically active chemically modified polyphenols (Fig. 1) obtained by acid cleavage of polymeric procyanidins in the presence of thiols have been reported [5,13]. They exert biological actions modulated by the non-phenolic part of the molecule. Recently, *O*-ethylcysteinyl catechin **7** and the galloylated derivative *O*-ethylcysteinyl epicatechin 3-*O*-gallate **9** have been described as potent antioxidants with high hydrogen donation capacity [15]. The later was also active at inducing apoptosis in HT29 colon carcinoma cells [15]. This compound, as well as other positively charged conjugates have been purified by cation-exchange chromatography combined with resin absorption and reversed-phase chromatography before and after the ion exchanger [12,16]. Because the retained conjugates elute together with uncharged polyphenolic material unspecifically retained by the matrix, the mixtures after cation exchange are still complex [13]. In the present work we show that anion exchange may be a convenient alternative if the target conjugates are excluded from the resin because most of the accompanying material (e.g. monomeric catechins and oligomers) is retained by the stationary phase. We suggest here a possible explanation for this behaviour involving cation–π interactions. Anion exchange appears to be a convenient option for the preparative purification of bioactive uncharged and cationic conjugates such as *N*-acetyl-

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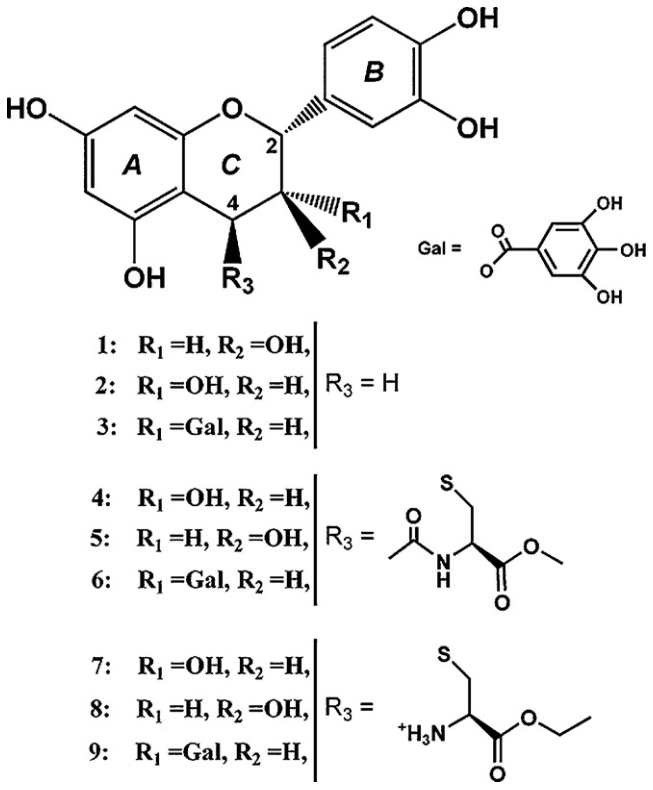


Fig. 1. Structures of monomeric catechins and their *N*-acetyl-L-cysteine and L-cysteine ethyl ester conjugates. (+)-catechin (**1**); (−)-epicatechin (**2**); (−)-epicatechin 3-*O*-gallate (**3**); 4$\beta$-[S-(*N*-acetyl-*O*-methyl-cysteinyl)]epicatechin (**4**); 4$\beta$-[S-(*N*-acetyl-*O*-methyl-cysteinyl)]catechin (**5**); 4$\beta$-[S-(*N*-acetyl-*O*-methyl-cysteinyl)]epicatechin 3-*O*-gallate (**6**); 4$\beta$-[S-(*O*-ethyl-cysteinyl)]epicatechin (**7**); 4$\beta$-[S-(*O*-ethyl-cysteinyl)]catechin (**8**) and 4$\beta$-[S-(*O*-ethyl-cysteinyl)]epicatechin 3-*O*-gallate (**9**).

*O*-methylcysteinyl and *O*-ethylcysteinyl derivatives of catechin and epicatechin.

## 2. Materials and methods

### 2.1. Chemicals

The primary source of procyanidins was the by-product from pressing destemmed Parellada grapes (*Vitis vinifera*) (Bodegas Miguel Torres, S.A., Vilafranca del Penedès, Spain) which were extracted and fractionated as described [12]. Water and solvents used were: analytical grade methanol (Panreac, Montcada i Reixac, Spain) for the acid cleavage reaction; deionised water and bulk ethanol (Momplet y Esteban, Barcelona, Spain) for semipreparative and preparative anion-exchange chromatography; milli-Q® water and HPLC grade acetonitrile (E. Merck, Darmstadt, Germany) for analytical RP-HPLC. L-Cysteine ethyl ester hydrochloride and *N*-acetyl-L-cysteine (Aldrich, Steinheim, Germany) were of synthesis grade. Thirty-seven percent HCl (E. Merck) and NaCl (Carlo Erba, Milano, Italy) were of analytical grade. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK) biotech grade was distilled in-house. (–)-Epicatechin was from Sigma Chemical Co. (Saint Louis, MO, USA).

### 2.2. Acid cleavage

The mixtures of catechin (flavanol) conjugates were obtained essentially as described in [13]. The procyanidin source (400 mL aqueous solution, 6 g estimated polyphenols by weight, coming from 3.2 kg of grape by-product) was dried. The resulting syrupy residue was dissolved in methanol (400 mL) and a solution of the appropriate cysteine derivative (20 g) and 37% HCl (10 mL) in methanol (400 mL) was added. The mixture was kept at 65 °C for 20 min under stirring. The reaction was then quenched with cold water (3.2 L). From the same procyanidin source, crude mixtures using L-cysteine ethyl ester hydrochloride (CEE) and *N*-acetyl-L-cysteine (NAC) were obtained.

### 2.3. Chromatography

Semipreparative anion-exchange chromatography was performed on a FPLC® system (Amersham-Pharmacia Biotech, Uppsala, Sweden) fitted with an Omnifit (Cambridge, UK) column (8 cm × 1 cm i.d., ∼6 mL bed volume) packed in house with MacroPrep™ High Q 50 μm (BioRad Laboratories, Hercules, CA). These semipreparative runs were used to set-up the separation conditions at milligram scale.

For the preparative separation of the *N*-acetyl-L-cysteine derived crude mixture, MacroPrep™ High Q 50 μm was packed into a flash chromatography type glass column (17 cm × 2.5 cm i.d., ∼333 mL bed volume). The eluents were [A]: 20 mM sodium phosphate, pH 4.75 buffer/ethanol (9:1), [B]: 20 mM sodium phosphate, pH 4.75 buffer/ethanol (7:3) and [C]: 20 mM sodium phosphate, pH 4.75 buffer/ethanol (3:2), 1 M NaCl. The column was equilibrated with [A] (3000 mL, nine bed volumes), loaded with the quenched depolymerised mixture (1000 mL) and washed with [A] (1200 mL, 3.6 bed volumes). The catechin and epicatechin derivatives were released with 2100 mL (6.3 bed volumes) of eluent [B]. Eluent [C] (1500 mL, 4.5 bed volumes) was required to elute the epicatechin gallate derivative and the three monomeric compounds, surprisingly retained by the resin. The operation was repeated (four times total) until the whole mixture was consumed.

The separation of the cationic mixture obtained in the presence of L-cysteine ethyl ester was performed using the same semipreparative anion-exchange chromatography and eluting conditions described before.

All the separation processes were monitored by analytical RP-HPLC on a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC™ (The Separations Group, Hesperia, USA) $C_{18}$, 300 Å pore size, 5 μm particle size, 250 mm × 4.6 mm i.d. column. The fractions collected (load 100 μL) were eluted using a binary system, [D] 0.10% (v/v) aqueous TFA, [E] 0.09% (v/v) TFA in water/acetonitrile (1:4) under different gradient conditions: 12 to 27% [E] over 45 min for the *N*-acetyl-*O*-methyl-L-cysteinyl derivatives and 10–25% [E] over 45 min for the L-cysteine ethyl ester derivatives at a flow rate of 1 mL/min with detection at 215 nm, 1.2 absorbance units full scale (aufs).

Semipreparative cation-exchange chromatography was performed on the FPLC® system described before and the 6 mL

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