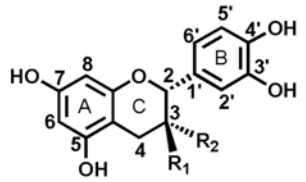
6. ANNEXOS

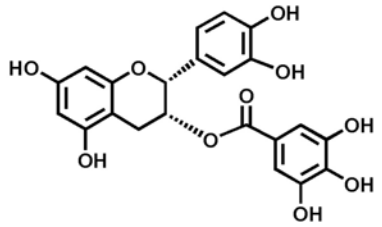
6.1 ANNEX I

Annex I.1. Estructura dels flavanols (—)-epicatequina **1**, (+)-catequina **2** i (—)-epicatequin 3-*O*-galat **3** i dels seus corresponents derivats amb CYA (**4**-**6**), CYS (**7**-**9**), OET (**10**-**12**) i NAmC (**13**-**15**), juntament amb la (—)-epigal·locatequin 3-*O*-galat (**16**).

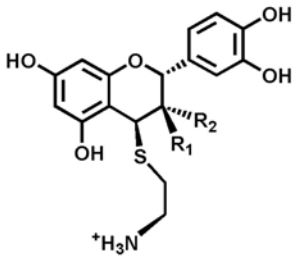


1, R_1=H, R_2=OH, (–)-epicatequina

2, R_1=OH, R_2=H, (+)-catequina



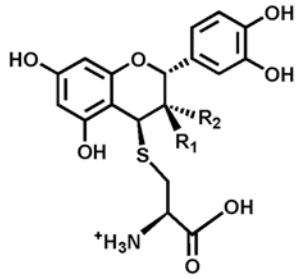
3, R_1=H, R_2=Galat, (–)-epicatequin 3-*O*-galat



4, R_1=H, R_2=OH, 4β-(2-aminoetiltio)epicatequina

5, R_1=OH, R_2=H, 4β-(2-aminoetiltio)catequina

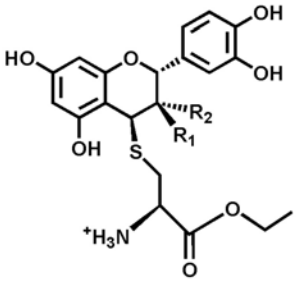
6, R_1=H, R_2=Galat, 4β-(2-aminoetiltio)epicatequin 3-*O*-galat



7, R_1=H, R_2=OH, 4β-(*S*-cisteinil)epicatequina

8, R_1=OH, R_2=H, 4β-(*S*-cisteinil)catequina

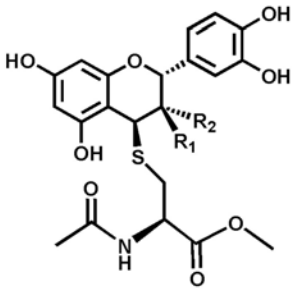
9, R_1=H, R_2=Galat, 4β-(*S*-cisteinil)epicatequin 3-*O*-galat



10, R_1=H, R_2=OH, 4β-[*S*-(*O*-etilcisteinil)]epicatequina

11, R_1=OH, R_2=H, 4β-[*S*-(*O*-etilcisteinil)]catequina

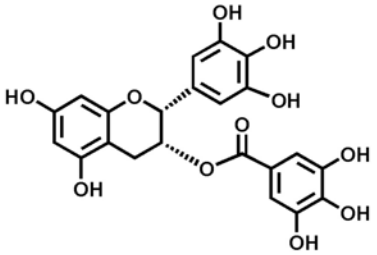
12, R_1=H, R_2=Galat, 4β-[*S*-(*O*-etilcisteinil)]epicatequin 3-O-galat



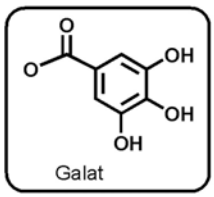
13, R_1=H, R_2=OH, 4β-[*S*-(*N*-acetil-*O*-metilcisteinil)]epicatequina

14, R_1=OH, R_2=H, 4β-[*S*-(*N*-acetil-*O*-metilcisteinil)]catequina

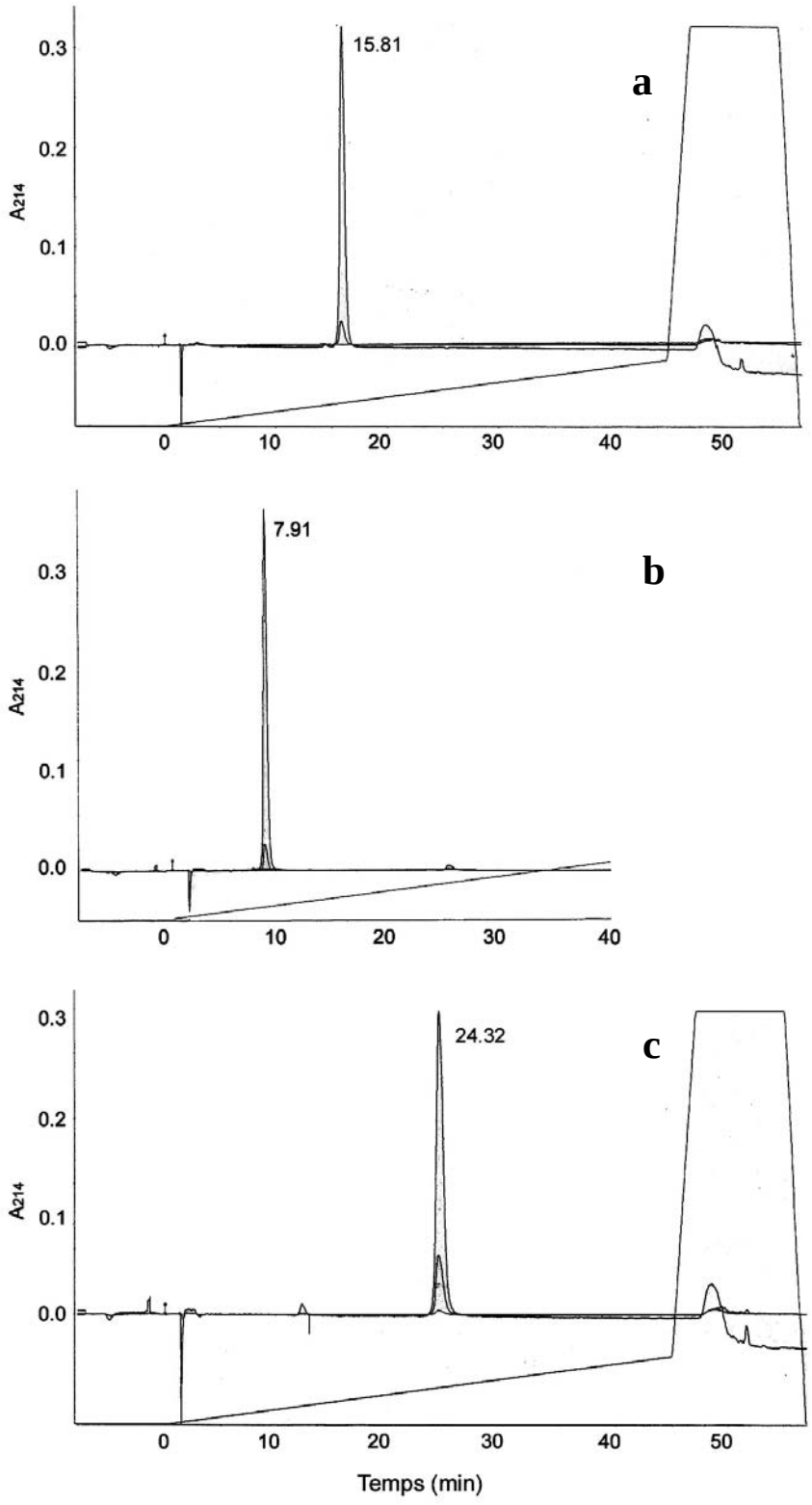
15, R_1=H, R_2=Galat, 4β-[*S*-(*N*-acetil-*O*-metilcisteinil)]epicatequin 3-*O*-galat



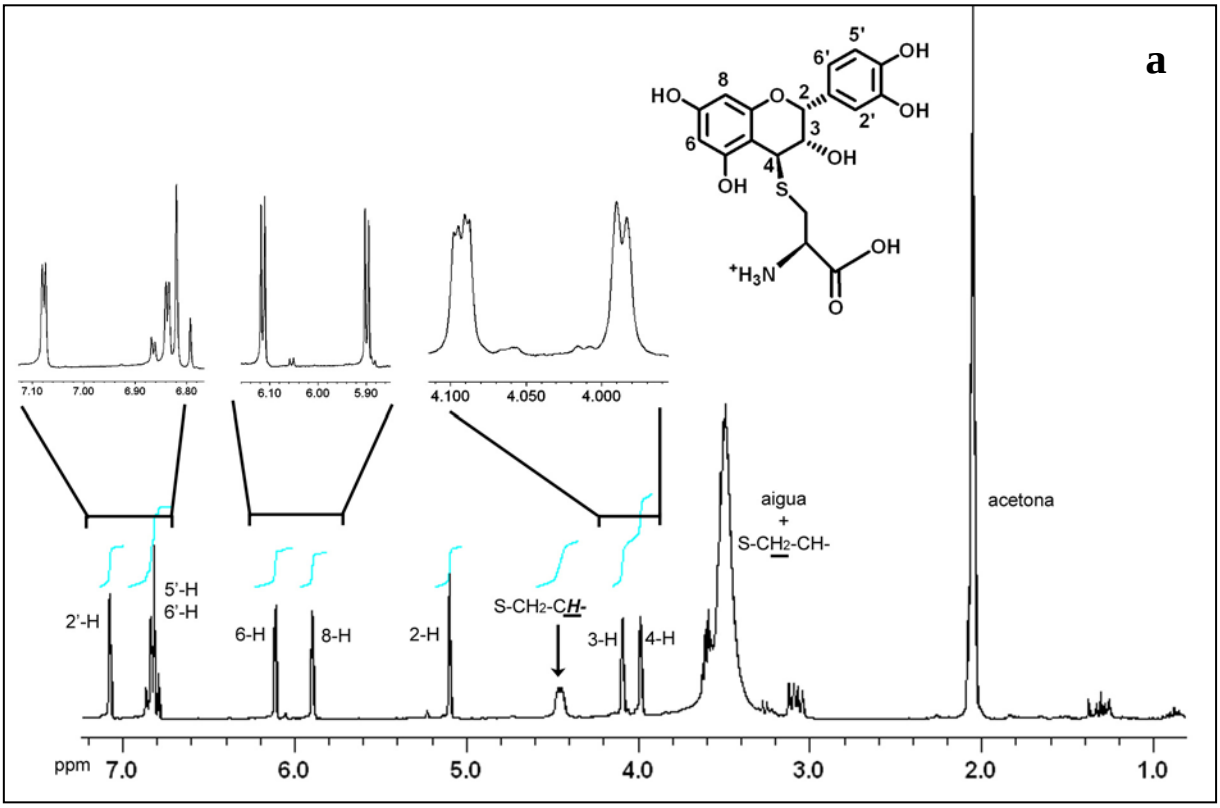
16, (–)-epigal·locatequin 3-*O*-galat

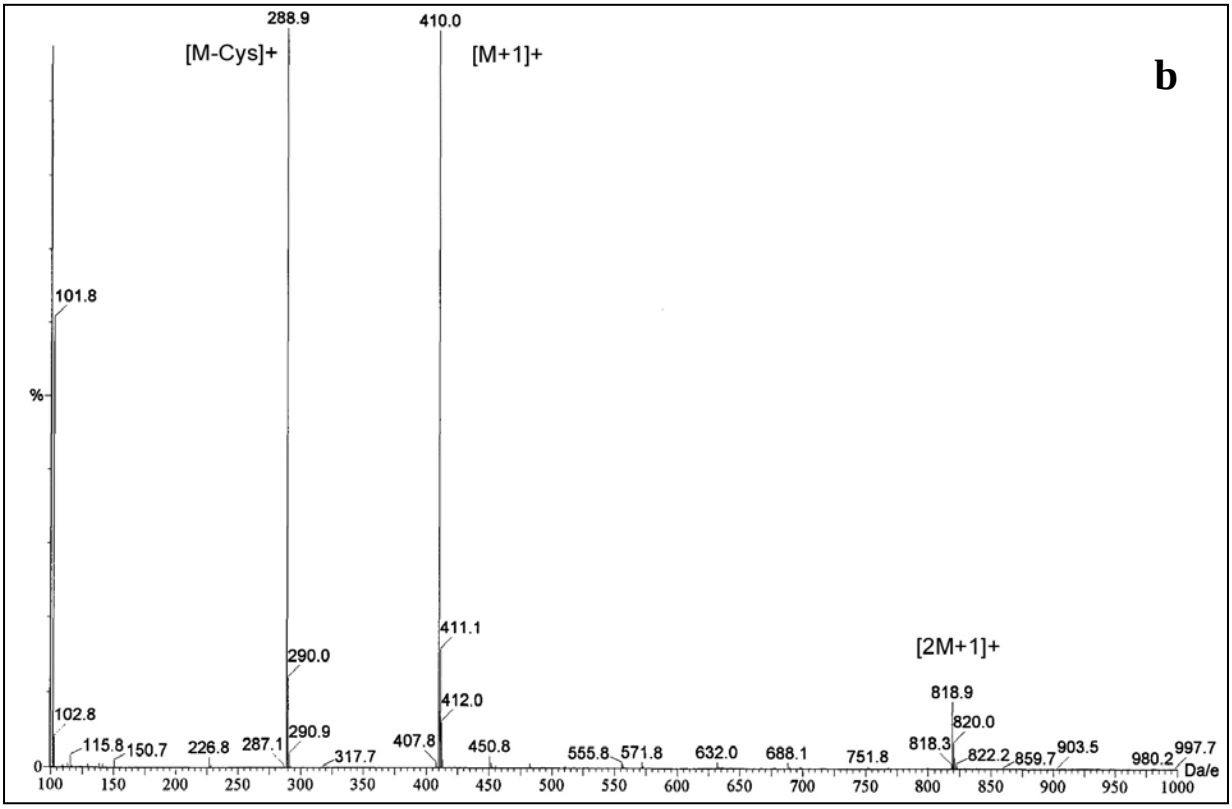


Annex I.2. Perfils de cromatografia en fase reversa on es mostren els productes derivats (**7**-**9**) després del procés de purificació de la fracció polimérica AQV. (**a**) 4β-(S-cisteinil)epicatequina **7**. (**b**) 4β-(S-cisteinil)catequina **8**. (**c**) 4β-(S-cisteinil)epicatequin 3-*O*-galat **9**. Columna, μRPC C2/C18 SC 2,1/10; 120 Å; 3 μM; 100×2,1 mm. Càrrega, 5 μL (~ 0,5 μg). Elució, de 8% a 23% de [D'] durant 45 min. Flux, 200 μL/min. Detecció 214, 280 i 320 nm.

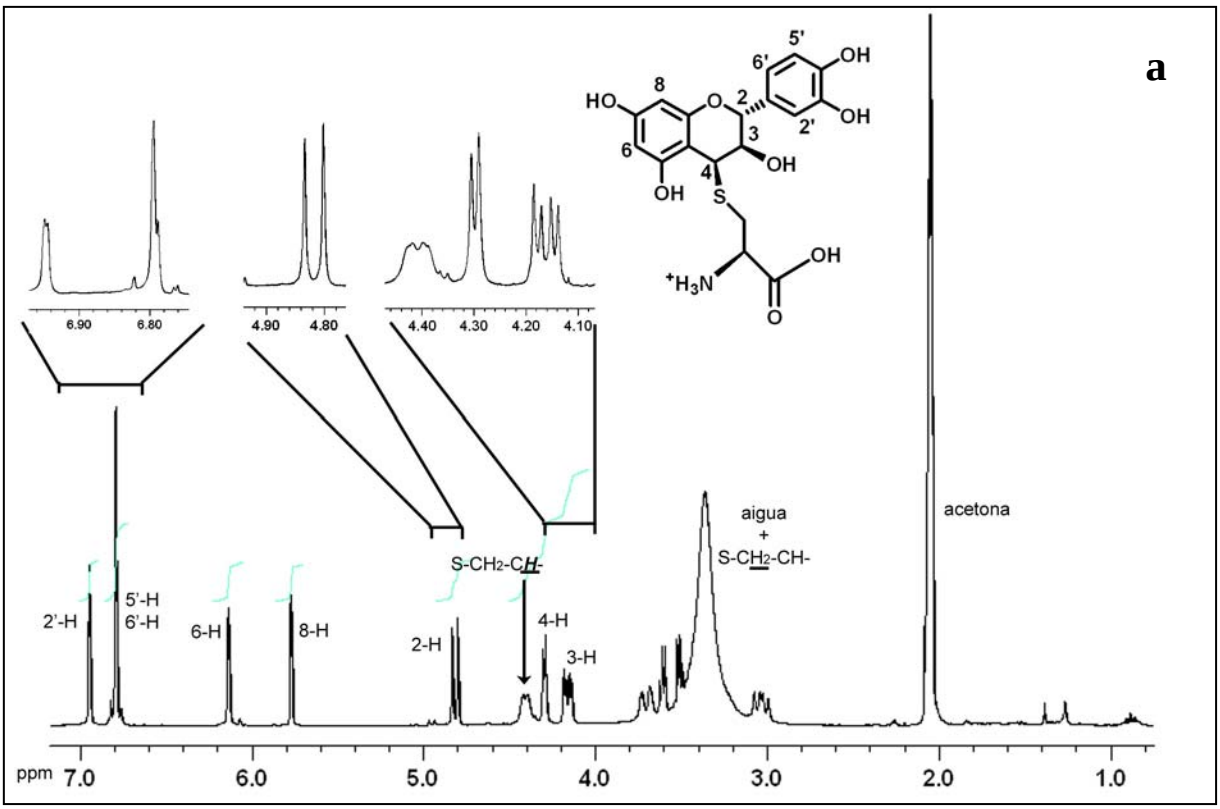


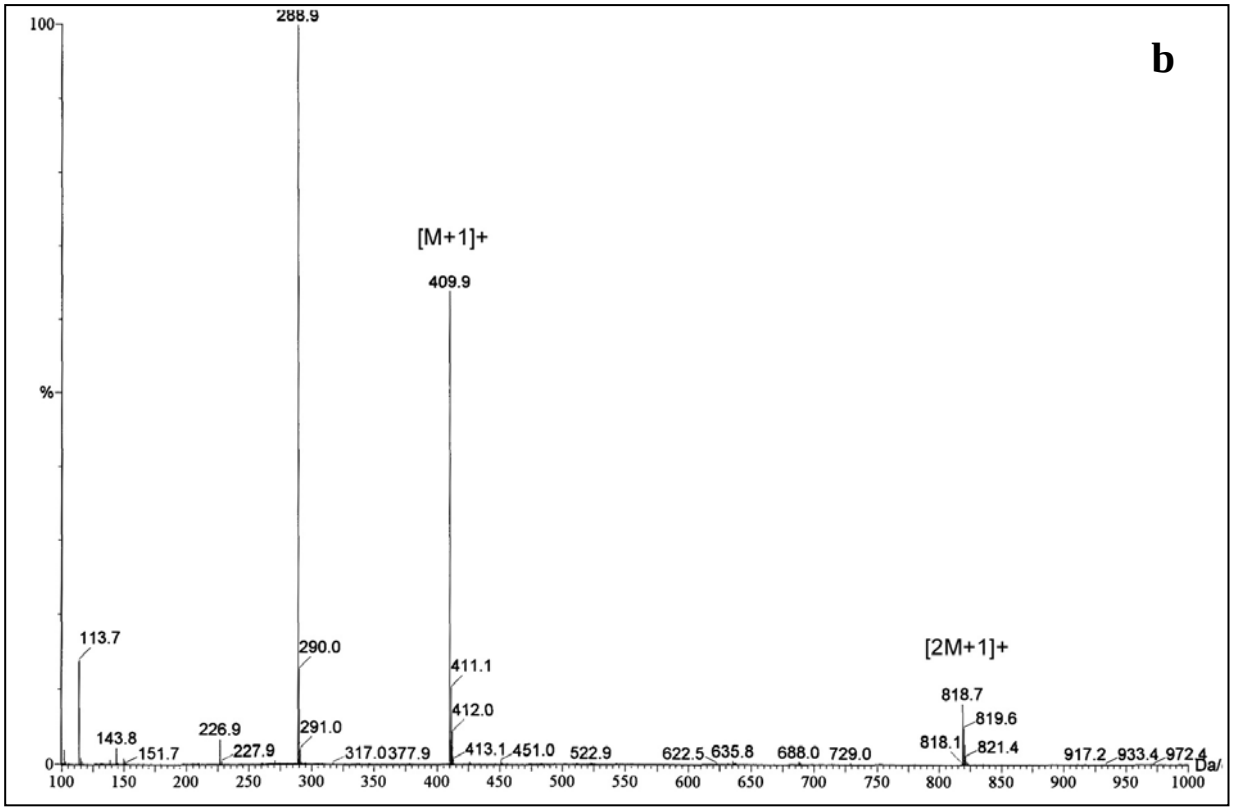
Annex I.3. (**a**) Espectre de RMN-^{1}H de la 4β-(S-cisteinil)epicatequina **7** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **7** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **7** amb mode d'ionització d'electrosprai positiu.



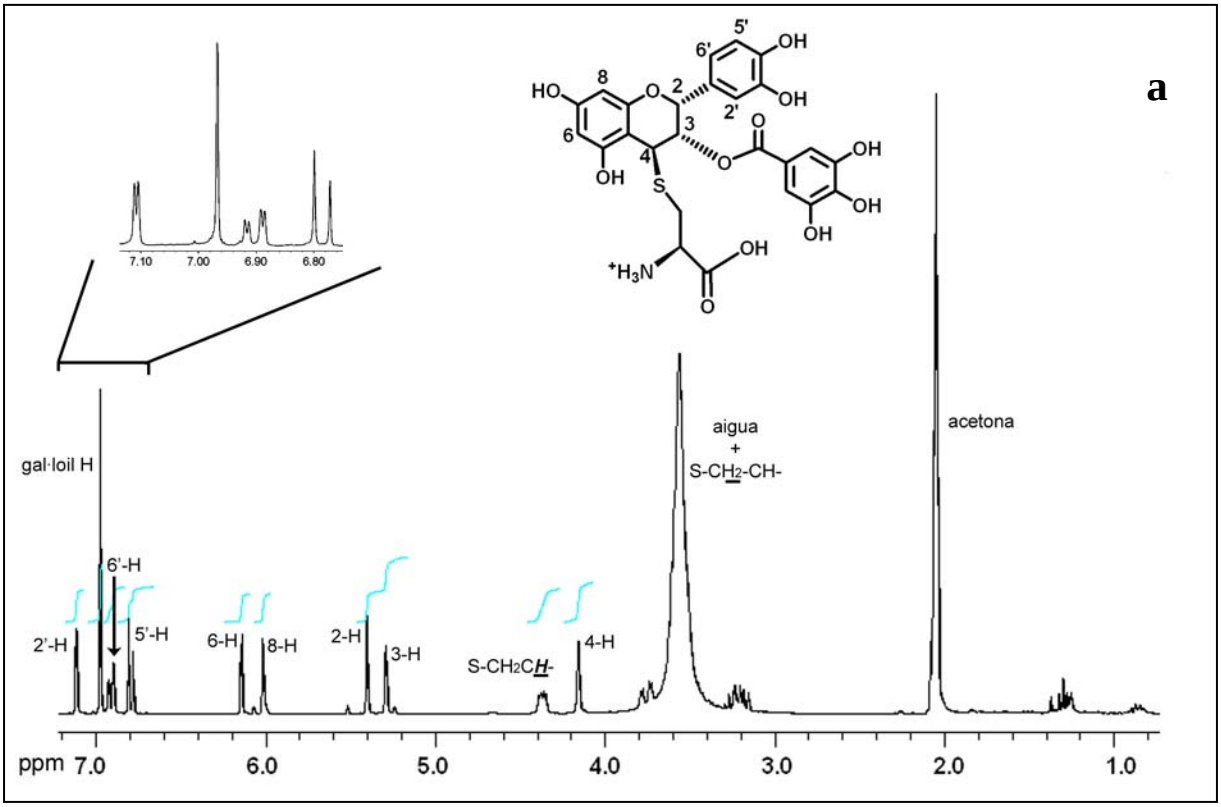


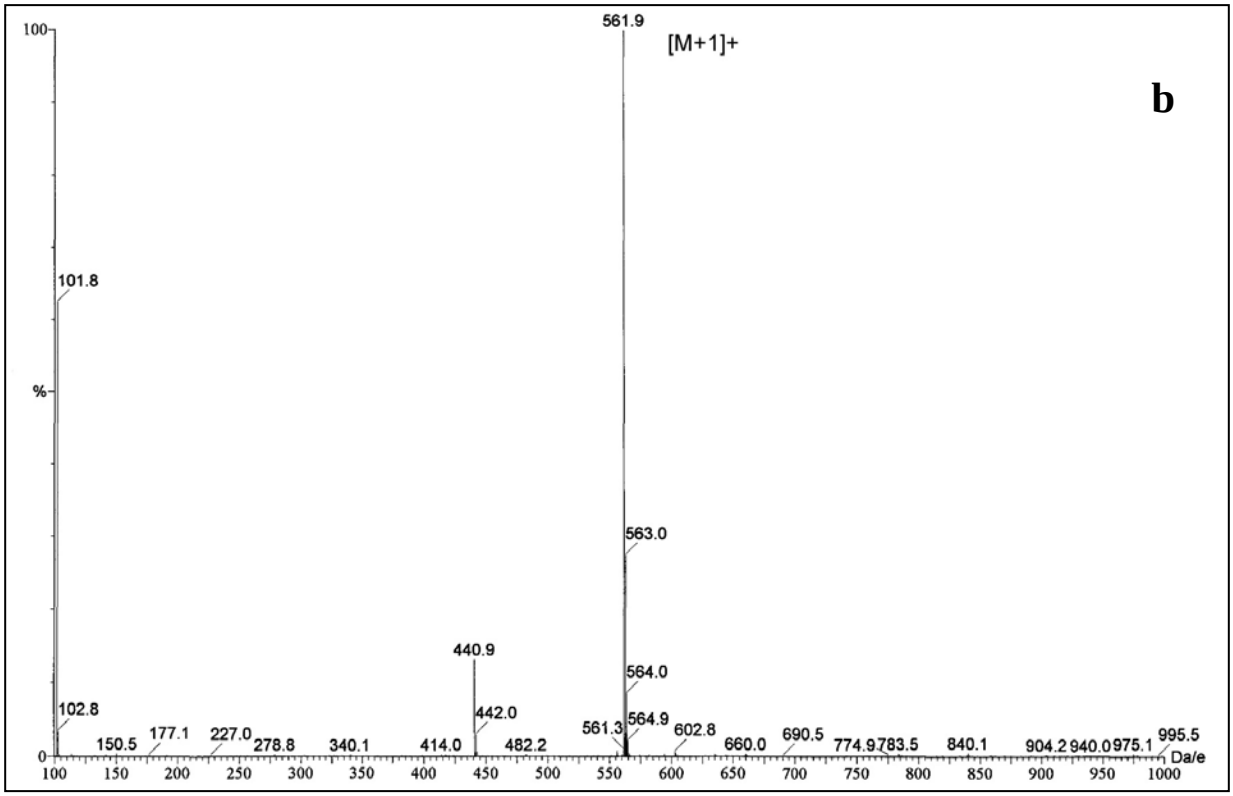
Annex I.4. (**a**) Espectre de RMN-^{1}H de la 4β-(S-cisteinil)catequina **8** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **8** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **8** amb mode d'ionització d'electrosprai positiu.



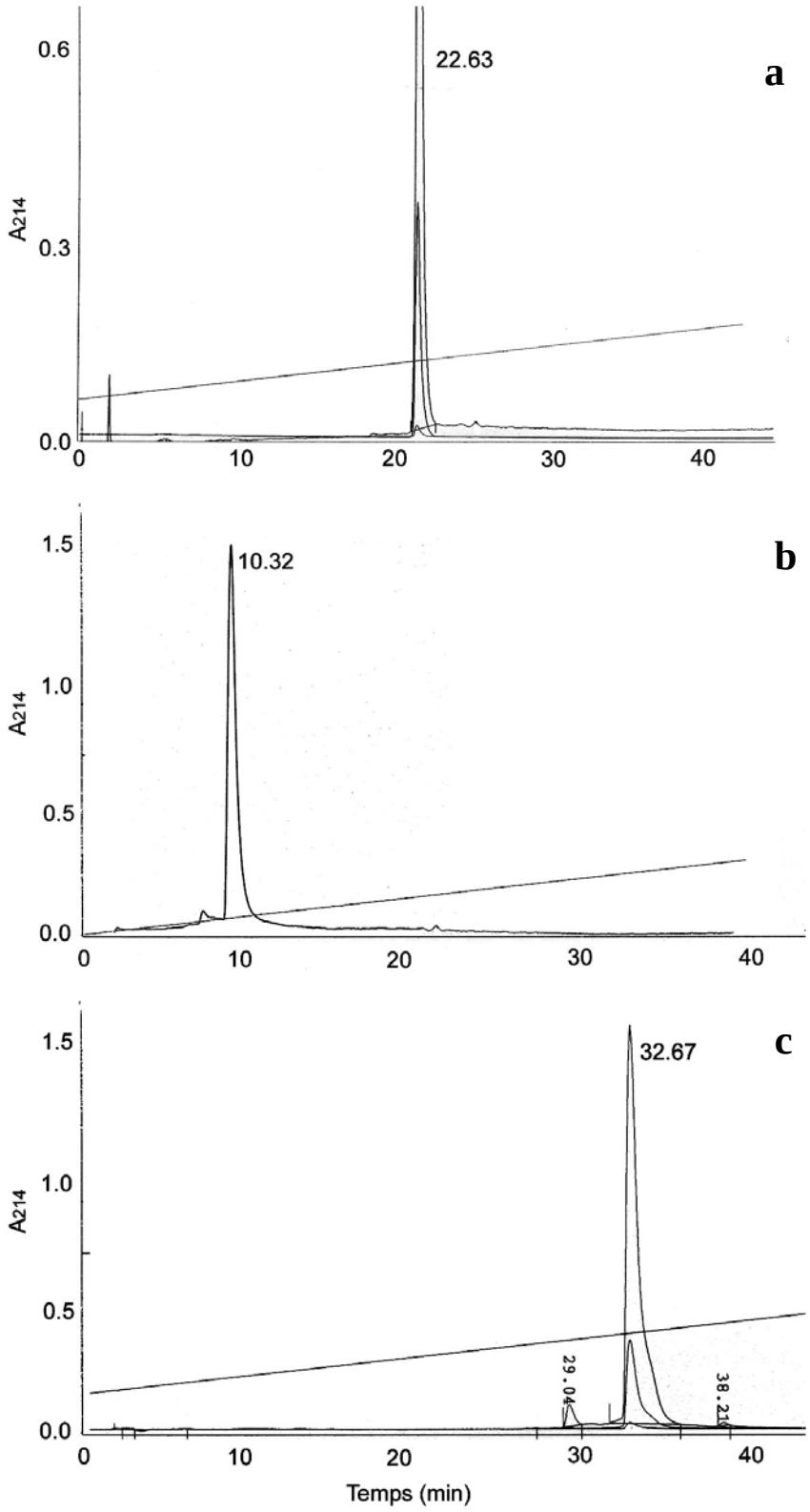


Annex I.5. (**a**) Espectre de RMN-^{1}H de la 4β-(S-cisteinil)epicatequin 3-*O*-galat **9** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **9** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **9** amb mode d'ionització d'electrosprai positiu.

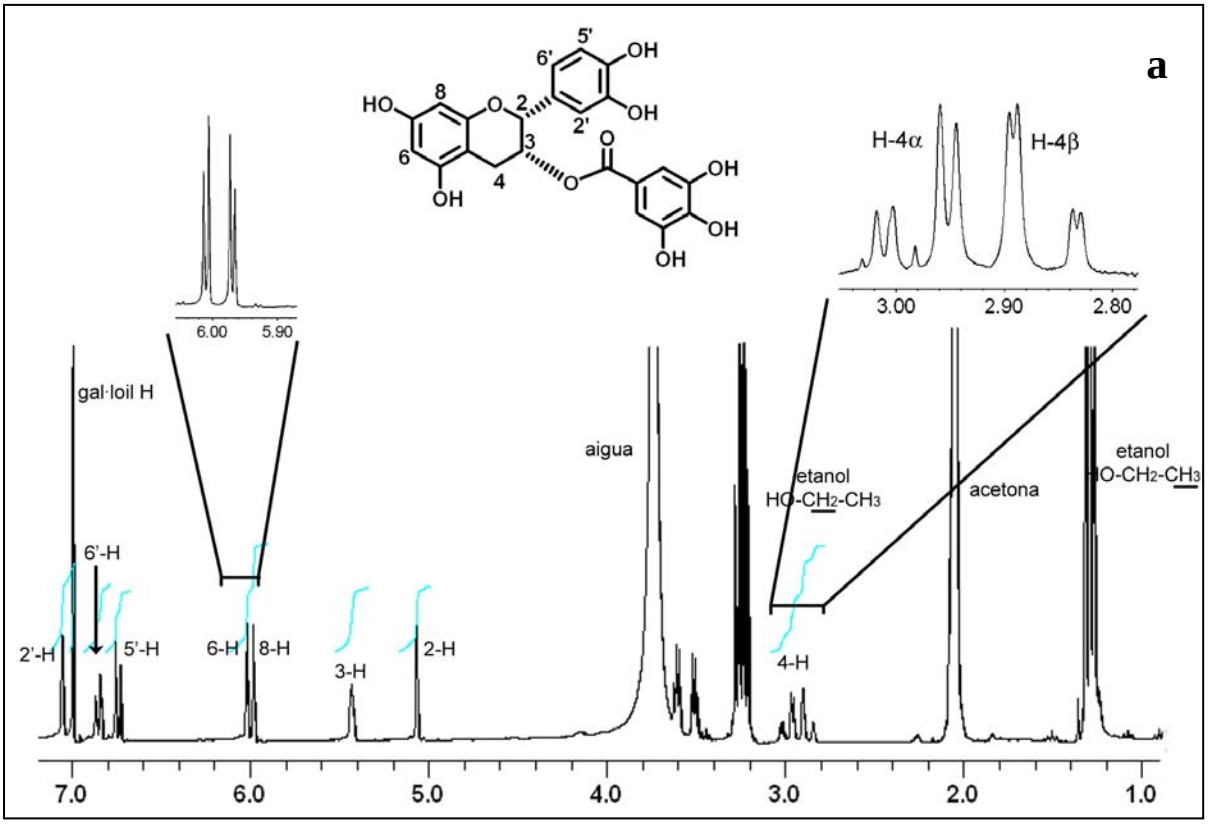


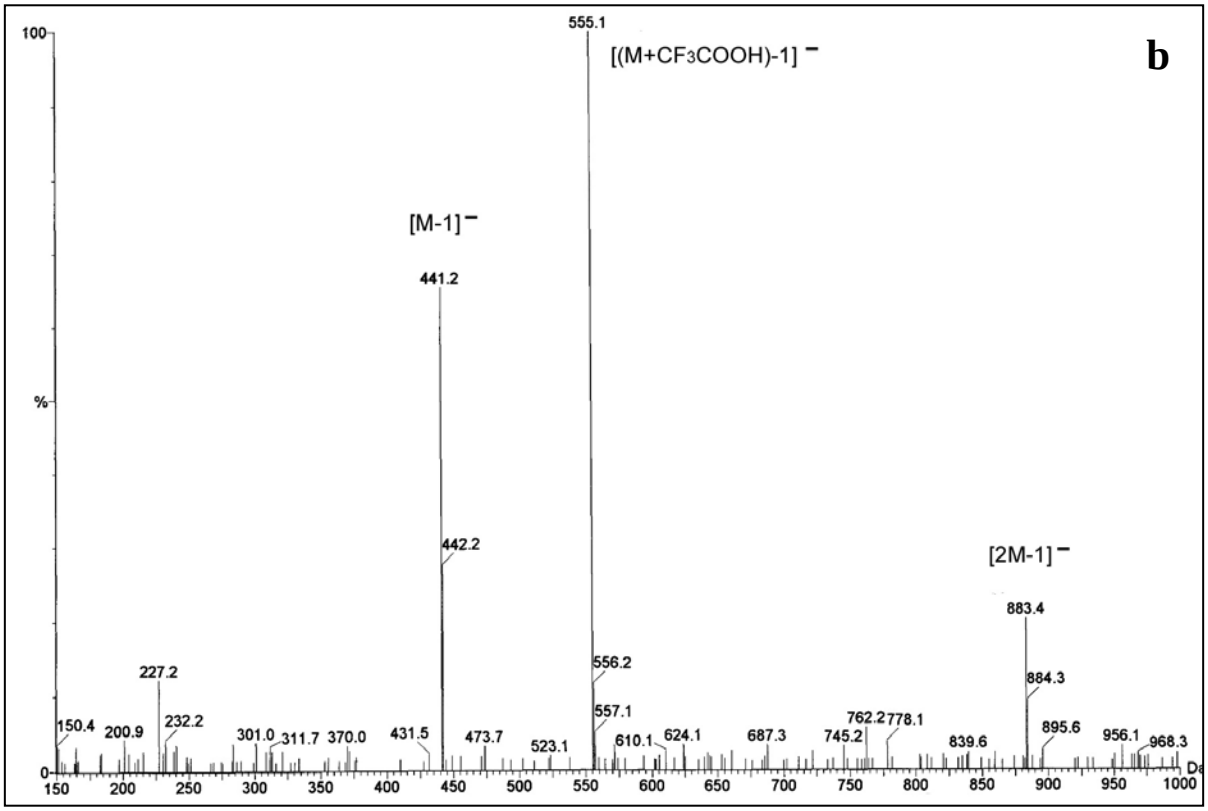


Annex I.6. Perfils de cromatografia en fase reversa on es mostra el monomer **3** purificat a partir del cru AQV, el producte derivat **7** purificat a partir del cru AQP i el producte **9** purificat a partir del cru AQH. (**a**) (—)-epicatequin 3-*O*-galat **3**. (**b**) 4β-(S-cisteinil)epicatequina **7**. (**c**) 4β-(S-cisteinil)epicatequin 3-*O*-galat **9**. Columna, μRPC C2/C18 SC 2,1/10; 120 Å; 3 μM; 100×2,1 mm. Càrrega, 10 μL (~ 1,5 μg). Elució, de 8% a 23% de [D'] durant 45 min. Flux, 200 μL/min. Detecció 214, 280 i 320 nm.

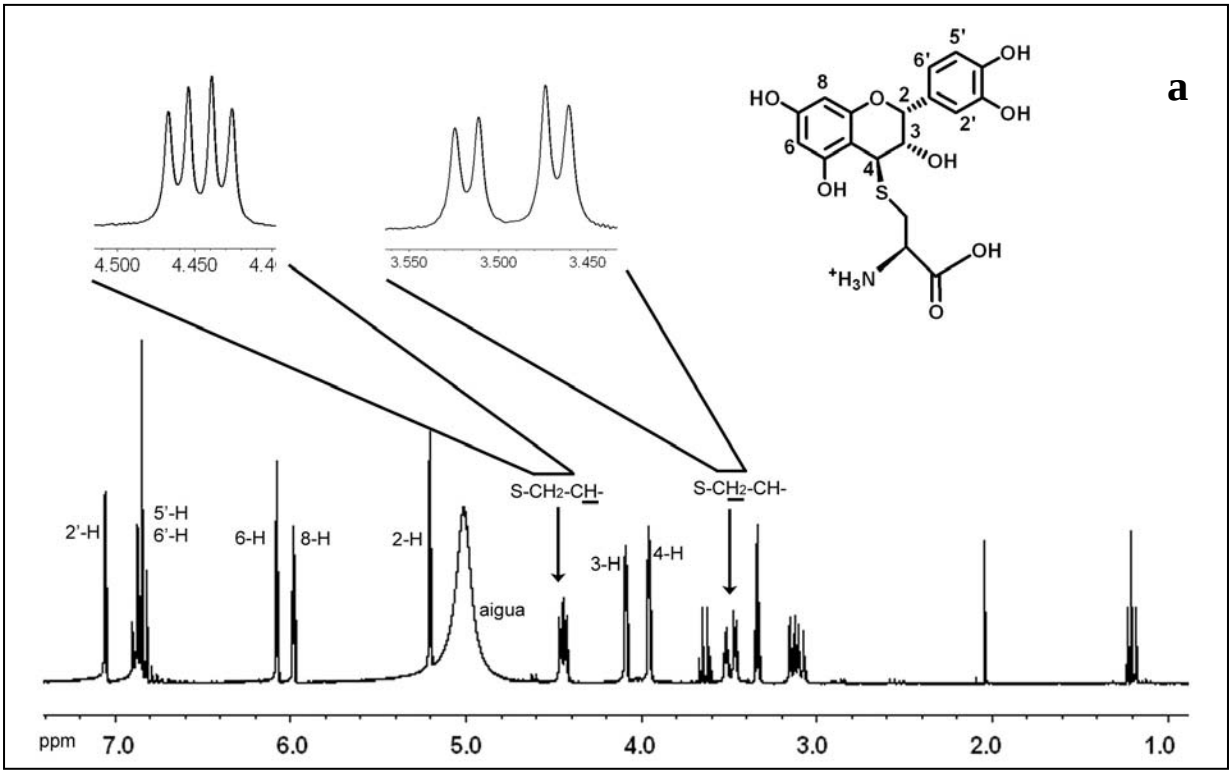


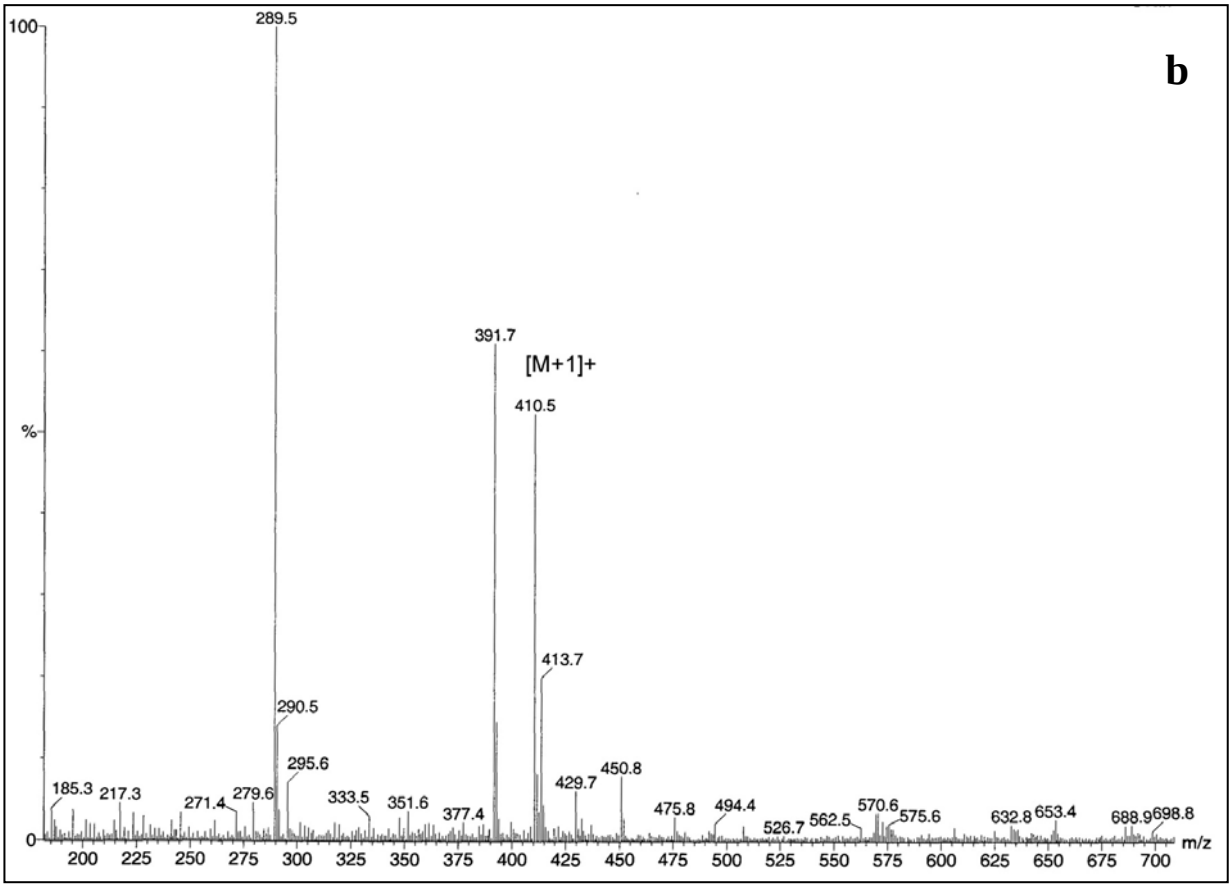
Annex I.7. (**a**) Espectre de RMN-^{1}H de la (−)-epicatequin 3-*O*-galat **3** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **3** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **3** amb mode d'ionització d'electrosprai negatiu.



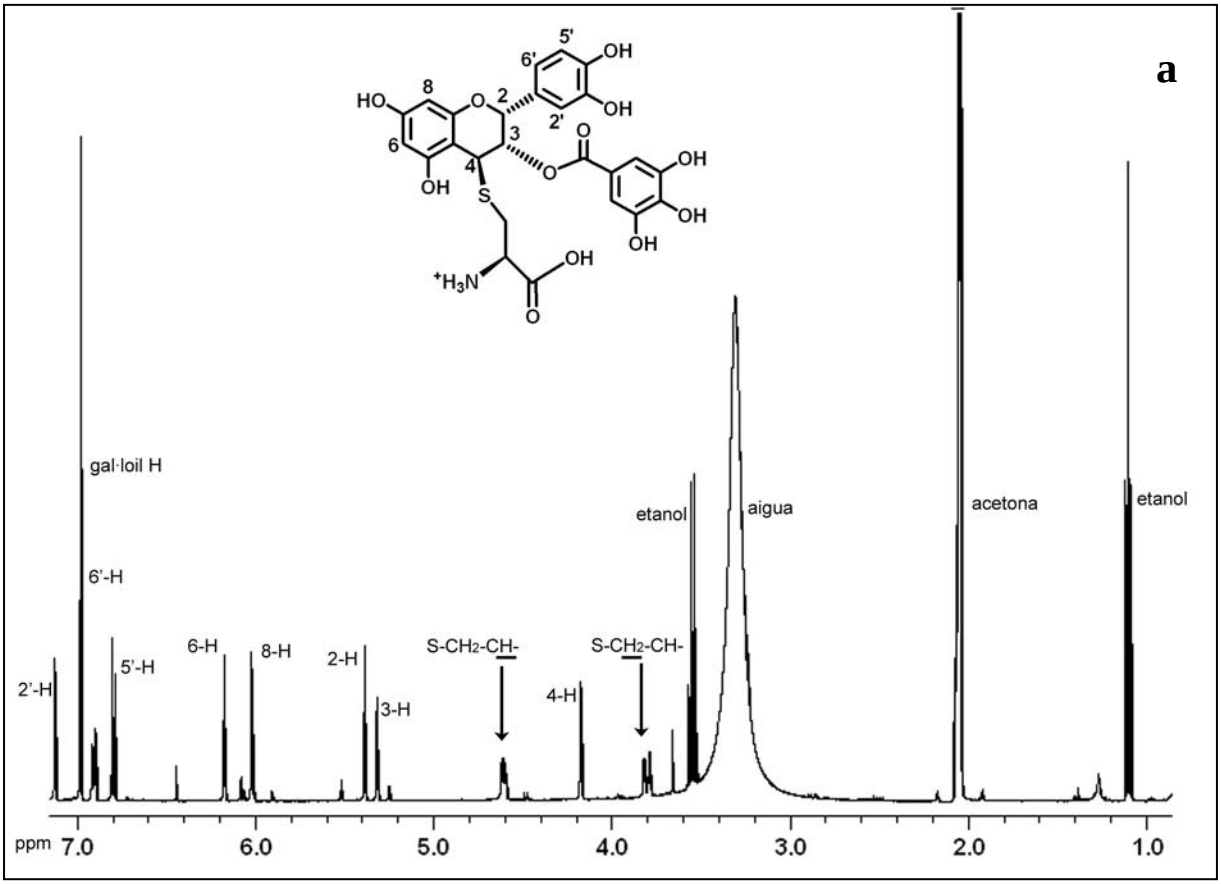


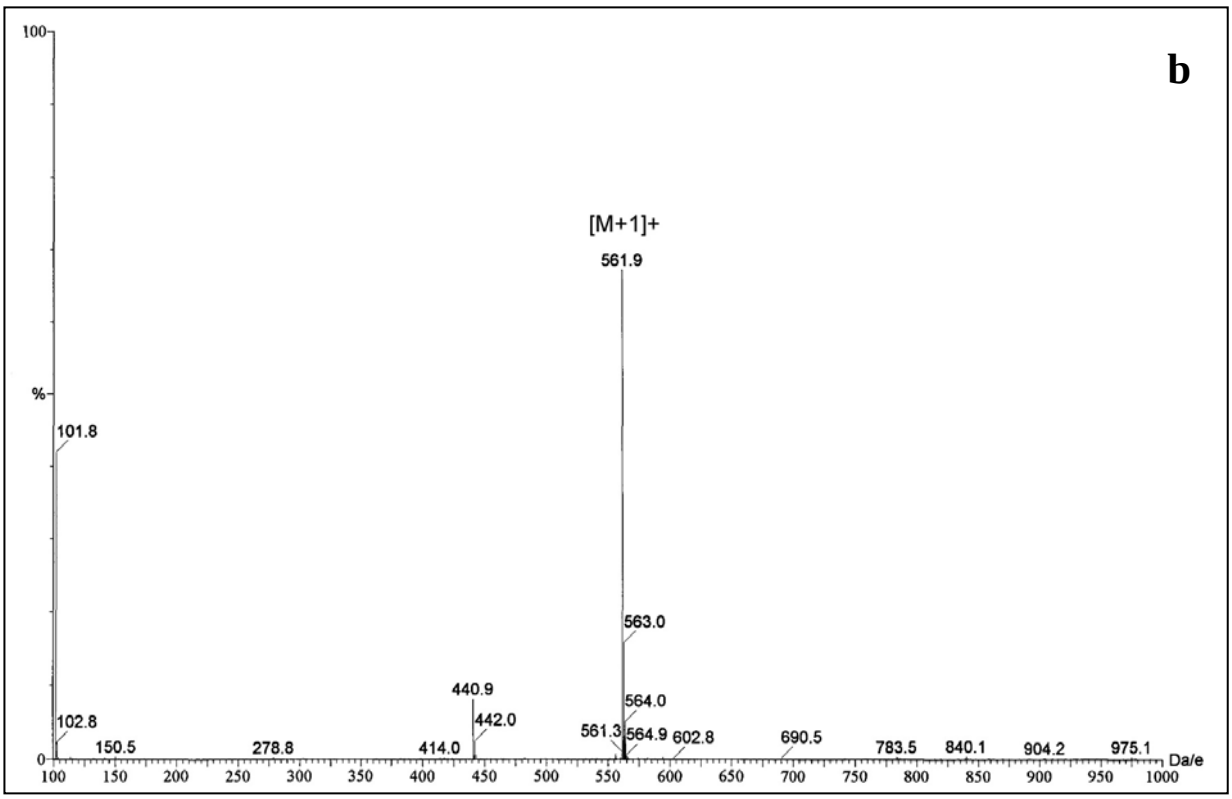
Annex I.8. (**a**) Espectre de RMN-^{1}H de la 4β-(S-cisteinil)epicatequin **7** a partir del cru AQP. Es va fer servir D_2O (0,7 mL) per dissoldre el producte **7** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **7** amb mode d'ionització d'electrosprai positiu.



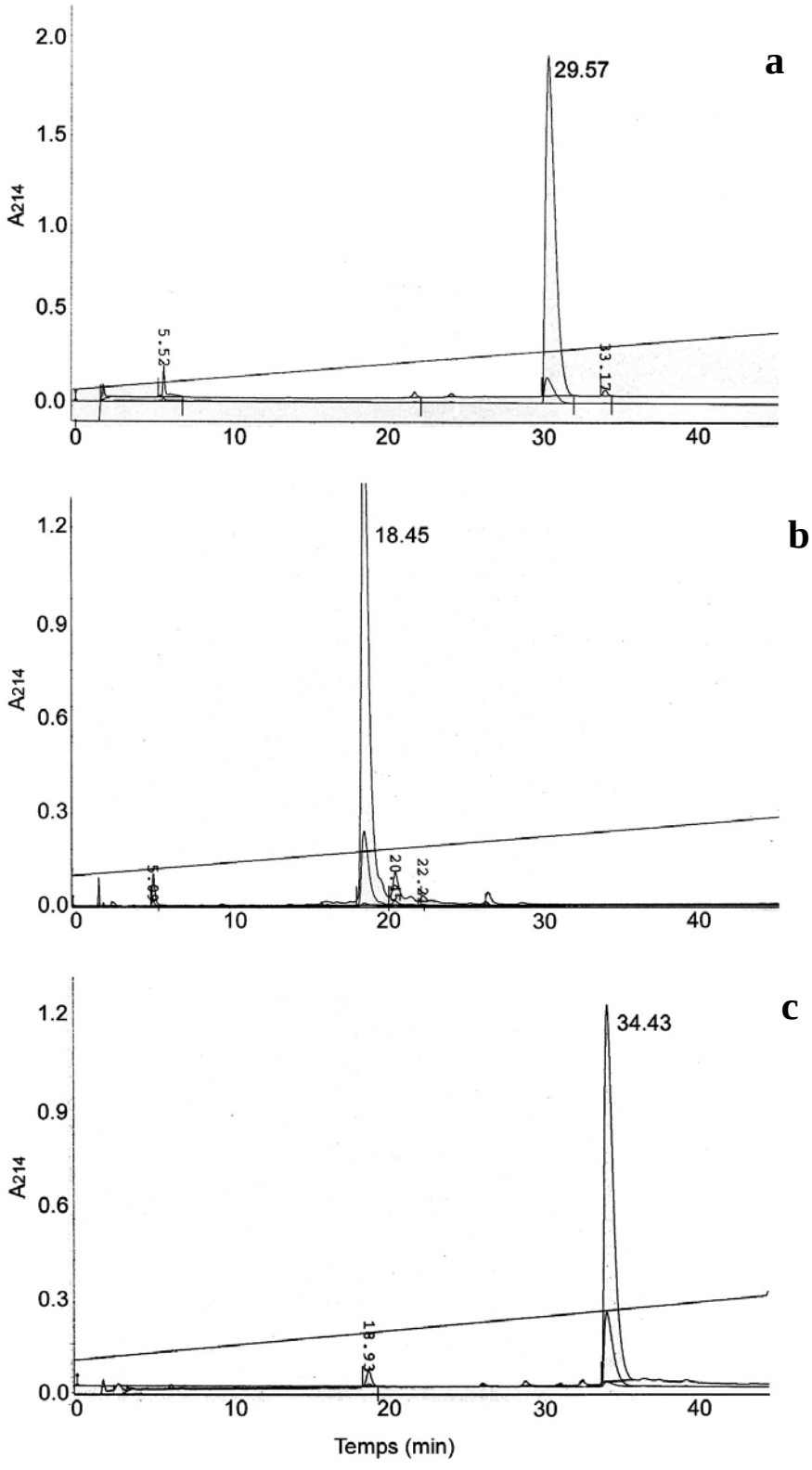


Annex I.9. (**a**) Espectre de RMN-^{1}H de la 4β-(S-cisteinil)epicatequin 3-*O*-galat **9** a partir del cru AQH. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 2 gotes de D_2O per dissoldre el producte **9** (6 mg) i TMS com a patró intern. Es realitzà a 500 MHz i 50 acumulacions. (**b**) Espectrometria de masses del producte **9** amb mode d'ionització d'electrosprai positiu.

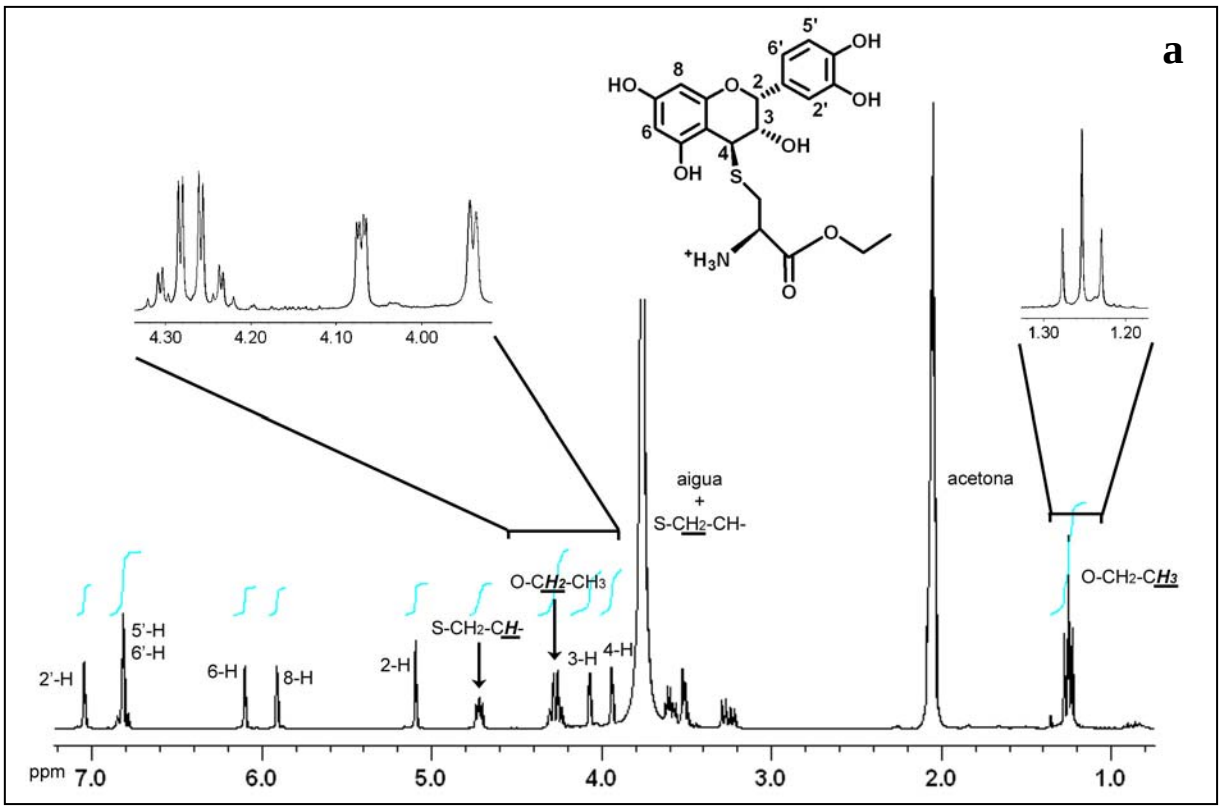


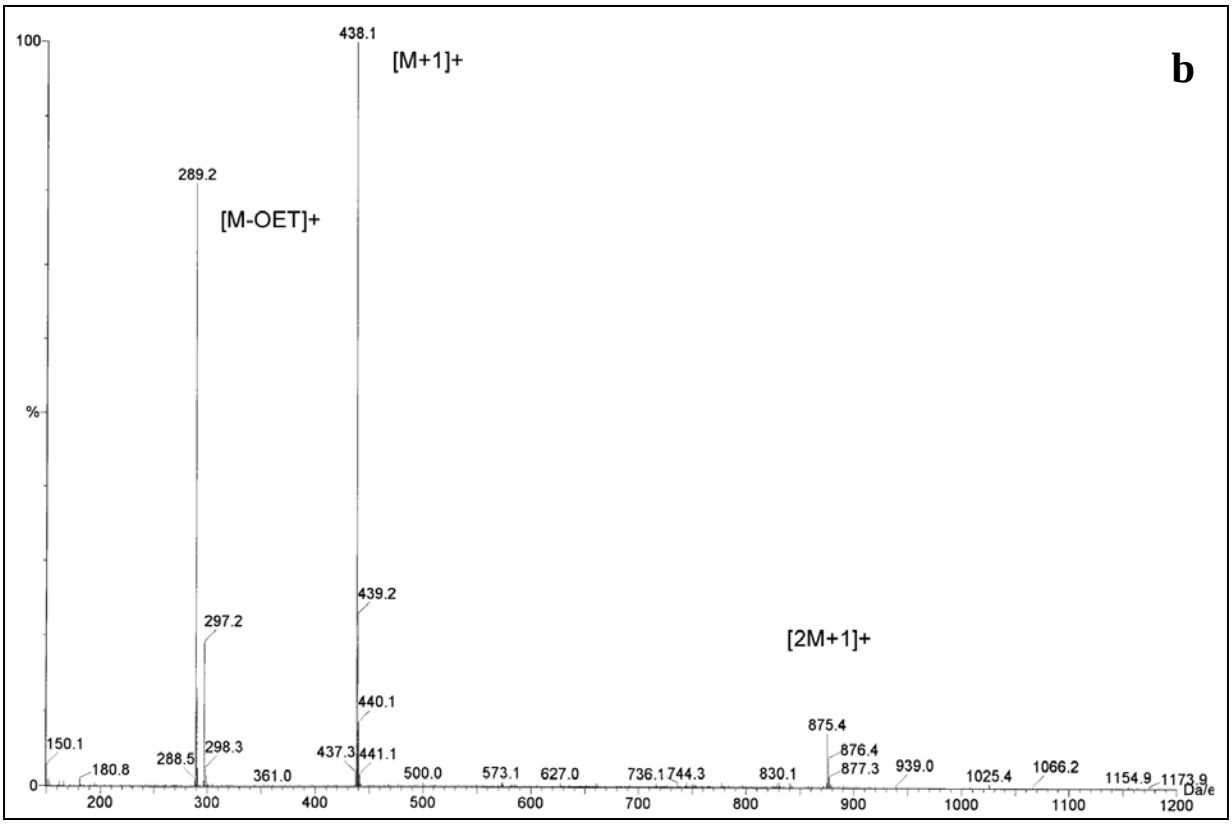


Annex I.10. Perfils de cromatografia en fase reversa on es mostren els productes derivats (**10**-**12**) després del procés de purificació de la fracció polimérica AQV. (**a**) 4β-[S-(*O*-etilcisteinil)]epicatequina **10**. (**b**) 4β-[S-(*O*-etilcisteinil)]catequina **11**. (**c**) 4β-[S-(*O*-etilcisteinil)]epicatequin 3-*O*-galat **12**. Columna, μRPC C2/C18 SC 2,1/10; 120 Å; 3 μM; 100×2,1 mm. Càrrega, 10 μL (~ 1,5 μg). Elució, de 8% a 23% de [D'] durant 45 min. Flux, 200 μL/min. Detecció 214, 280 i 320 nm.

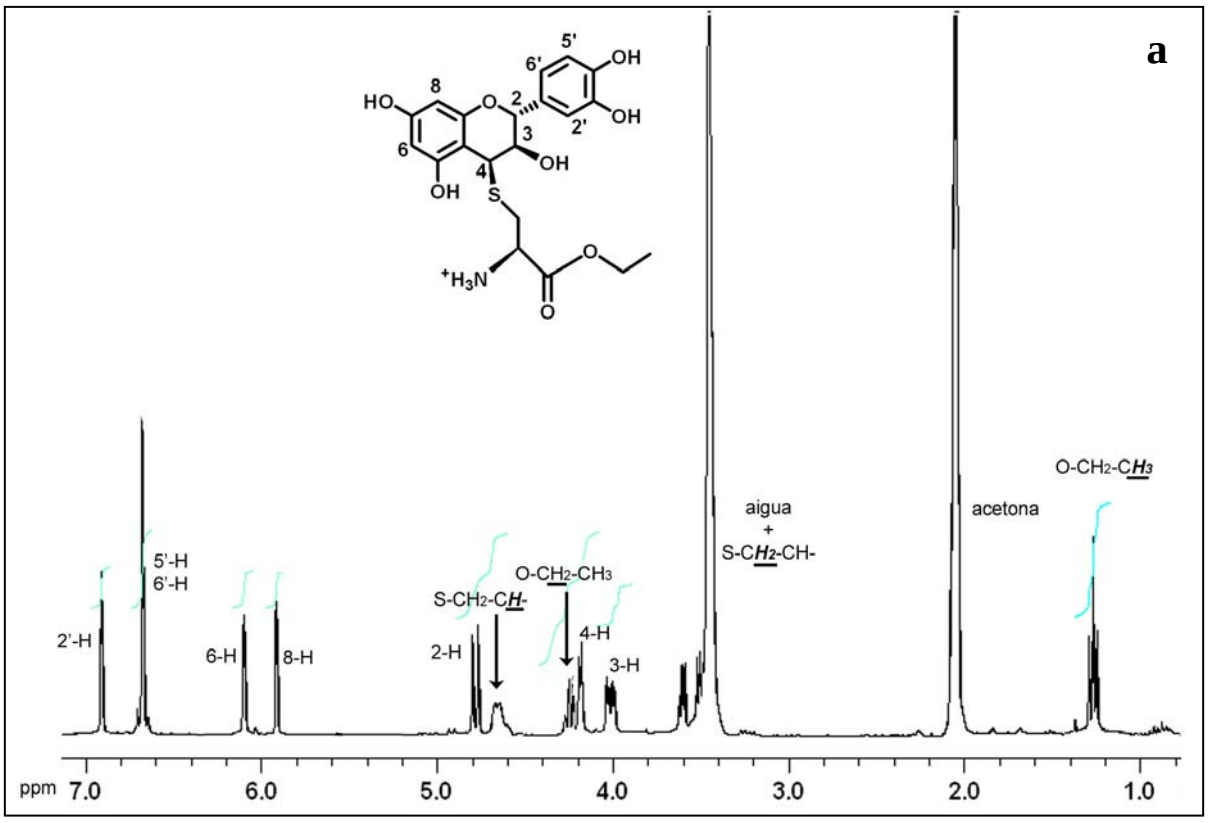


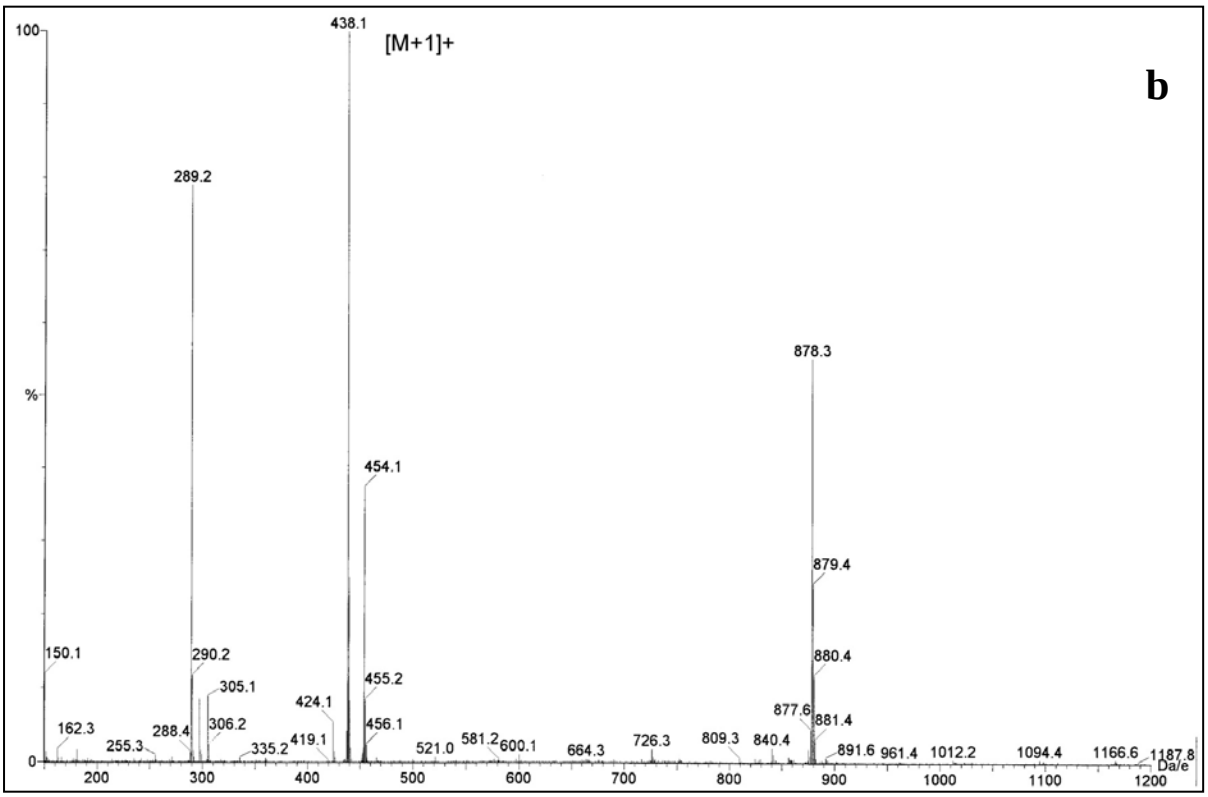
Annex I.11. (**a**) Espectre de RMN-^{1}H de la 4β-[S-(*O*-etilcisteinil)]epicatequina **10** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **10** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **10** amb mode d'ionització d'electrosprai positiu.



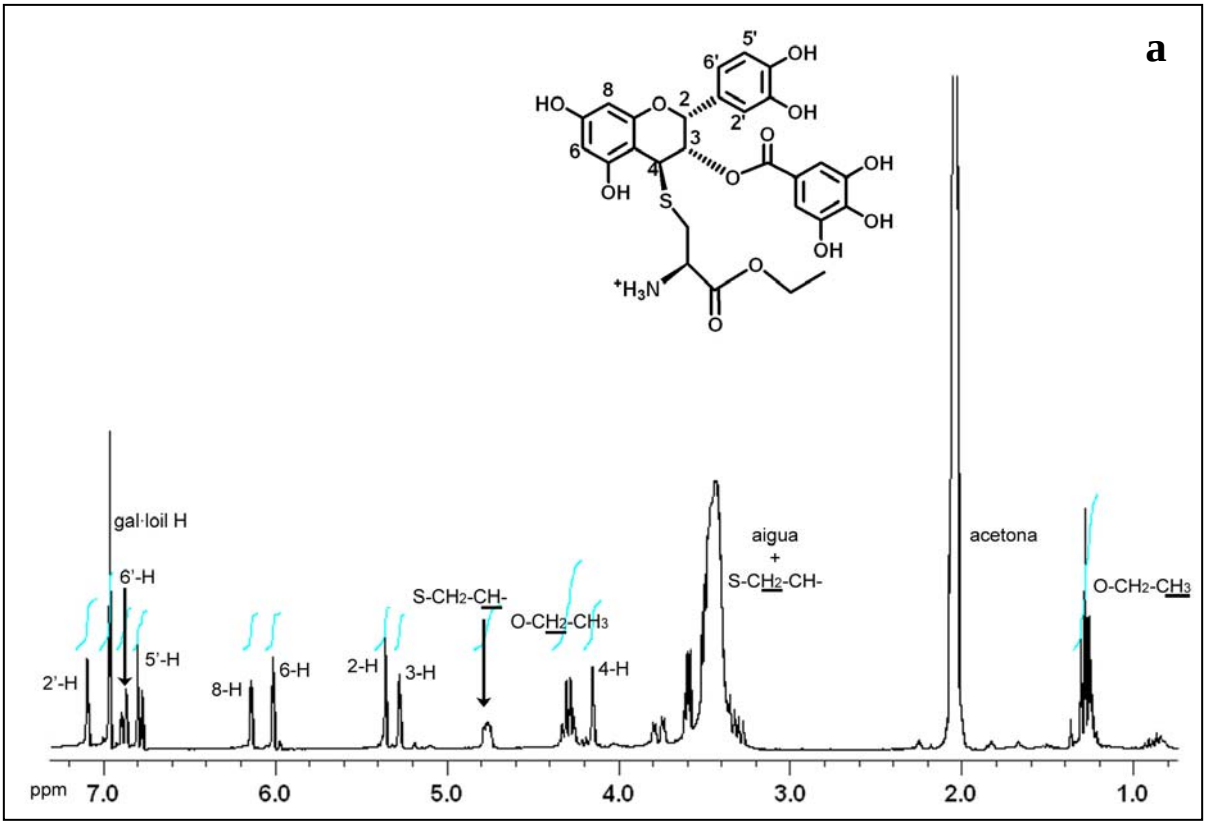


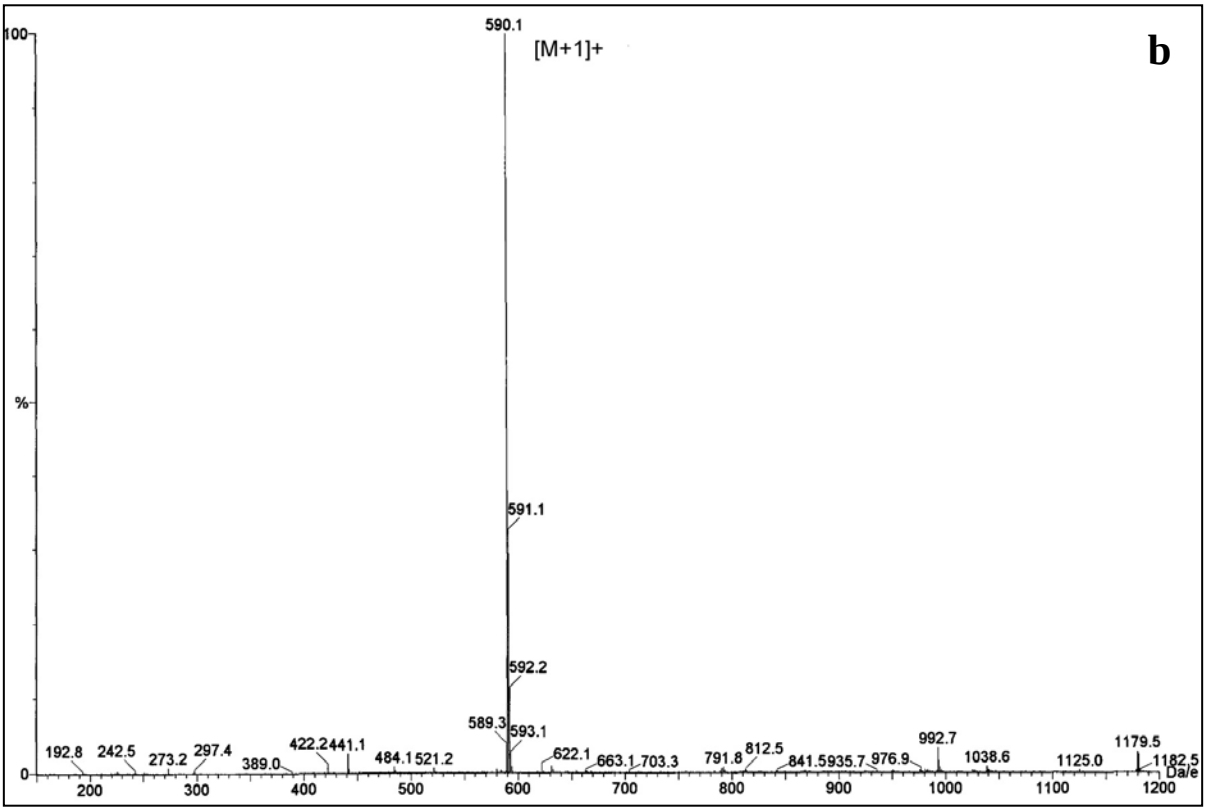
Annex I.12. (**a**) Espectre de RMN-^{1}H de la 4β-[S-(*O*-etilcisteinil)]catequina **11** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **11** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 512 acumulacions. (**b**) Espectrometria de masses del producte **11** amb mode d'ionització d'electrosprai positiu.



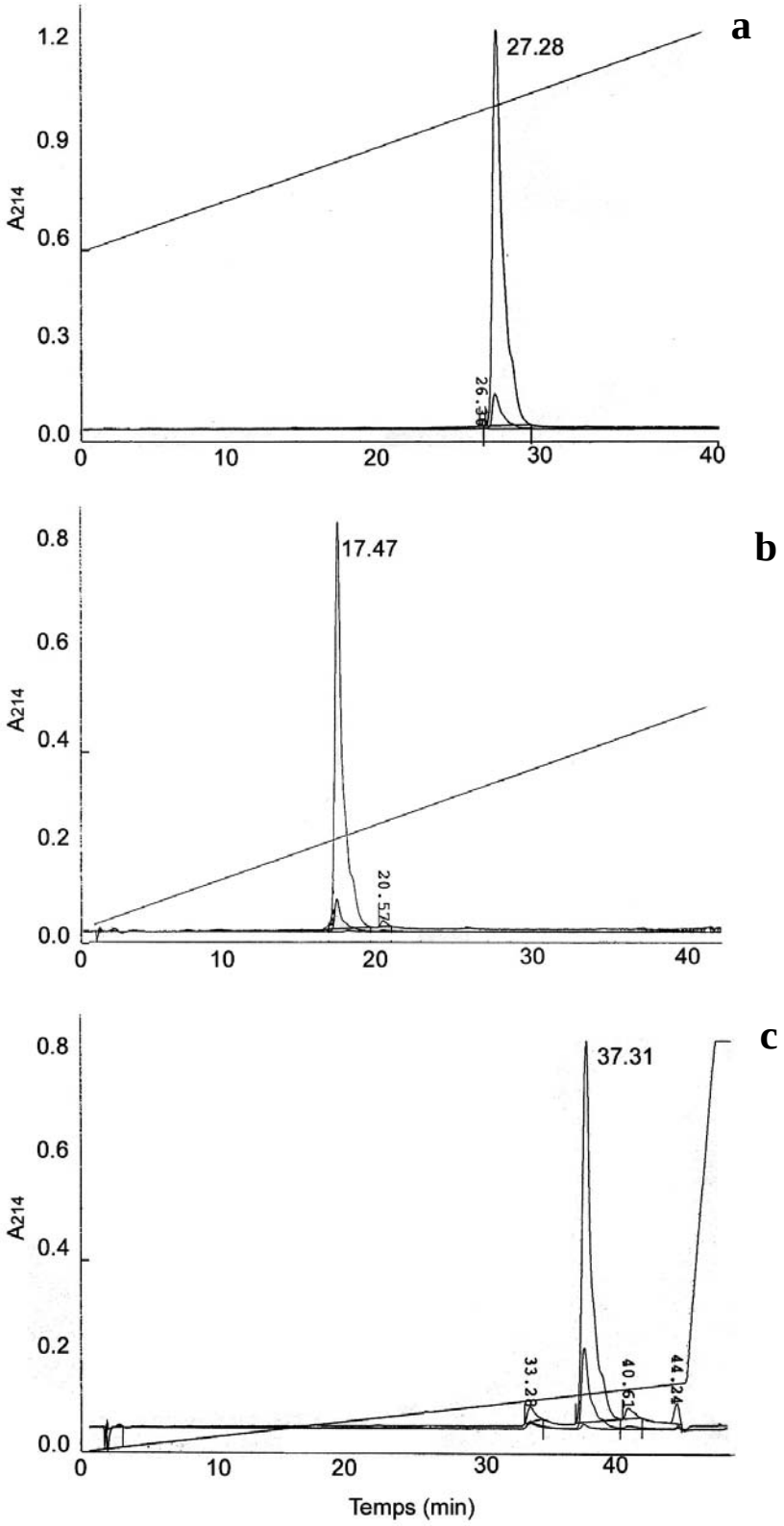


Annex I.13. (**a**) Espectre de RMN-^{1}H de la 4β-[S-(*O*-etilcisteinil)]epicatequin 3-O-galat **12** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **12** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **12** amb mode d'ionització d'electrosprai positiu.

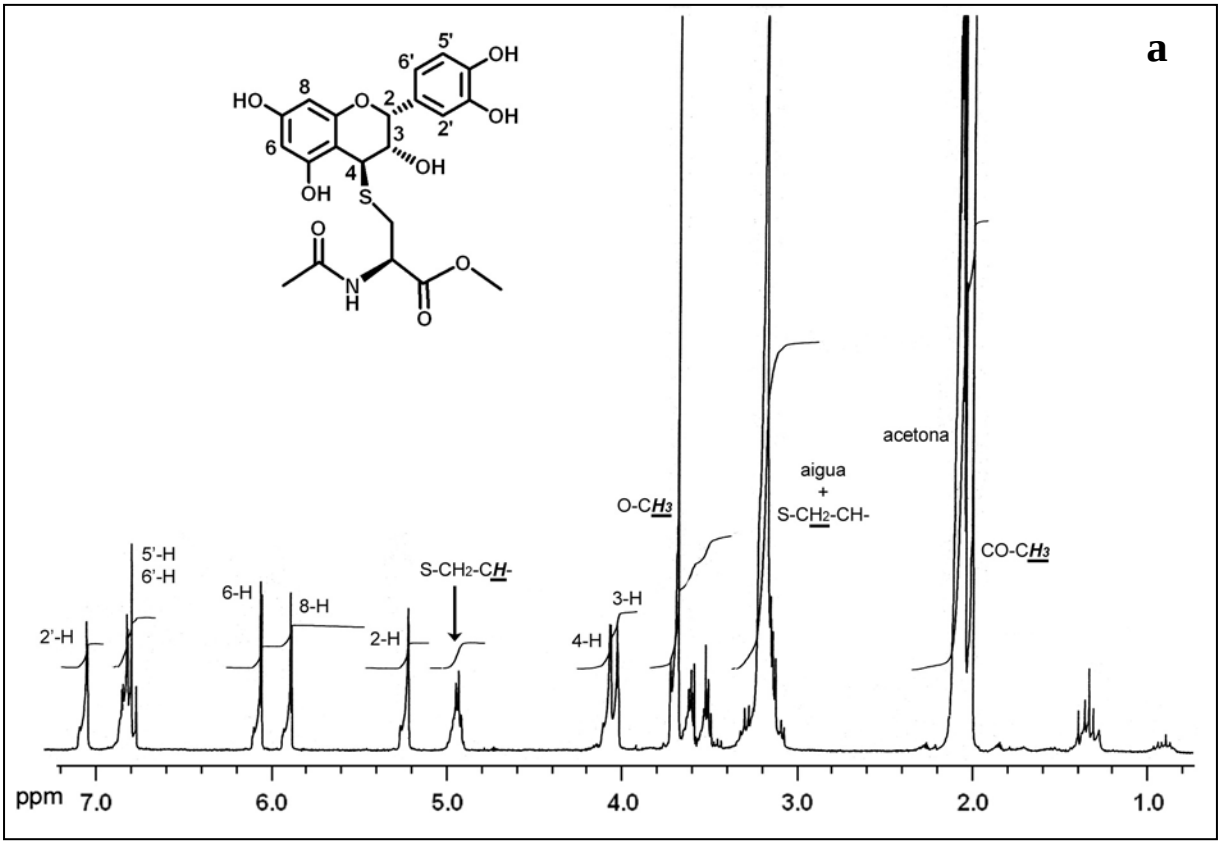


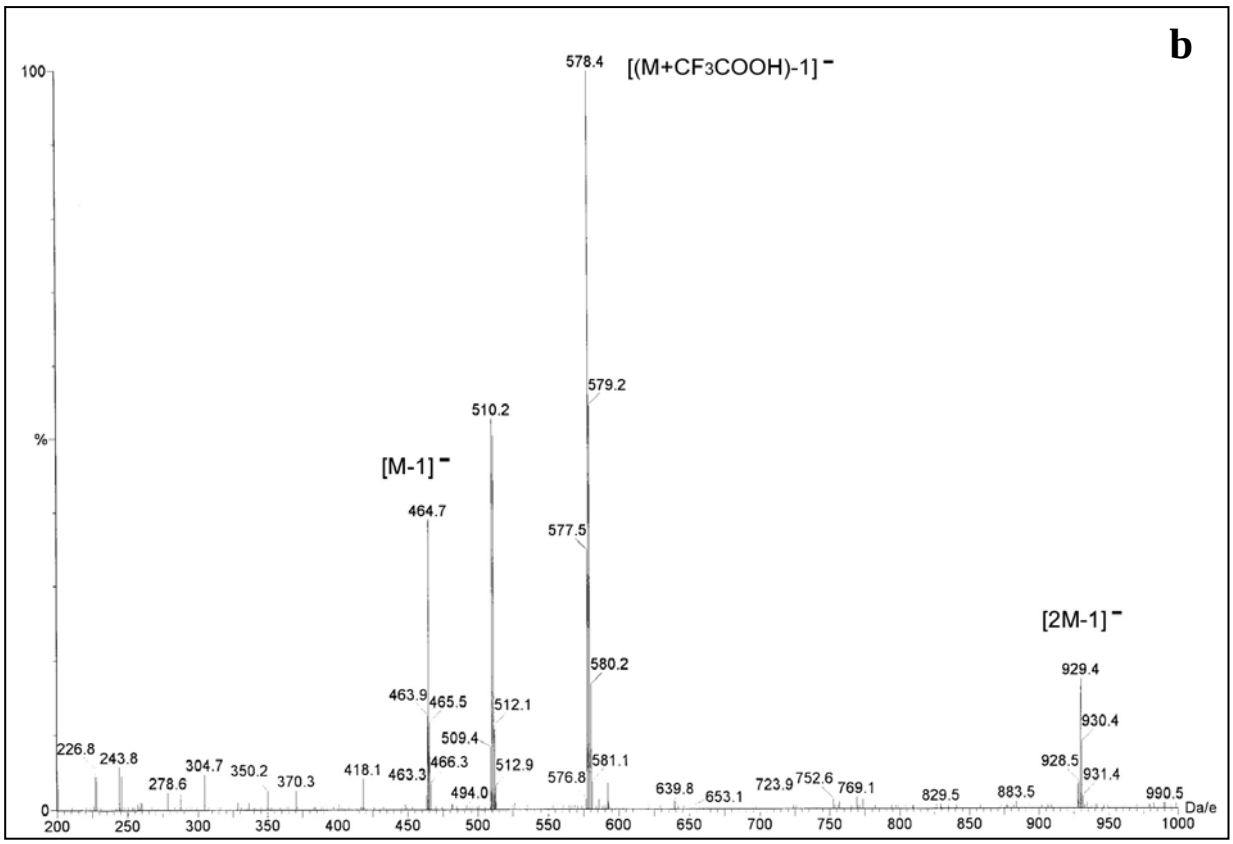


Annex I.14. Perfils de cromatografia en fase reversa on es mostren els productes derivats (**13**-**15**) després del procés de purificació de la fracció polimérica AQV. (**a**) 4β-[S-(*N*-acetil-*O*-metilcisteinil)]epicatequina **13**. (**b**) 4β-[S-(*N*-acetil-*O*-metilcisteinil)]catequina **14**. (**c**) 4β-[S-(*N*-acetil-*O*-metilcisteinil)]epicatequin 3-*O*-galat **15**. Columna, μRPC C2/C18 SC 2,1/10; 120 Å; 3 μM; 100×2,1 mm. Càrrega, 10 μL (~ 1,2 μg). Elució, de 12% a 27% de [D] durant 45 min. Flux, 200 μL/min. Detecció 214, 280 i 320 nm.

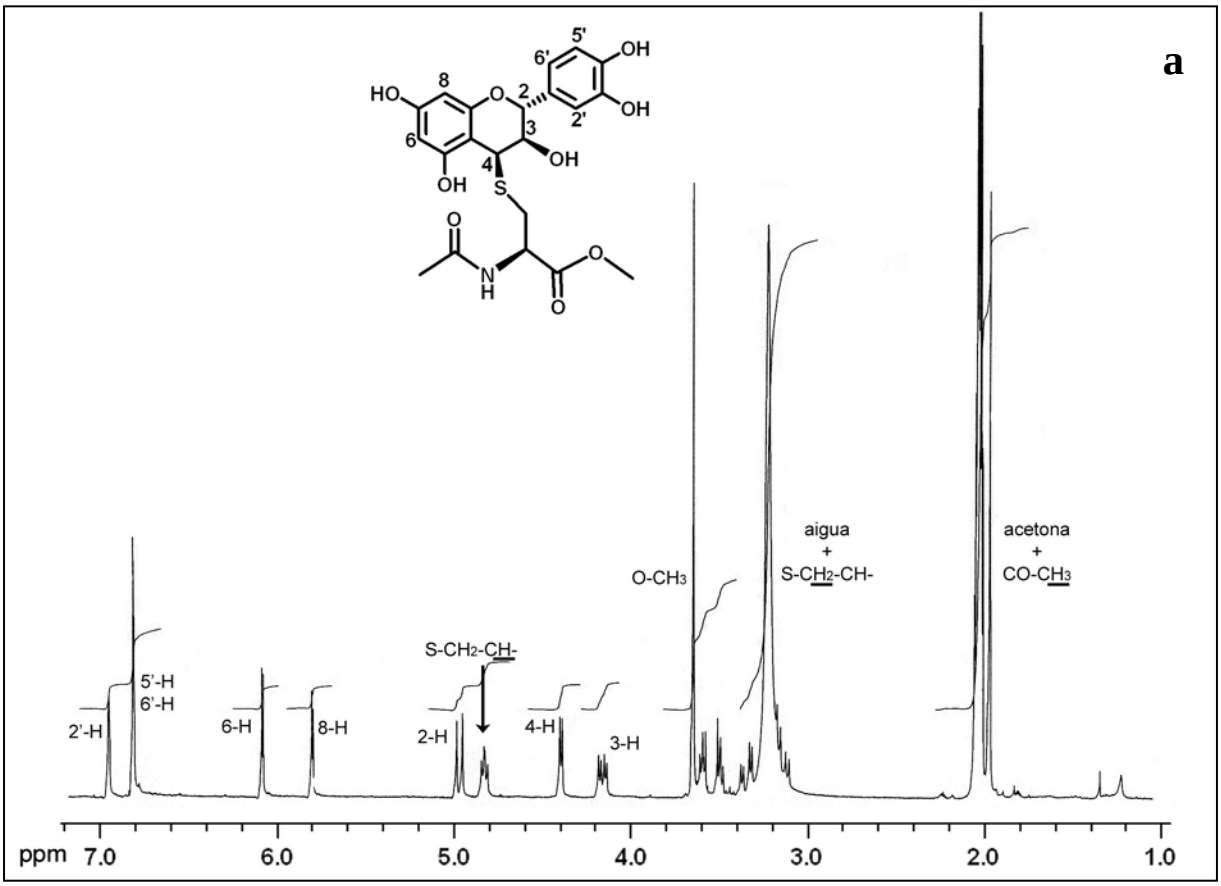


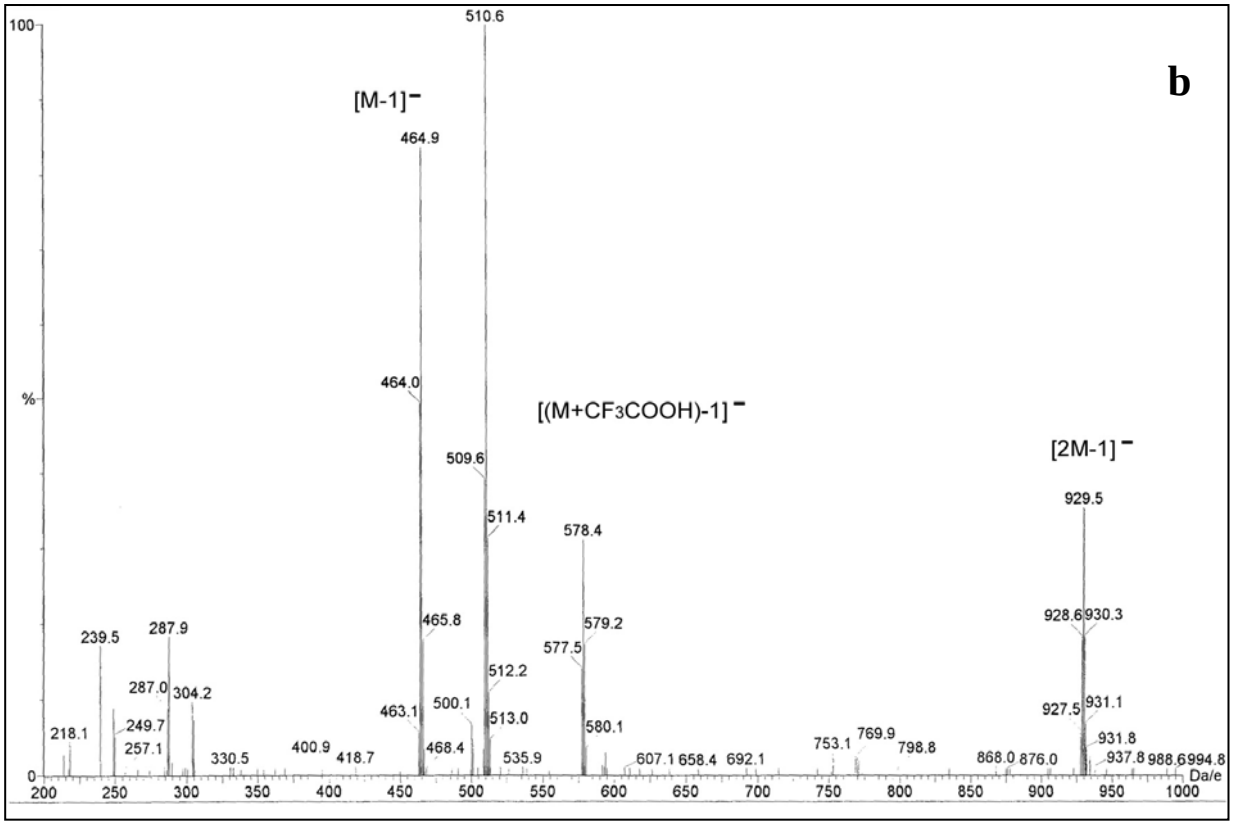
Annex I.15. (**a**) Espectre de RMN-^{1}H de la 4β-[S-(*N*-acetil-*O*-metilcisteinil)]epicatequina **13** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **13** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **13** amb mode d'ionització d'electrosprai negatiu.



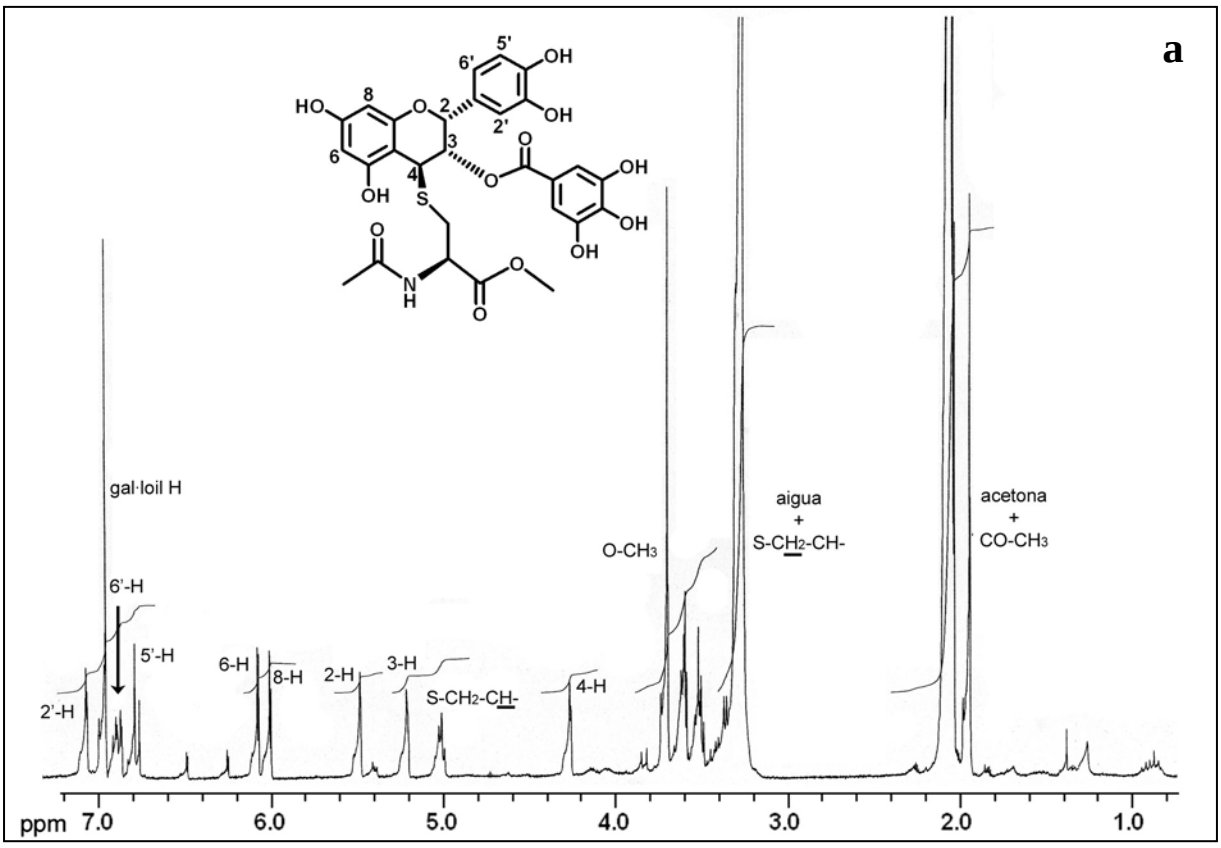


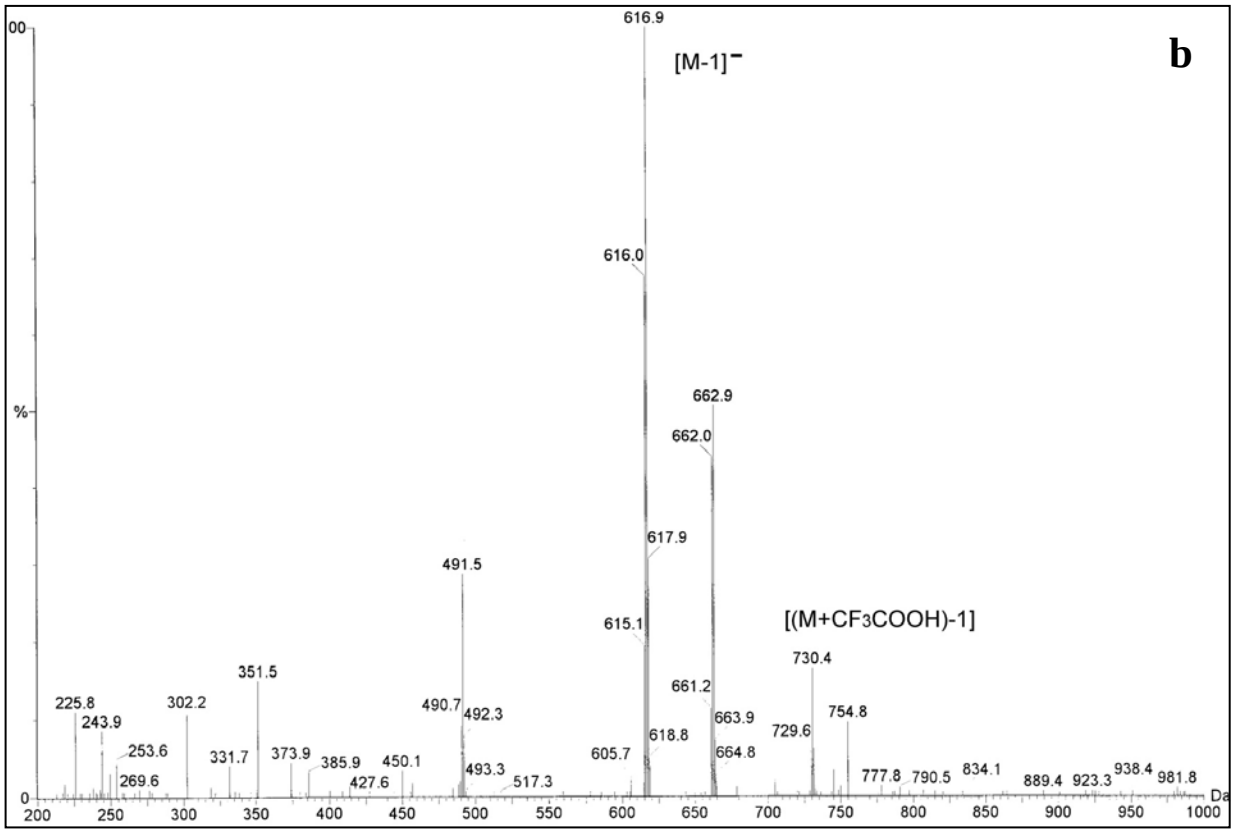
Annex I.16. (**a**) Espectre de RMN-^{1}H de la 4β-[S-(*N*-acetil-*O*-metilcisteinil)]catequina **14** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **14** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **14** amb mode d'ionització d'electrosprai negatiu.



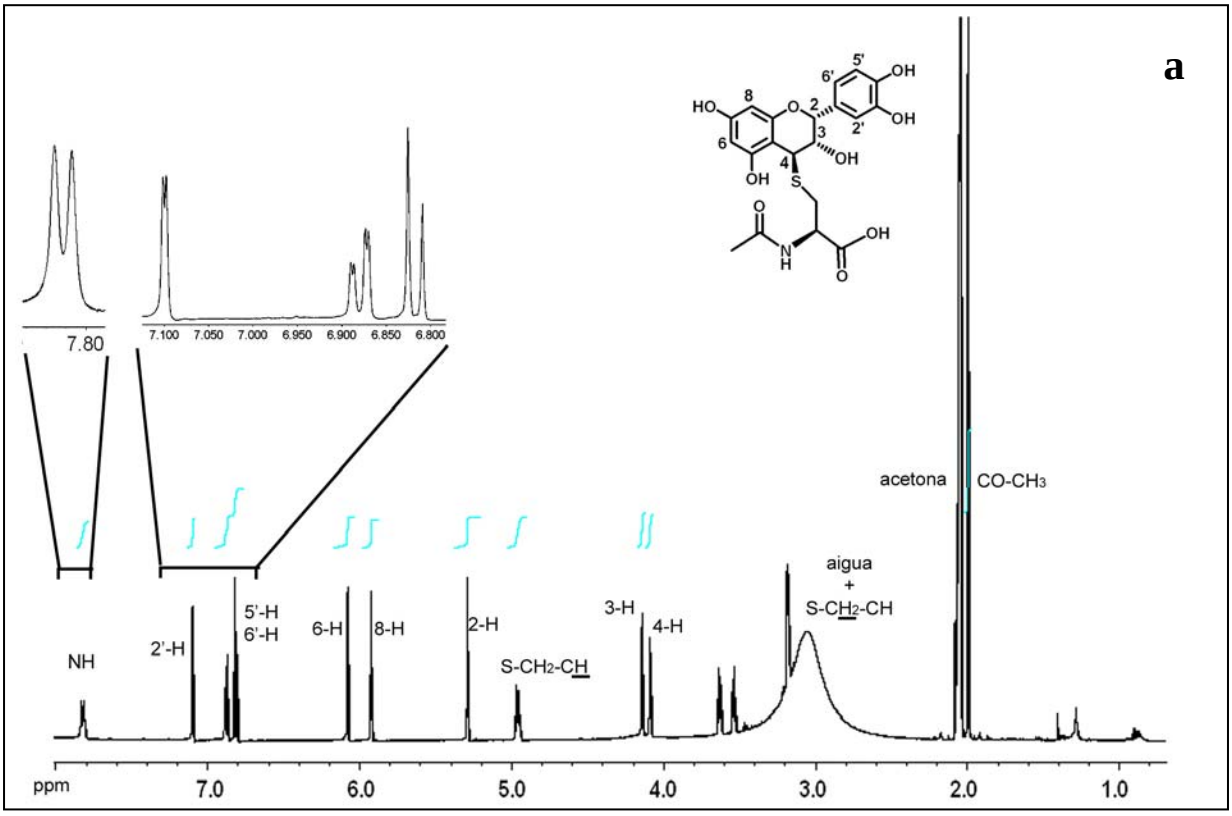


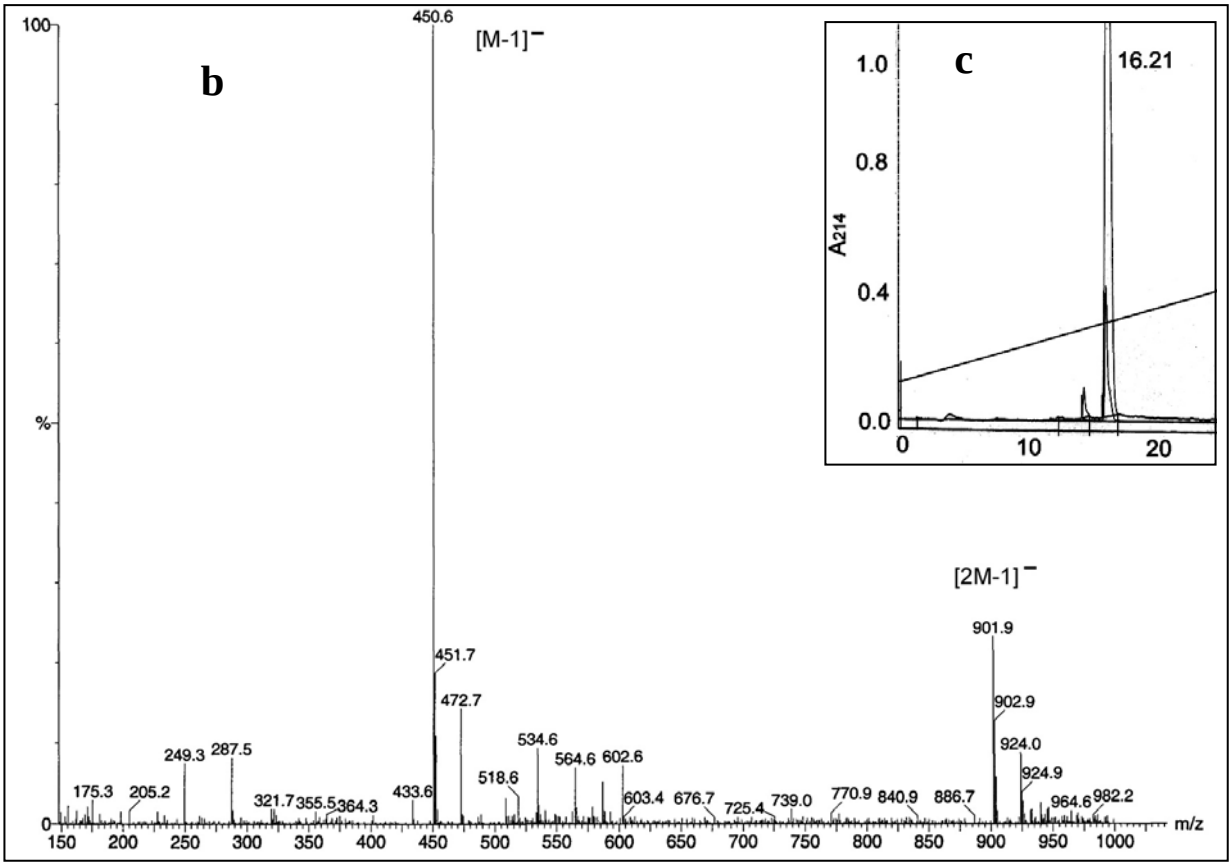
Annex I.17. (**a**) Espectre de RMN-^{1}H de la 4β-[S-(*N*-acetil-*O*-metilcisteinil)]epicatequin 3-*O*-galat **15** a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) + 3 gotes de D_2O per dissoldre el producte **15** (7 mg) i TMS com a patró intern. Es realitzà a 300 MHz i 128 acumulacions. (**b**) Espectrometria de masses del producte **15** amb mode d'ionització d'electrosprai negatiu.





Annex I.18. (**a**) Espectre de RMN-^{1}H de la 4β-[S-(*N*-acetilcisteinil)]epicatequina a partir del cru AQV. Es va fer servir $(CD_3)_2CO$ (0,7 mL) per dissoldre el conjugat (5 mg) i TMS com a patró intern. Es realitzà a 500 MHz i 50 acumulacions. (**b**) Espectrometria de masses del conjugat amb mode d'ionització d'electrosprai negatiu. (**c**) La puresa per HPLC es determinà com en els anteriors Annexes amb un gradient de 10% a 30% [D] en 45 min





6.2 ANNEX II : PUBLICACIONS ORIGINADES

Els resultats derivats de la realització d'aquest treball de recerca han donat lloc a quatre articles principals publicats en revistes científiques i que es recullen en **l'Annex II**:

Annex II.1

Chromatographic characterization of proanthocyanidins after thiolysis with cysteamine.

J.L. TORRES AND C. LOZANO.

Chromatographia, *54*, 523-526 (2001)

Annex II.2

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

C. LOZANO, M. CASCANTE AND J.L. TORRES.

Journal of Chromatography A, *973*, 229-234 (2002)

Annex II.3

Cysteinyl-flavan-3-ol conjugates from grape procyanidins. Antioxidant and antiproliferative properties.

J.L. TORRES, C. LOZANO, L. JULIÀ, F.J. SÁNCHEZ-BAEZA, J.M. ANGLADA, J.J. CENTELLES AND M. CASCANTE.

Bioorganic & Medicinal Chemistry, *10*, 2497-2509 (2002)

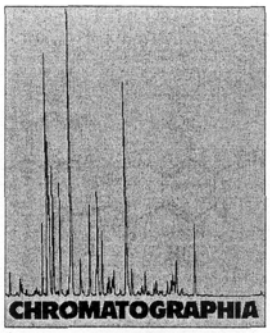
Annex II.4

Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells.

CARLES LOZANO, JOSEP LLUÍS TORRES, LLUÍS JULIÀ, AURORA JIMENEZ, JOSEP JOAN CENTELLES, M. CASCANTE.

FEBS Letters, *579 (20)*, *4219-4225 (2005)*

Chromatographic Characterization of Proanthocyanidins after Thiolysis with Cysteamine



2001, *54*, 523–526

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Key Words

Column liquid chromatography
Proanthocyanidins
Thiolysis with cysteamine
Flavan-3-ols
Polyphenols

Summary

Cysteamine is proposed as a user-friendly thiol donor with application to the analysis of proanthocyanidins by thiolysis. Oligomeric proanthocyanidins are potent antioxidants and disease-preventing agents. The efficiency of which depends on their composition and size. The degree of polymerization of proanthocyanidins is usually estimated by thiolysis then reversed-phase high-performance liquid chromatography. The new derivatization procedure is an alternative to the use of toluene-α-thiol as thiol donor. In addition to enabling the direct chromatographic analysis of crude material, the amino function introduced facilitates prior discrimination between terminal and extension flavanoid moieties by means of cation-exchange chromatography.

Introduction

Polyphenols are potent free-radical scavengers with applications as preventative agents against cancer, cardiovascular diseases, and other disorders [1–5]. Among the polyphenols, oligomeric proanthocyanidins (polymers of flavan-3-ol units), are particularly appreciated. It is becoming evident that the efficiency of these molecules as antioxidants in different environments depends on the size and composition of the oligomeric structures [6, 7].

The degree of polymerisation of flavanols in extracts is usually estimated by thiolysis, with toluene-α-thiol as the thiol donor [8]. The terminal flavan-3-ol units are released unchanged whereas the extension moieties are released as the benzylthio derivatives on position four of the flavanolic system. The mixtures are analysed by reversed-phase high-performance liquid chromatography (RPHPLC) [9, 10]. By use of this method the degree of polymerisation and/or galloylation of proanthocyanidins from grape seeds, skins, and stems [10–12] and from apples [13, 14], has been estimated. The major components of the oligomeric and polymeric proanthocyanidins from grape are (–)-epicatechin, (–)-epicatechin-3-*O*-gallate and (+)-catechin [10–12]. Toluene-α-thiol is toxic, an irritant, and a lachrymator, and handling of this reagent is also extremely unpleasant because of its strong and persistent smell. We have recently reported recovery of antioxidant activity from residual polymeric proanthocyanidins by thiolysis in the presence of cysteamine then cation-exchange chromatography [15]. Here we propose the analytical application of cysteamine as an alternative thiol donor for characterization of proanthocyanidins. No only is cysteamine incomparably more user-friendly and much less toxic than toluene-α-thiol, the introduction of an amino function enables separation of terminal units from the extension moieties of the polymers by cation-exchange chromatography.

Experimental

Materials

The source of the proanthocyanidins (supplied by Bodegas Miguel Torres S.A., Vilafranca del Penedes, Spain) were the skins, the seeds, and a small amount of the stems obtained as a by-product from the pressing of grapes (variety Parellada). Analytical grade methanol (Panreac, Montcada i Reixac, Spain) was used for the thiolysis reaction; absolute ethanol (E. Merck, Darmstadt, Germany) for micropreparative cation-exchange chromatography; HPLC-grade CH_3CN (E. Merck) for analytical HPLC; and milli-Q water for all chromatography. Cysteamine hy-

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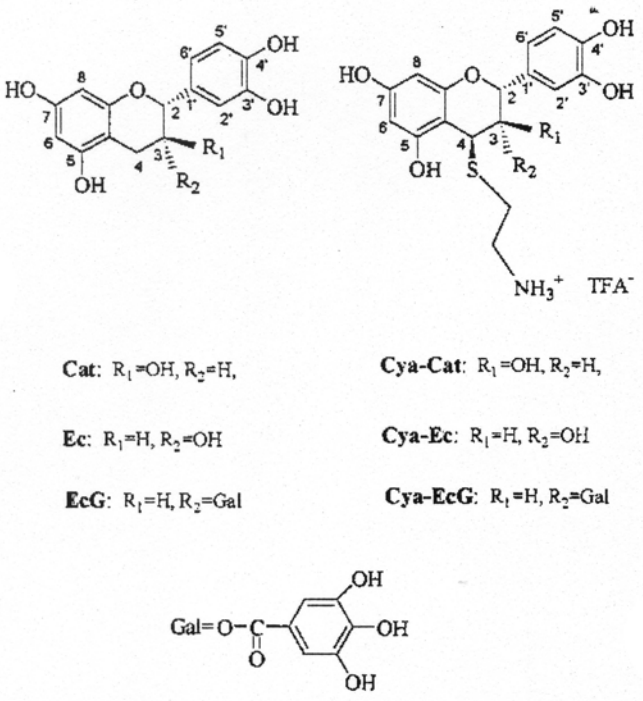

Figure 1. The structures of the monomeric catechins and their aminomethylthio derivatives.

drochloride (E. Merck) was of synthesis grade. Acetic acid and 37% hydrochloric acid (E. Merck), and sodium chloride (Carlo Erba, Milano, Italy) were of analytical grade. Trifluoroacetic acid (TFA; Fluorochem, Derbyshire, UK), biotech grade, was distilled in house.

Sample Preparation

The crude thiolysis product was generated from a lyophilized polyphenol fraction of grape origin. The fraction contained species, soluble both in water and ethyl acetate, from a grape by-product [15]. The lyophilizate (1 mg) was dissolved in MeOH (1 mL). An aliquot (200 µL) was added to the thiolysis mixture (200 µL), which consisted of cysteamine hydrochloride (50 mg) and 37% hydrochloric acid (20 µL) dissolved in MeOH (930 µL). The mixture (400 µL) was kept at 60 °C for 15 min, when the reaction was then quenched with 0.1% (*v*/*v*) aqueous TFA (1.2 mL).

Chromatography

Analytical HPLC was performed either on Kontron Instruments (Basel, Switzerland) equipment fitted with a 4.6 mm × 250 mm Vydac peptide and protein C_{18}, 300-Å pore size, 5-µm particle size column (The Separations Group, Hesperia, USA), or on an Amersham-Pharmacia Biotech (Uppsala, Sweden) Smart System equipped with a µPeak Monitor and fitted with a 2.1 mm × 100 mm µRPC C2/C18 SC 2.1/10, 3 µm particle size column (both from Amersham-Pharmacia Biotech). With both systems the crude material and fractions were chromatographed with a binary mobile phase gradient prepared from 0.10% (*v*/*v*) aqueous TFA (component A) and 0.09% (*v*/*v*) TFA in 2:3 water-CH_3CN (component B); the gradient was from 8 to 23% B over 45 min. The flow rate was 1.5 mL min^{-1} on the Vydac column and 0.2 mL min^{-1} on the µRPC column. Detection was performed at 215 nm on the Kontron system, and at three wavelengths, 214, 280, and 320 nm, on the Smart system.

The major components of the thiolyzed mixtures were identified by comparison with standards. (+)-Catechin, (–)-epicatechin, and (–)-epicatechin-3-*O*-gallate were purchased from Sigma-Aldrich (St Louis, MO, USA). The cysteamine conjugates were isolated from the polyphenol fraction by preparative chromatography and identified by electrospray-mass spectrometry and nuclear magnetic resonance spectroscopy.

Micropreparative cation-exchange chromatography was performed with the Smart system fitted with a 1.6 mm × 50 mm Mono-S PC 1.6/5 (0.1 mL bed volume) column (Amersham-Pharmacia Biotech). The crude material was eluted with a binary mobile phase prepared from 7:3 sodium phosphate buffer (20 mM, pH 2.27)-EtOH (Component C) and 13:7 sodium phosphate buffer (20 mM, pH 2.27)-EtOH containing 1 M NaCl (Component D). Compounds were eluted with 1 mL (10 bed volumes) C followed by 2 mL (20 bed volumes) D. The flow rate was 0.1 mL min^{-1}. Detection was performed at three wavelengths, 214, 280, and 320 nm on the µPeak Monitor. Two fractions (1 mL each) eluted with C and D, respectively, were collected. After solvent evaporation each sample was dissolved in 0.1% (*v*/*v*) aqueous TFA (0.4 mL).

Results

A model fraction that contained polyphenols soluble in both water and ethyl acetate was submitted to thiolysis with hydrochloric acid and cysteamine in methanol. The fraction contained flavan-3-ols (monomers, oligomers) and glycosylated flavonols. The oligomers were completely consumed after 15 min at 60 °C.

The crude product obtained after thiolysis was chromatographed on two different reversed-phase columns, µRPC C2/C18 and Vydac protein and peptide C_{18}. The major components of the thiolysate were (+)-catechin (Cat), (–)-epicatechin (Ec), (–)-epicatechin-3-*O*-gallate (EcG), and their cysteamine conjugates 4β-(2-aminoethylthio) catechin (Cya-Cat), 4β-(2-aminoethylthio) epicatechin (Cya-Ec), and 4β-(2-aminoethylthio)epicatechin-3-*O*-gallate (Cya-EcG) (Figure 1). The major thiolysis products were resolved on µRPC C2/C18 (Figure 2). Figure 2 also shows that the flavonol-related materials were eluted later and did not interfere with the analysis.

The crude thiolysis product was also chromatographed on a Vydac protein and peptide C_{18} column (Figure 3a). Separation between the underivatized and derivatized flavan-3-ols depended on the stationary phase (Figures 2 and 3a). Ec and Cya-Ec eluted as one peak on Vydac C_{18} whereas EcG and Cya-EcG were separated better on Vydac C_{18} than on µRPC C2/C18.

The amino group of cysteamine facilitated identification of the thiolysis products. Thus, micropreparative cation-exchange chromatography enabled fractionation of the crude material into two

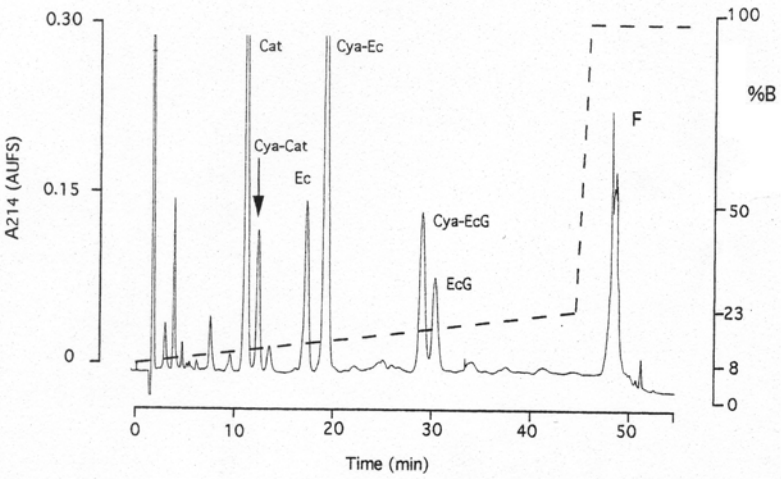

Figure 2. Reversed-phase HPLC-UV profile of flavan-3-ols generated by thiolysis in the presence of cysteamine. Conditions: 3-μm μRPC C2/C18 column; load, 20 μL thiolysis mixture after quenching with 0.1% (*v*/*v*) TFA; elution with gradient from 8 to 23% B in A over 45 min; flow rate 200 μL min^{-1}; detection at 214 nm on the μPeak Monitor. Peaks are labelled in accordance with the abbreviations used in Figure 1. **F** is flavonol-related material absorbing at 365 nm.

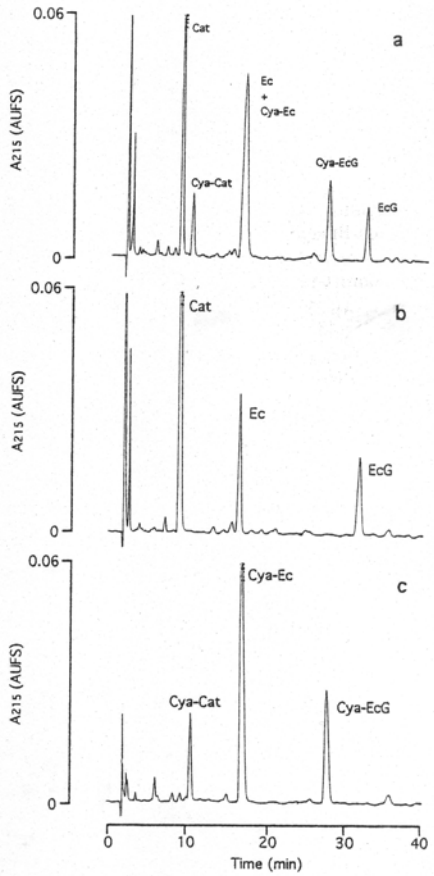

Figure 3. Reversed-phase HPLC UV profiles of flavan-3-ols generated by thiolysis in the presence of cysteamine: (**a**) crude thiolysis mixture; (**b**) unretained fraction from cation-exchange chromatography on the Mono-S column; (**c**) retained fraction from cation-exchange chromatography. Conditions: 5-μm Vydac peptide and protein C_{18} column; load, (**a**) 15 μL thiolysis mixture after quenching with 0.1% (*v*/*v*) TFA, (**b**) 30 μL unretained fraction from cation-exchange chromatography, (**c**) 30 μL retained fraction from cation-exchange chromatography (the concentration of loads **b** and **c** was approximately the same as that of load **a**); elution with gradient from 8 to 23% B in A over 45 min; flow rate 1.5 mL min^{-1}; detection at 215 nm. Peaks are labelled in accordance with the abbreviations used in Figure 1.

groups of molecules, unretained underivatized monomers and the polymers' terminal moieties (mainly Cat, Ec, and EcG) and retained aminated polymers' extension moieties, which were eluted with 1 M NaCl in the eluent buffer. This is shown in Figure 3.

Discussion

The procedure described here is a rapid, efficient, user-friendly alternative to thiolysis with toluene-α-thiol. Results have been generated with a model fraction containing a balanced amount of monomeric and oligomeric flavanols. A short (15 min) treatment at 60 °C was used to depolymerize the sample. The same conditions have resulted in the depolymerization of fractions rich in polymeric proanthocyanidins even in the presence of amounts of cysteamine ten times lower [15]. The efficiency of the procedure does not seems to depend on the source of the proanthocyanidins.

The main advantage of the method is the user-friendly character of the thiol donor. Phloroglucinol has previously been proposed as an odorless alternative nucleophile, but it is less efficient than thiols in the depolymerization of proanthocyanidins [16].

Compared with toluene-α-thiol, cysteamine yields more hydrophilic derivatives that elute earlier in RPHPLC. This might be detrimental to their separation from their underivatized counterparts, depending on the RPHPLC stationary phase available. In our hands, all major peaks were resolved on μRPC C2/C18 and the amino group introduced enabled fractionation of the thiolyzates into terminal and extension proanthocyanidin constituent moieties by cation-exchange chromatography, which facilitated identification of the thio derivatives and the purification of standards. This previous separation will also be especially useful for difficult mixtures, e. g. crude samples or fractions rich in highly polymerized proanthocyanidins, when a wide thio-epicatechin (Cya-Ec) peak might overlap with the epicatechin (Ec) peak.

Another advantage of the new procedure is that flavonols do not interfere with the analysis and so need not be eliminated before the thiolysis reaction. This is especially convenient for grape skin and apple extracts.

Conclusions

Cysteamine may be used as a user-friendly alternative to toluene-α-thiol in the characterization of procyanidins by thiolysis and RPHPLC. Introduction of the amino function enables the separation of monomers and the polymers' terminal flavan-3-ol moieties from the polymers' extension moieties by cation-exchange chromatography. Moreover, previous sample clean-up from interfering polyphenols, e. g. glycosylated flavonols, is unnecessary.

Acknowledgments

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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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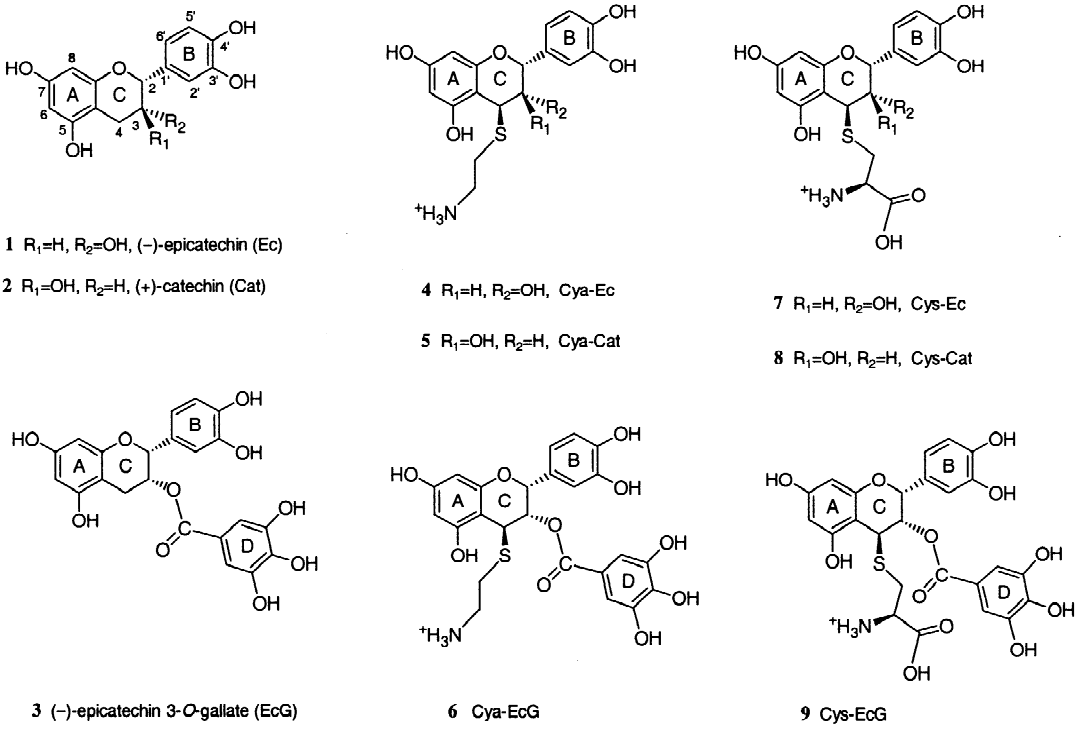

Fig. 1. Structures of monomeric catechins and their cysteamine and cysteine conjugates. Cya-Ec: 4β-(2-aminoethylthio)epicatechin; Cya-Cat: 4β-(2-aminoethylthio)catechin; Cya-EcG: 4β-(2-aminoethylthio)epicatechin 3-*O*-gallate; Cys-Ec: 4β-(*S*-cysteinyl)epicatechin; Cys-Cat: 4β-(*S*-cysteinyl)catechin;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

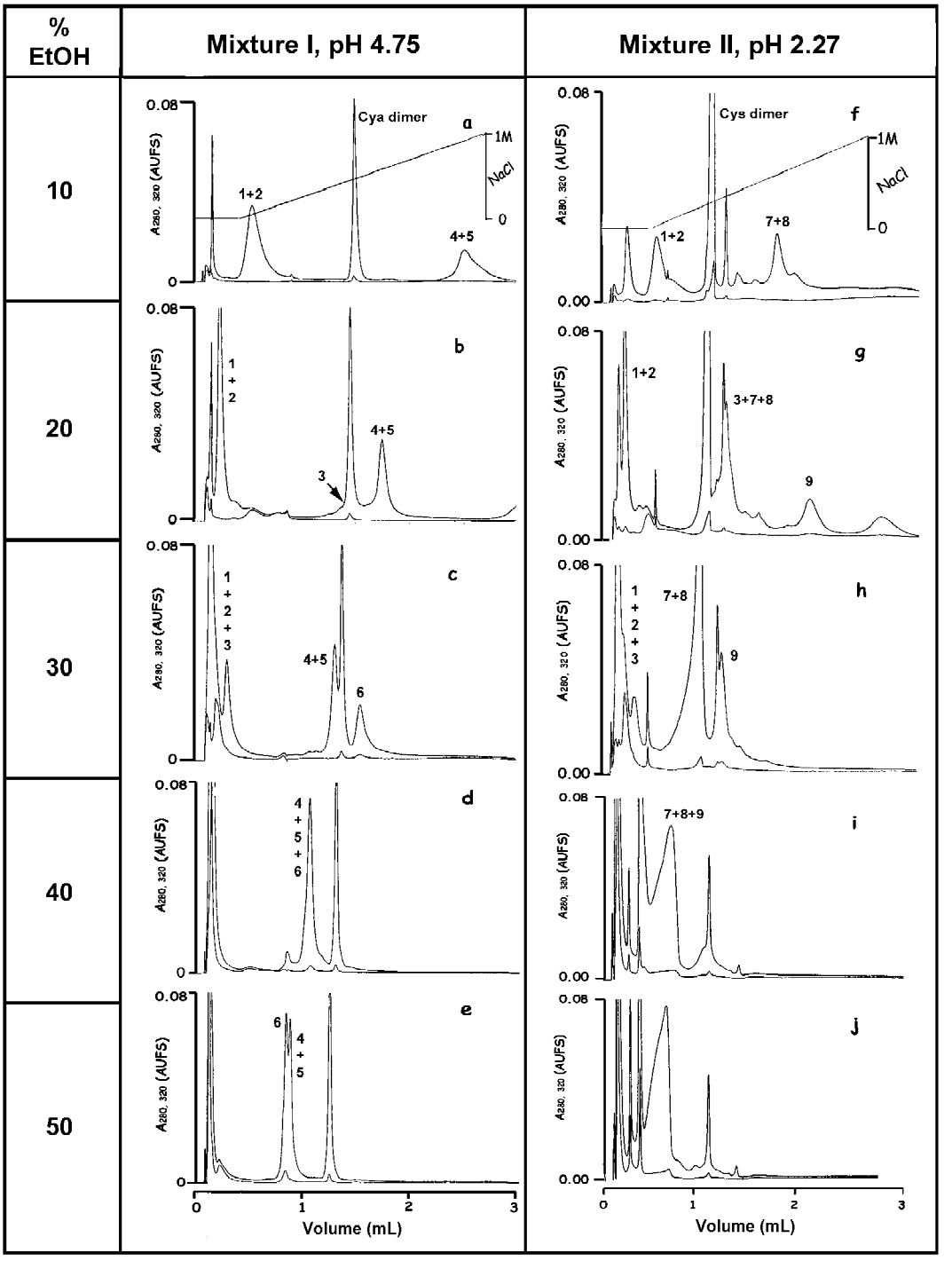

Fig. 2. Cation-exchange chromatograms corresponding to the elution of depolymerised mixtures I (compounds **1**–**6**) and II (compounds **1**–**3**, **7**–**9**). Load: 20 μl, 2 μg catechins. Column: Mono-S® PC 1.6/5. Eluent A: sodium acetate 20 m*M*, pH 4.75 buffer–EtOH or sodium phosphate 20 m*M*, 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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Cysteinyl-flavan-3-ol Conjugates from Grape Procyanidins. Antioxidant and Antiproliferative Properties

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Abstract—New bio-based antioxidant compounds have been obtained by depolymerisation of grape polymeric flavanols in the presence of cysteine. Their preparation and purification, as well as their antiradical/antioxidant and antiproliferative properties are reported. 4β-(*S*-cysteinyl)epicatechin **5**, 4β-(*S*-cysteinyl)catechin **6** and 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate **7** were efficiently purified from the crude depolymerised mixture by cation-exchange chromatography and preparative reversed-phase chromatography. The new compounds were more efficient than the underivatised (−)-epicatechin **1** as scavengers of the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) and weak growth inhibitors of human colon carcinoma HT29 cells. The order of antiradical and antiproliferative efficiency was **7** > **5** ∼**6** > **1**, the same for both assays.

Introduction

Much attention is currently being paid to the putative benefits of plant polyphenols as complements to the organism's antioxidant defence systems. While evolving from anaerobic microorganisms, aerobic life forms had to establish a delicate balance between the benefits and risks of using oxygen to obtain energy. The respiratory chain inevitably produces reactive intermediates such as the superoxide anion radical ($O_2^{\cdot-}$) which lead to other reactive species potentially harmful to biological molecules such as DNA, lipids and proteins.[1] Life organisms have developed enzymatic systems to regulate the levels of all these reactive oxygen species (ROS). Superoxide dismutase, catalase and glutathione peroxidase which scavenge the superoxide anion, H_2O_2 and organic hydroperoxides, respectively, are major detoxifying enzymes.[1] Occasionally, the defence systems are overwhelmed by an excess of ROS produced by illnesses, aging or by external factors such as air pollution, smoking or UV radiation.

In humans, the excess of ROS, which can be either radicals or non-radical compounds, has been related to numerous diseases and dietary plant antioxidants such as carotenoids and flavonoids are considered to help the internal defence mechanisms against unwanted oxidations.[1,2] Particularly flavanols from tea, grape or pine bark are potent free radical scavengers of interest as preventative agents against cancer and cardiovascular diseases.[3–7] The capacity to arrest the cell cycle and/or promote apoptosis may contribute to the chemopreventative effect of some members of the flavanoid family.[8,9]

The major flavanols in green tea are monomeric catechins, that is (−)-epigallocatechin 3-*O*-gallate, (−)-epigallocatechin, (−)-epicatechin (**1**) and (−)-epicatechin 3-*O*-gallate (**3**).[10] Grape and pine bark extracts also contain monomers and are rich in oligomeric (∼2–7 residues) and polymeric (>7 residues) flavanols.[7,11–14] Polymerised flavanols are called proanthocyanidins because they yield anthocyanidins upon depolymerisation under acidic conditions. In the presence of a strong nucleophile, proanthocyanidins yield two kinds of compounds: flavan-3-ols and their derivatives at position 4, coming from the polymer's terminal and extension

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units, respectively[15] (Scheme 1). This procedure, with toluene-α-thiol as nucleophile, is currently used to estimate the degree of polymerisation and galloylation of proanthocyanidins.[11,12,16,17]

The acid cleavage of proanthocyanidins in the presence of other thiols (2-hydroxyethylthiol, 2-aminoethylthiol, Scheme 1) has been recently used to obtain new compounds with antiradical/antioxidant activity and modified physico-chemical properties.[18,19] The combination of antioxidant polyphenols with other natural products is a promising approach in the search for new safe compounds with diversified applications.

Here, we describe the preparation and purification of a new family of bio-based thio-derivatives of flavan-3-ols (**5**–**7**) obtained by depolymerisation of procyanidins[20] in the presence of the natural amino acid cysteine. The reaction also yielded the underivatised terminal units **1**–**3**. The cleavage conditions used were essentially those described before for aminoethylthio-derivatives such as **4**.[19] The antiradical/antioxidant activity in the DPPH assay and the antiproliferative activitity on a human colon carcinoma cell line of the new derivatives are evaluated.

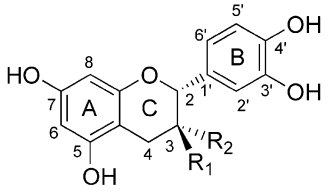
Results

Purification

The cysteinyl flavan-3-ols were separated from the crude depolymerisation mixture on a strong cation-exchange resin (MacroPrep™ High S 50 μm) by taking advantage of the amino function on the cysteinyl moiety. The goal was to have the underivatised monomers and the cysteinyl conjugates elute in two separate groups by means of two consecutive isocratic steps, namely elution with initial buffer and with NaCl containing buffer, respectively.

First, the elution conditions were optimised at semi-preparative scale (6 mL bed volume). The buffers pH was adjusted to 2.25 to make sure that the carboxylic acid function was completely protonated, and the conjugates were retained on the resin through their net positive charges. Hydrophobic interactions with the stationary phase were eliminated with 30% (v/v) EtOH added as co-solvent. More ethanol resulted in early release of the charged conjugates. Moreover, the amount of salt necessary to elute the charged conjugates was minimised. The final optimal conditions were: [A], 4.75 bed volumes of 20 mM sodium phosphate pH 2.25 buffer/EtOH (7:3) for the elution of monomers; [B], 4.75 bed volumes of 20 mM sodium phosphate pH 2.25 buffer/EtOH (13:7) 100 mM NaCl for the elution of the conjugates together with the excess of cysteine from the cleavage reaction.

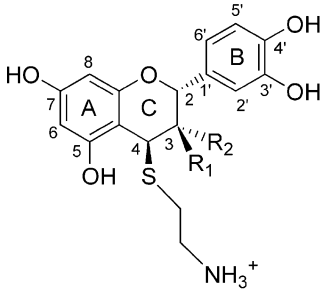
The cation-exchange separation was scaled-up on a glass column (21 × 2.5 i.d., 105 mL bed volume) packed with the same stationary phase. The maximum load was found to be 500 mL (ca. 500 mg GAE, gallic acid equivalents by the Folin–Ciocalteu's method, initial polyphenols) of crude cleavage mixture which was processed in seven repetitive runs. Subsequently, compounds **5**–**7** were purified by preparative reversed-phase



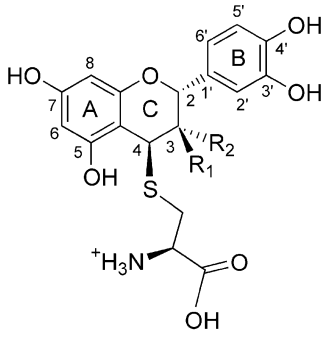
1 R_1=H, R_2=OH, (-)-epicatechin

2 R_1=OH, R_2=H, (+)-catechin

3 R_1=H, R_2=Gal, (-)-epicatechin 3-*O*-gallate



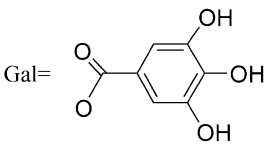
4 R_1=H, R_2=OH, 4β-(2-aminoethylthio)epicatechin

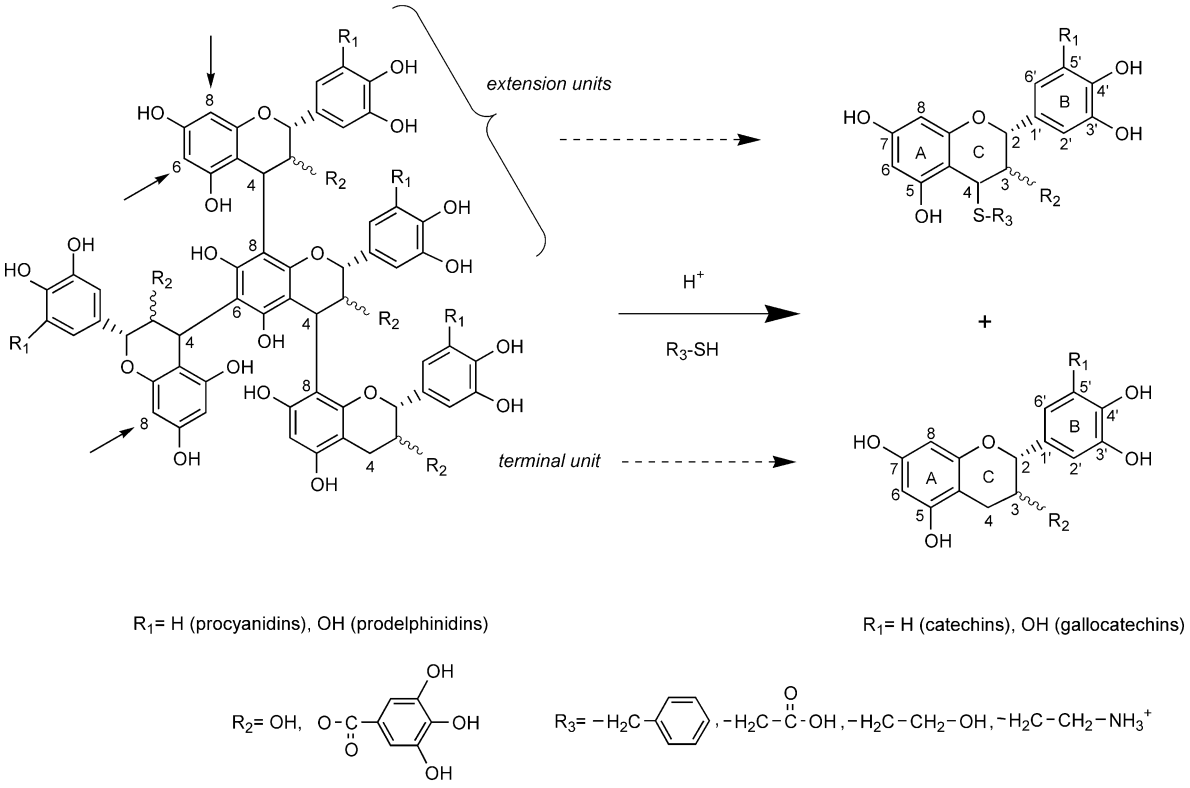


5 R_1=H, R_2=OH, 4β-(*S*-cysteinyl)epicatechin

6 R_1=OH, R_2=H, 4β-(*S*-cysteinyl)catechin

7 R_1=H, R_2=Gal, 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate





Scheme 1. Depolymerisation of proanthocyanidins (polymeric flavan-3-ols). The arrows indicate putative polymerisation positions.

high-performance liquid chromatography (RP-HPLC) as stated in the experimental section. Briefly, the mixture obtained by cation exchange was desalted and fractionated by a CH_3CN gradient in 0.1% (v/v) trifluoroacetic acid (TFA). Then, each fraction, rich in one of the cysteinyl derivatives, was re-chromatographed with CH_3CN gradients in triethylamine phosphate (TEAP) buffers and 0.1% (v/v) TFA to yield 99.5% pure (by RP-HPLC) compounds. The stereochemistry at C-2, C-3 and C-4 of compounds **5**–**7** was assigned from the hydrogen coupling constants measured by ^1H NMR and following Haslam and co-workers.[15] In agreement with the literature, the 4β derivatives were the major isomers obtained irrespective of 2,3-stereochemistry.[15,21]

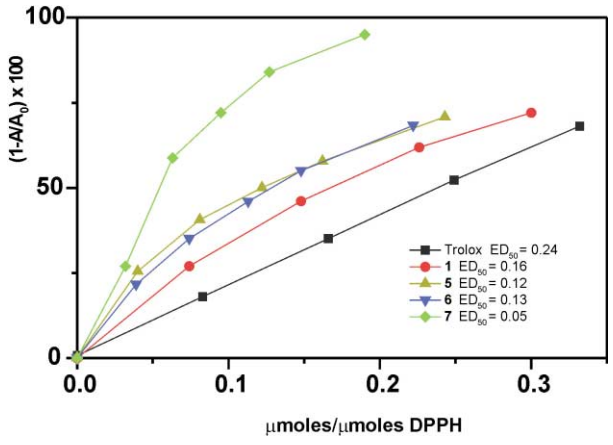


Figure 1. Free radical scavenging activity of the cysteinyl derivatives of flavan-3-ols in the DPPH assay. Absorbance (A) at 517 nm is a measure of the amount of free radical remaining in solution. $(1-A/A_0)\times100$ represents the percentage of DPPH already reacted with the antioxidant. Each point is the mean of three determinations.

Free radical scavenging activity

The new cysteinyl flavan-3-ol derivatives are potent free radical scavenging agents (Fig. 1) in the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay.[22,23] The results are expressed as ED_{50} as defined in the experimental section. The inverse of ED_{50} is a measure of the antiradical power (ARP). By multiplying the ED_{50} by two, the stoichiometric value (theoretical concentration of antioxidant to reduce 100% of the DPPH) is obtained. The inverse of this value represents the moles of DPPH reduced by one mole of antioxidant and gives an estimate of the number of hydrogen atoms involved in the process. Table 1 summarises the parameters obtained from the DPPH assay. The new conjugates were more efficient than (−)-epicatechin **1** and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), being the order of antiradical power

Table 1. Antiradical power and stoichiometry from the DPPH assay

Compound	ARP ($1/ED_{50}$)	Stoichiometric value	H atoms per molecule[a]
Trolox	4.2	0.48	2.1
1	6.2	0.32	3.1
5	8.3	0.24	4.2
6	7.7	0.26	3.8
7	20	0.10	10

[a]Moles reduced DPPH per mole antioxidant.

7 > **5** ~**6** > **1** > Trolox.[24] We obtained a stoichiometric value of 0.32 for (−)-epicatechin, corresponding to the reduction of ca. three DPPH molecules per molecule of **1**. The cysteinyl derivatives of epicatechin (**5**) and catechin (**6**) were able to reduce roughly one more molecule of DPPH than the underivatised (−)-epicatechin **1**. Gallate containing compound **7** is clearly the most efficient of the new molecules.

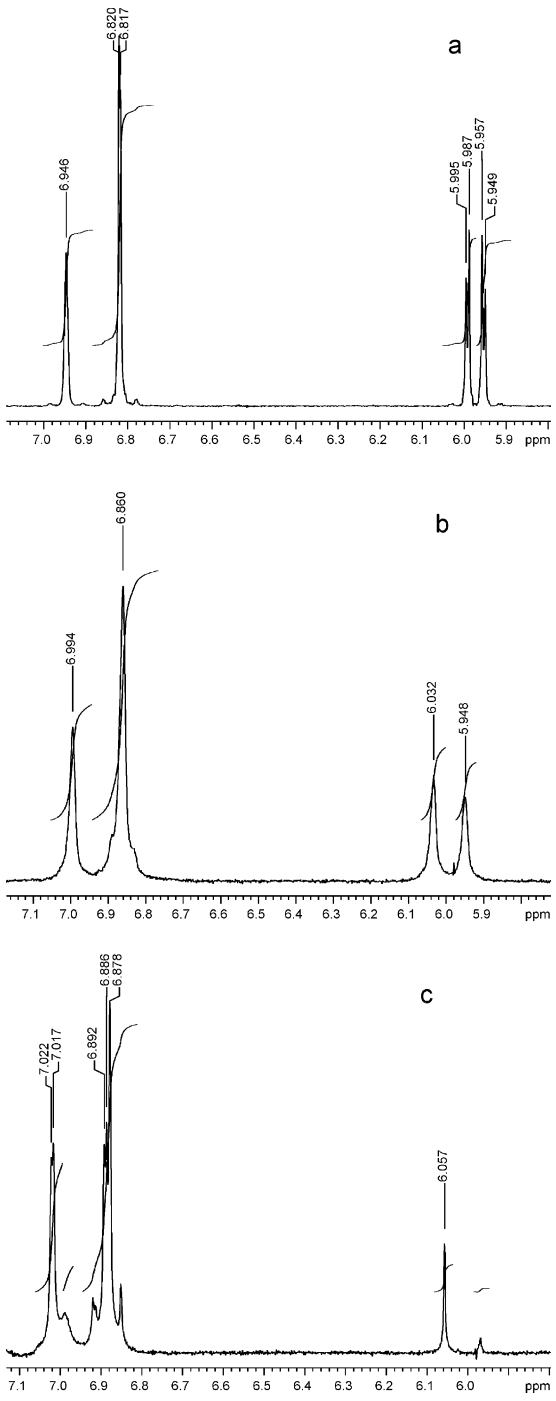


Figure 2. ^{1}H NMR spectra expansion of the aromatic hydrogen region. (a) Compound **1**; (b) compound **4** and (c) compound **5**. All three spectra were recorded under identical conditions after 6 h in D_2O/acetone-d_6 (3:1 v/v) at pD = 6 (ammonium chloride buffer). Signal at ca. 6.05 ppm corresponds to H-6 and signal at ca. 5.95 ppm corresponds to H-8.[25,26] The signals at lower field and insensitive to the deuterium exchange correspond to H-2′, H-5′ and H-6′.

Nuclear magnetic resonance (NMR) deuterium exchange

When recording the standard ^{1}H NMR spectra of derivatives **4**–**7** using acetone-d_6/D_2O as solvent, we observed a systematic discrepancy in the area corresponding to the signals of hydrogen atoms at positions 6 and 8 which we attributed to deuterium exchange with the solvent. In order to confirm this, we recorded the ^{1}H and ^{13}C NMR spectra of two freshly prepared samples, one using regular compound **4** and a second sample using a preparation of the same compound generated by three successive operations of dilution in D_2O and lyophilisation. The spectrum corresponding to the first sample showed the normal signals with only a little decrease in the integral for H-6 and H-8. In the second sample no signal for H-6, H-8, C-6 and C-8 was detected while the rest of the spectrum did not show any other change. A closer look at the ^{13}C NMR spectrum revealed two low intensity signals with the characteristic pattern for monodeuterated carbons near the positions of C-6 and C-8.

The observed hydrogen exchange was unexpectedly fast and we decided to follow the kinetics of the deuteration process by ^{1}H NMR under controlled conditions (solvent, concentration, temperature, pD) using (−)-epicatechin **1** as a control. The spectra were obtained using a mixture of D_2O/acetone-d_6 buffered at pD = 6 (see details in Experimental). For compounds **4** and **5** the deuterium exchange at positions H-6 and particularly H-8 was very fast compared to that of **1** (Figs 2 and 3). The deuteration first order pseudokinetic data were fitted using a simple exponential decay model and a good correlation (R^2 higher than 0.96) for the six sets of data was obtained. The observed half life for H-8 and H-6 signals was respectively of 3851 and 5369 min for compound **1**; 511 and 911 min for compound **4**; 57 and 150 min for compound **5**. In all cases the exchange process proceeded until total hydrogen exchange.

Bond dissociation enthalpies (BDE)

We have performed theoretical calculations at B3LYP level of theory[27,28] to estimate the BDEs of several X–H

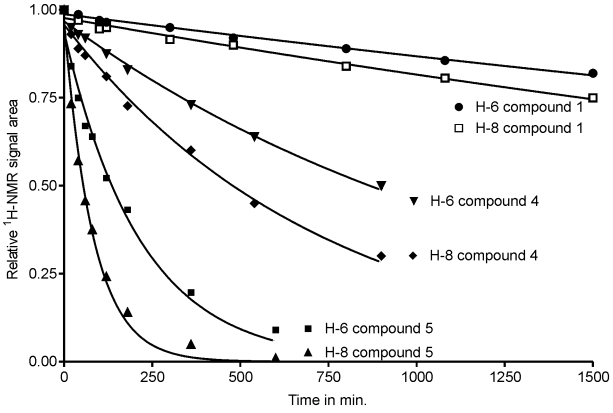


Figure 3. Time evolution for the H-6 and H-8 ^{1}H NMR signal areas for compounds **1**, **4**, **5**. Relative signal is the signal area referred to the average one-hydrogen area for the further hydrogen signals. Data were adjusted using a single exponential decay. R^2 was higher than 0.96 in all cases.

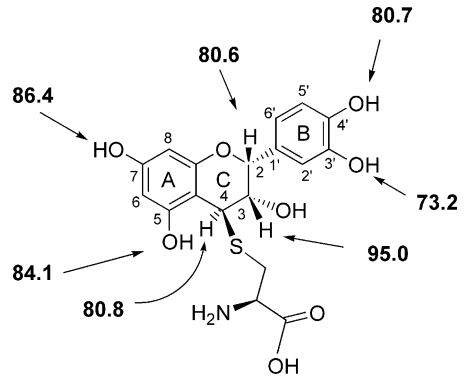


Figure 4. Calculated bond dissociation enthalpies for compound **5** obtained at B3LYP level of theory.

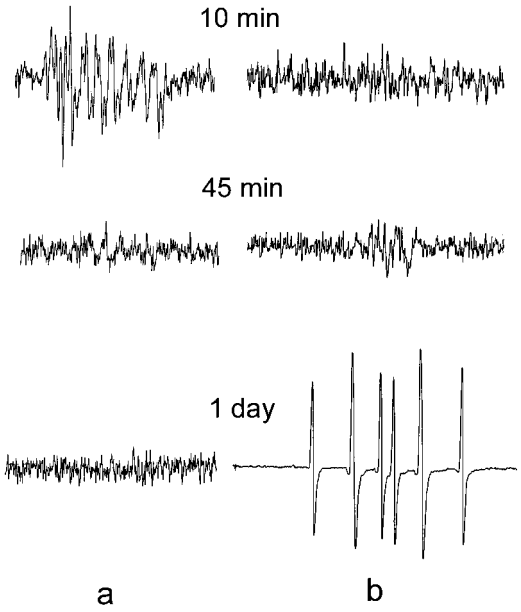


Figure 5. EPR spectra of NaOH (1 M) aqueous solutions of (−)-epicatechin **1** (10 mM) (a) and 4β-(*S*-cysteinyl)epicatechin **5** (10 mM) (b) at different times.

bonds of compound **5**. These BDEs are expected to be directly related to the ability of flavanols to scavenge reactive oxygen species and radicals such as DPPH by donating hydrogen atoms. Our computed values for compound **5** are displayed in Figure 4, which shows that the BDEs at the C-2 and C-4 positions (80.6 and 80.8 kcal/mol, respectively) are of the same order than the BDE of the OH at C-4′ (80.7 kcal/mol).

Formation of free radicals

Flavanols are air oxidised under aqueous basic conditions and the intermediate radicals formed may be detected by electron paramagnetic resonance (EPR).[29–31] The EPR spectra of diluted (10 mM) solutions of (−)-epicatechin **1** and its cysteinyl derivative **5** in 1 M aqueous NaOH, obtained at different times, revealed significant differences (Fig. 5). While **1** generated

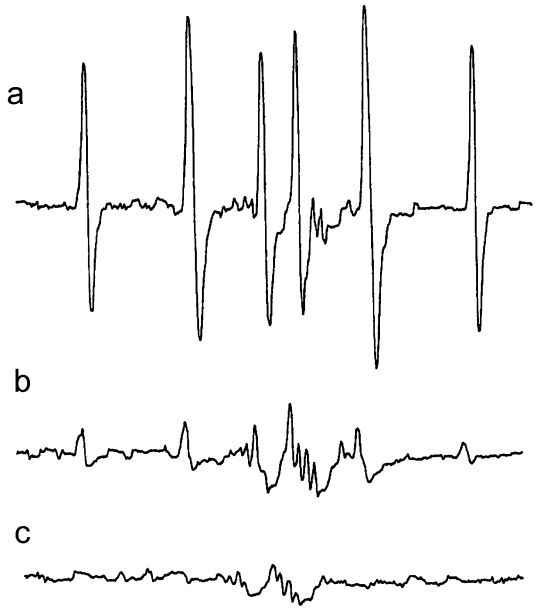


Figure 6. EPR spectra of the stable radical generated from 4β-(*S*-cysteinyl)epicatechin **5** (10 mM) by a 1-day treatment with 1 M aqueous NaOH (a) and after ultraviolet irradiation with a high-pressure mercury lamp for 2 min (b) and 10 min (c).

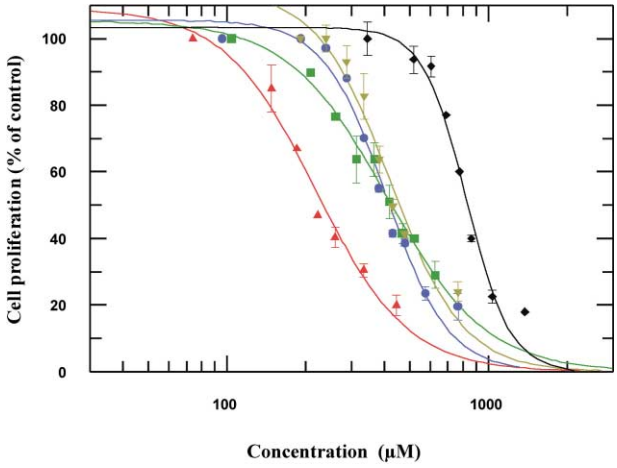


Figure 7. HT29 colorectal adenocarcinoma cell proliferation in response to treatment with increasing doses (*X* axis) of 2-aminoethylthio and cysteinyl derivatives of flavan-3-ols. ■ (**4**), ● (**5**), ▼ (**6**), ▲ (**7**) and ◆ (**1**). The viability and proliferation are expressed as per cent of untreated control cells (mean ± SD).

radicals detected within minutes which rapidly disappeared and were coincident with those observed by other authors[29] (Fig. 5a), **5** generated within hours a simple and much more stable radical which presents a six-line pattern with relative intensities that correspond to hfs constants from three protons with values of 2.67, 1.55 and 1.62 G (Fig. 5b). These signals slowly decreased over a period of several days and were reduced significantly upon irradiation with UV light (Fig. 6). The same radical was detected for the 2-aminoethylthio derivative **4**. In no case, this paramagnetic species was detected from (−)-epicatechin **1**.

Table 2. Antiproliferative potency against human colorectal adenocarcinoma HT29 cells

Compound	n[a]	Mean IC_{50} (μM)	SD
1	4	825.7	12.2
4	6	408.0	11.6
5	6	406.6	21.0
6	6	421.2	26.5
7	6	218.9	6.7

[a]*n*, number of experiments performed; SD, standard deviation.

Antiproliferative activity

The effect of compounds **4**–**7** and (−)-epicatechin **1** on the proliferation of a human carcinoma cell line (HT29) using an MTT assay was examined. The results showed a reduction in cell proliferation in a dose-dependent fashion after treatment with the flavan-3-ols at the reported concentrations for 72 h (Fig. 7). Table 2 shows the mean IC_{50} values and corresponding SD obtained. It should be noted that IC_{50} was calculated with respect to the total number of cells in the control after 72 h of proliferation (20,000 cells/well). At the IC_{50}, the final number of treated cells was approximately 10,000 cells/well, which was 4 times higher than the number of cells at the beginning of the experiment. Thus, the IC_{50} calculated in this paper is indicative of the concentration of product that inhibits the proliferation by 50%. The lower the IC_{50}, the more potent the compound is. The order of antiproliferative potency followed the same pattern obtained from the antiradical activity assay: **7** $>$**4** $\sim$**5** $\sim$**6** $>$**1**.

Discussion

Purification

The efficient cation-exchange separation of the cysteinyl conjugates from the complex crude cleavage mixture depends on several factors, particularly on how successfully the hydrophobic interactions with the stationary phase are handled. If no interaction other than electrostatic were to take place, the underivatised monomers together with any other uncharged material would be washed away with the starting aqueous buffer while positively charged amine containing derivatives would be retained and subsequently eluted with salt. In many instances though, some amount of an organic co-solvent is needed to either lessen or eliminate hydrophobic interactions. In our hands, when no solvent was added to the eluents all molecules were hydrophobically retained on the resin. Particularly, more than 10 bed volumes of washing buffer were needed to release the uncharged (−)-epicatechin 3-*O*-gallate **3**. Moreover, other materials, including coloured species from the grape extract, were also retained and gradually released over the entire chromatographic process. Addressing this point is crucial for preparative purposes when volumes must be kept to a minimum and cleaner mixtures mean easier purifications. We have previously reported the use of an agarose-based gel (SP Sepharose™ high performance) and acetonitrile (CH_3CN) as co-solvent for the separation of flavanol conjugates with cysteamine.[19] The monomers were washed away with 10% (v/v) CH_3CN in the starting eluent and the amine containing conjugates were eluted with a gradient of salt to eliminate the electrostatic interaction with the resin and a simultaneous gradient of CH_3CN. The co-solvent gradient allowed the separation of the epicatechin and catechin derivatives from the more hydrophobic gallate containg conjugate. This facilitated the subsequent purification of the products. The main disadvantage of this procedure is the high eluting volumes needed to obtain a baseline separation. In the present case we decided to minimise the eluting volume by raising the amount of co-solvent at the cost of having all conjugates eluting together. Further modifications introduced in this step were the use of a bulk stationary phase (MacroPrep™ High S 50 μm) based on a methacrylate co-polymer and EtOH instead of CH_3CN. The appropriate amount of co-solvent in the starting eluent was found to be 30% (v/v) EtOH. Less co-solvent resulted in unacceptable retention of (−)-epicatechin 3-*O*-gallate **3** and more EtOH resulted in early elution of the charged conjugates **5** and **6**. This effect has also been observed in the cation-exchange purification of hydrophobic cationic surfactants[32] and might be due to the influence of the co-solvent on the stabilisation of ions pairs with the buffer. After elution of the monomers we took advantage of this effect and increased the amount of EtOH to help recover the conjugates in the presence of salt (100 mM NaCl). At the end we came out with a set of conditions that yielded two groups of compounds, namely underivatised monomers and cysteinyl conjugates, with minimum buffer, co-solvent and salt consumption.

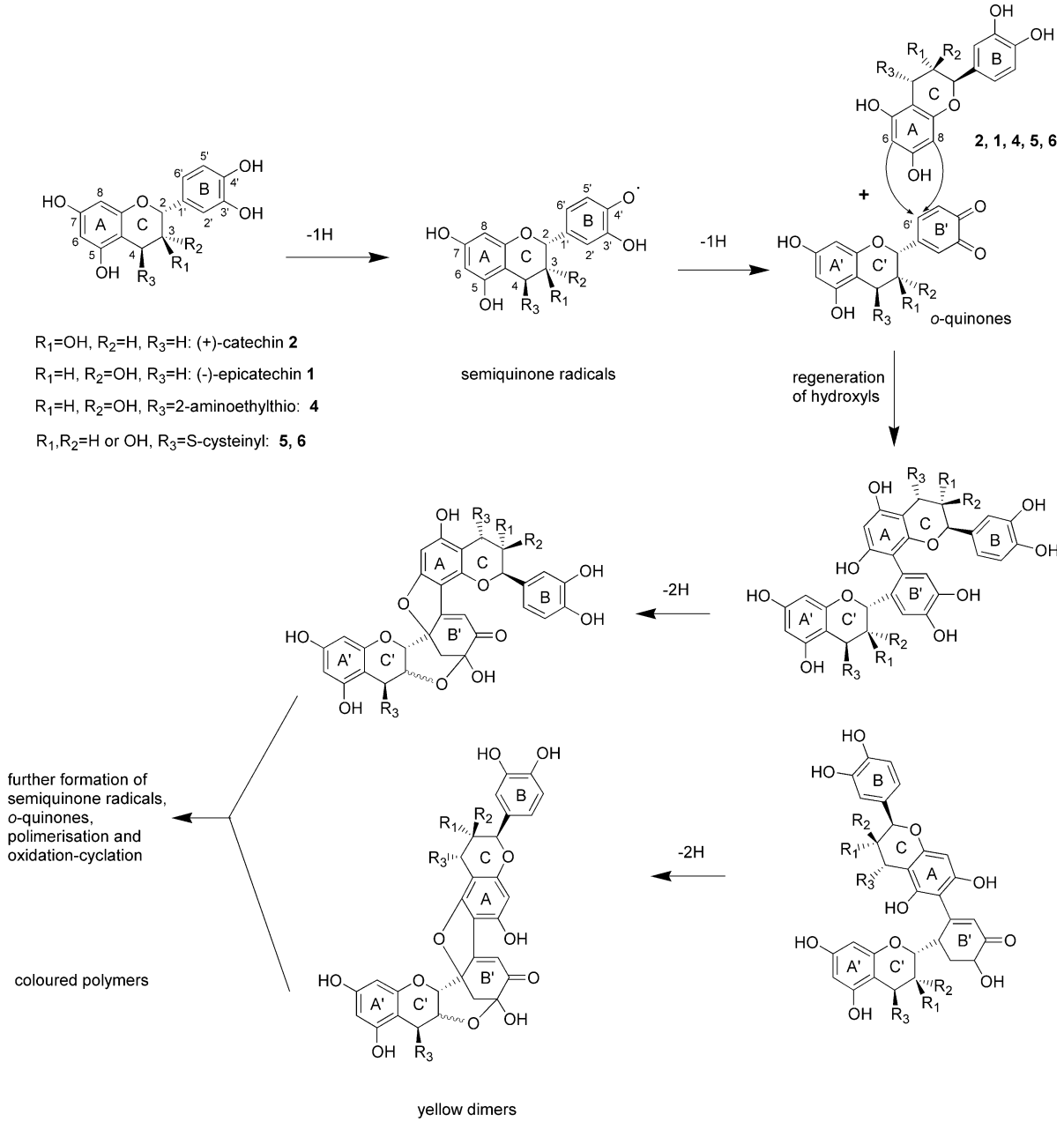
Antiradical/antioxidant activity

In the present paper, we have focused on the free radical scavenging activity of the cysteinyl derivatives in solution using a well known stable free radical (DPPH). While the overall efficiency of polyphenolic antioxidants against lipid and protein oxidation may depend on many factors including the physico-chemical properties of the environment, the DPPH test provides a simple measure of intrinsic antioxidant efficiencies.[23,33,34]

It has been described that the oxidation of (+)-catechin **2** by DPPH takes place in two kinetic steps, one fast step when the most labile hydrogen atoms are abstracted and simple dimers formed, and a second slow step when dimers are further oxidised and polymerised (Scheme 2, catechin).[33] The electron-rich positions 6 and 8 are putatively involved in the polymerisation process by nucleophilic additions on flavanol derived *o*-quinones.[33] Altogether the process results in the reduction of more than two molecules of DPPH per molecule of (+)-catechin. Regeneration of phenolic hydroxyls (Scheme 2, third step) by polymerisation appears to be the reason for the high number of H atoms or electrons involved in the scavenging activity of polyphenols under a variety of other experimental setups such as enzyme catalysed oxidation followed by

either product purification[35] or EPR measurements,[30] and electrochemical oxidation.[36]

The introduction of sulphur atom containing moieties into position 4 of the flavan system have resulted in derivatives (**4**–**7**) able to reduce at least one more molecule of DPPH than (−)-epicatechin **1**. The differences in antiradical efficiencies may be related to an enhanced nucleophilic character of positions 6 and 8 in ring A. Our evidence comes from NMR studies. While the ^{1}H NMR spectrum of (−)-epicatechin **1** remained almost unaltered in acetone-d_6/D_2O, the signals corresponding to protons 6 and 8 from compounds **4**, **5** disappeared progressively over a period of 1–72 h, suggesting that these protons were exchanged by deuterium. To rule out detection problems arising from long relaxation times of those atoms or due to a dynamic chemical exchange process, we introduced changes in the appropriate acquisition parameters with the same outcome: H-6 and H-8 signals had completely vanished from the old samples. The exchange was corroborated by ^{13}C NMR with a sample of **4** previously lyophilised in the presence of D_2O as described in Experimental and by a kinetic study at controlled pD for compounds **1**, **4** and **5** (Figs 2 and 3). It is known that phloroglucinol derivatives are sensitive to the presence of Lewis and protic (low pH) acids reactive towards electrophiles.[37] When spectra of those compounds were recorded in solvents with interchangeable deuterium atoms (e.g., D_2O) the aromatic ring hydrogen atoms could be exchanged by deuterium. In flavan-3-ols hydrogen atoms at positions 6 and 8 have similar properties but deuterium exchange is more difficult.[37] This exchange may be related to the well-known aromatic electrophilic substitution or keto-enol

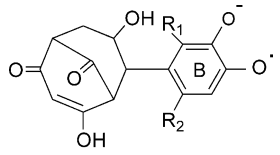


Scheme 2.

tautomerism applied to the case of aromatic phenol rings with highly increased π-electron density. Our data show clearly that the substitution of the 4β hydrogen of flavanols by cysteamine or cysteine through a thioether bond exerts a dramatic influence over the properties of the contiguous aromatic ring A. In the pseudo first order kinetic conditions used (great excess of the electrophile reagent), the difference in exchange half-lives may be directly related to variations in the activation energy barriers. This effect may be mediated by the formation of intramolecular hydrogen bonds between the phenol groups and the functional groups on the sulphur containing moiety. Other mechanisms directly related with the sulphur atom may also be involved. In any case our results show that positions 6 and particularly 8 of compounds **4**–**7** posses an increased ability to react with electrophilic species (e.g., water). This may result in a higher capacity to regenerate polyphenolic hydroxyls through polymerisation (Scheme 2) that would explain why derivatives **4**–**6** are able to provide more H atoms than (−)-epicatechin **1**. Compound **7** is the most effective of the conjugates as expected by the presence of the pyrogallol group containing gallate ester moiety, which provides additional H atoms and polymerisation positions.[29,38]

An alternative explanation for the enhanced capacity of the new derivatives to transfer hydrogen atoms would involve the substituted position 4 on ring C. Mechanistic studies on radical oxidation of catechins by 2,2′-azobis(2-aminopropane)hydrochloride (AAPH) and semiempirical calculations show that hydrogen atoms other than phenolic (i.e., H-2 on ring C) may be abstracted from flavanols.[38,39] Using more accurate theoretical calculations, which have been shown to compare quite well with the experimental values,[28] we obtained a BDE for the C–H bond at position 4 of compound **5** similar to the values for the C–H bond at position 2 and the O–H bond at position 4′ (Fig. 4). This result is suggesting that the hydrogen atom on C-4 may be abstracted by oxidising agents such as DPPH and might contribute to the enhanced antiradical power of the thiol containig derivatives.

EPR experiments of spontaneous air oxidation of **4**, **5** and (−)-epicatechin **1** in 1 M aqueous NaOH also evidenced differences between derivatised and underivatised flavanols. The multiplet (Fig. 5b), with a Landé factor $g = 2.0041$, detected when **4** and **5** (10 mM) were analysed corresponds to the coupling of the unpaired electron with three protons with constant values of 2.67, 1.55 and 1.62 G. In a very extensive work by Jensen and Pedersen[31] about oxidation of (−)-epicatechin **1**, (+)-catechin **2** and catechinic acid in basic solutions they concluded that oxidation of (−)-epicatechin led to the stable radicals **8** and **9** in alkaline aqueous DMSO solution, and the generation of only **8** in alkaline aqueous EtOH solution. These radicals appeared to be species resulting from hydroxylation on ring B and rearrangement reactions on rings A and C of (−)-epicatechin. We have confirmed that oxidation of (−)-epicatechin **1** in concentrated (50 mM) 1 M aqueous NaOH solution generates radical **8**, and in no case gives the six-line



8 R_1=H, R_2=O^-

9 R_1=O^-, R_2=H

pattern multiplet coming from **4** and **5**. This pattern differs from the spectra of radicals generated from 1-substituted 2,3,4-trihydroxybenzenes, 1-substituted 3,4,6-trihydroxybenzenes and 1-substituted 3,4,5-trihydroxybenzenes.[40] The stable radical detected for the sulphur containing derivatives might alternatively be formed on ring A or C. In view of the bond dissociation energies of the different carbon-hydrogen bonds obtained by theoretical calculations (Fig. 4), a radical on C-4 is likely to be formed under our experimental conditions. This radical would be coupled to three protons at positions 3, 6 and 8 which might explain the EPR pattern recorded (Figs 5b and 6a). This point is currently being looked into in our Laboratories. Apart from those described so far, other reactions may occur in an aqueous basic medium, namely oxidation to sulfoxides and nucleophilic attack of the amino group onto quinones, which may influence the formation and/or stabilisation of radicals. In any case the thio derivatives tend to generate relatively stable radicals that last for days and remain sensitive to further oxidation triggered by UV light (Fig. 6) and, presumably, by reactive oxygen species.

Antiproliferative activity

It has been reported that flavan-3-ols are active against proliferation of cancer cells including colon carcinoma cell lines, being (−)-epicatechin **1** and (+)-catechin **2** the least efficient of the monomers.[4,41,42] The novel 2-aminoethylthio and cysteinyl derivatives **4**–**7** proved to be more efficient than **1** in HT29 human carcinoma cells. It has been proposed that the antiproliferative activity of catechins (flavanols) and other flavonoids comes from the inhibition of the kinase activity of proteins involved in cell cycle regulation[43–46] rather than any interaction with any antioxidant defence mechanism. The conjugates might have higher affinity than intact flavan-3-ols for the appropriate protein binding site. Alternatively, conjugation might influence the transport of the flavanols into the cell to reach putative intracellular binding sites. This point is also being looked into in our Laboratories. Curiously, the order of efficiency of the molecules on both the antiradical and antiproliferative assays was identical. Whether this is just a coincidence or not is something that will have to be clarified. Thorough studies on cell cycle disruption and apoptosis are also under way.

Conclusions

Potent cysteine conjugates with natural antioxidants of the flavanol type have been obtained by acid cleavage of polymeric procyanidins followed by cation-exchange

and RP-HPLC purification. The derivatisation appears to improve the free radical scavenging capacity of flavanols by fostering the regeneration of active hydroxyl groups upon polymerisation and/or by facilitating the abstraction of the hydrogen atom geminal to the sulphur atom. The new conjugates are also weak inhibitors of colon carcinoma cells proliferation. The cysteinyl flavanols, together with the previously described cysteamine conjugates incorporate chemical functions (amine, carboxylic acid) and include activated positions (8 and 6 on ring A) which may facilitate the preparation of other products with improved properties.

Experimental

Materials

The primary source of procyanidins was the byproduct from pressing destemmed Parellada grapes (*Vitis vinifera*) (Bodegas Miguel Torres, S.A., Vilafranca del Penedès, Spain) which were extracted and fractionated as described.[19,20] Water and solvents used were: analytical grade MeOH (Panreac, Montcada i Reixac, Spain) for the acid cleavage reaction and DPPH assay; deionised water and bulk EtOH (Momplet y Esteban, Barcelona, Spain) for semipreparative and preparative cation-exchange chromatography; milli-Q® water and HPLC grade CH_3CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC; deionised water and preparative grade CH_3CN (Scharlau, Barcelona, Spain) for preparative and semipreparative RP-HPLC; Milli-Q® water for electron paramagnetic resonance (EPR) studies. Deuterated solvents for nuclear magnetic resonance (NMR) were from SDS (Peypin, France). Cysteine hydrochloride (Aldrich, Steinheim, Germany) was of synthesis grade. Acetic acid, 37% HCl (E. Merck) and NaCl (Carlo Erba, Milano, Italy) were of analytical grade. Triethylamine (E. Merck) was of buffer grade. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK) biotech grade was distilled in-house. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) and gallic acid (97%) were from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) from Aldrich (Milwaukee, USA) and (−)-epicatechin from Sigma Chemical CO, (Saint Louis, MO, USA).

Fetal bovine serum (FBS) was purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Dulbecco's phosphate buffer saline (PBS) and trypsin-EDTA solution C (0.05% trypsin & EDTA 1:5000 in PBS) were from Biological Industries (Kibbutz Beit Haemek, Israel). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and DMSO (dimethyl sulfoxide) were from Sigma Chemical CO (Saint Louis, MO, USA).

Chromatographic equipment and columns

Semi-preparative cation-exchange chromatography was performed on a FPLC® system (Amersham-Pharmacia Biotech, Uppsala, Sweeden) fitted with a Omnifit (Cambridge, UK) column (8 × 1 cm i.d., ca. 6 mL bed volume) packed in-house with MacroPrep™ High S 50 μm (BioRad Laboratories, Hercules, CA, USA). For preparative purposes, the same stationary phase was packed into a flash chromatography type glass column (21 × 2.5 cm i.d., ca. 105 mL bed volume). Preparative RP-HPLC chromatography was performed on a Waters (Milford, USA) Prep LC 4000 pumping system with a Waters PrepPack® 1000 module fitted with a PrepPack® Waters cartridge (30 × 4.7 cm i.d.) filled with VYDAC™ (The Separations Group, Hesperia, USA) C_{18}, 300 Å pore size, 15–20 μm particle size stationary phase. A flow splitter was placed after the column and detection was done by an analytical Merck-Hitachi (Darmstadt, Germany) L-4000 UV detector. Semipreparative runs were performed on the same pumping system fitted with a Perkin–Elmer C_{18} column (25 × 2 cm i.d.). Analytical RP-HPLC was performed on either a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC™ C_{18}, 300 Å pore size, 5 μm particle size, 250 × 4.6 mm i.d. column, or a Smart® System (Amersham-Pharmacia Biotech) equipped with a μ Peak Monitor (Amersham-Pharmacia Biotech) and fitted with a μRPC C2/C18 SC 2.1/10 (100 × 2.1 mm i.d.) column (Amersham-Pharmacia Biotech).

Mass spectrometry, NMR and EPR measurements

Electrospray–mass spectrometry (ES–MS) analyses were recorded on a VG-Quattro® system from Fisons Instruments (Altricham, UK). The carrier solution was water/CH_3CN (1:1) containing 1% (v/v) formic acid. ^1H and ^{13}C NMR spectra were observed with a Unity-300 spectrometer from Varian (Palo Alto, CA, USA) for acetone-d_6/D_2O solutions using standard acquisition conditions at a regulated temperature of 25 °C. Controlled deuterium exchange experiments were performed in D_2O/acetone-d_6 (100% deuteration degree, 3:1 v/v) buffered at pD = 6 (measured with standard pH electrode) with deuterated ammonium chloride (150 mM final concentration in the solvent mixture). All the glass material used in the experiment was thoroughly washed with the deuterated solvent mixture to remove all traces of labile hydrogen. Compounds (ca. 5 mg) were dissolved in 0.75 mL of the buffered solvent mixture (concentration between 15 and 20 mM) just before starting the NMR acquisition. Spectra were acquired at 30 °C using short pulse widths (15°) and 32 scans (for each kinetic data point) with 4 s interpulse time in order to assure a similar integral to concentration factor for all spectral signals. In the case of deuterium substituted samples, the carbon spectra were acquired using an interpulse delay of 5 s and a pulse of 25° until a minimum signal:noise ratio of 40:1 for the quaternary aromatic ring carbons signals was achieved.

EPR measurements were performed on a Varian (Palo Alto, CA, USA) E-109 spectrometer. The freshly prepared basic solutions (1 M NaOH) of the samples were degassed and transferred to a quartz capillary tube, fitted into the cavity of the spectrometer working in the X band at room temperature, and the changes in the spectra were recorded with time. *g* Values of the radicals were

determined with DPPH ($g = 2.0037$) as standard. Measurement conditions: microwave power, 5 mW; modulation amplitude, 0.1 G; modulation 100 kHz; scan width, 20 G. High values of gain were necessary to detect very low concentrations of magnetic species.

Theoretical calculations

The program Gaussian 98[47] was used to perform all calculations described in this paper. The bond dissociation enthalpies were computed following the theoretical approach pointed out by Wright et al.[28] In a first step, we carried out geometry optimisations and vibration frequencies calculations for X–H and X radicals by using the semiempirical PM3 method. In a second step, the electronic energies were obtained by performing single point calculations at the optimised geometries by means of density functional theory (DFT) using the B3LYP functional.[27] The bond dissociation enthalpies at 298 K were then computed as the difference between the B3LYP energy of the X + H radicals minus the B3LYP energy of the XH molecule, plus the corresponding thermochemical corrections obtained at the PM3 level of theory. The B3LYP calculations were performed using the 6–31G(d,p) basis set,[48] with the p-exponent on hydrogen changed to 1.0 as optimised by Wright et al.[28] Moreover, in computing the thermochemical corrections, we have also scaled the zero point energy (ZPE) by 0.947 as suggested by Wright et al.[28] It is worth noting that our results show that the PM3 derived BDEs are systematically underestimated in the 8–30 kcal/mol range, with respect to the more accurate B3LYP values. We obtained computed BDEs of 69.2, 65.9, 60.3, 65.0, 67.5, 71.4 and 71.7 kcal/mol for positions 2, 3, 4, 3′, 4′, 5 and 7, respectively, at PM3 level of theory, which are lower than those displayed in Fig. 4, calculated at B3LYP level of theory. The reason for this discrepancy is that the high level of delocalisation of the radicals is not accurately described at the semiempirical level of theory.

Treatment of procyanidins with acid in the presence of cysteine

The solvent (water saturated with EtOAc) was eliminated from an aliquot (400 mL, 4 g GAE, 6 g estimated polyphenols by weight, coming from 3.2 kg of grape byproduct) of the source of procyanidins.[20] The pellet was then dissolved in MeOH (400 mL) and dried. This operation was repeated three times in order to eliminate moisture. The resulting syrupy residue was dissolved in MeOH (400 mL) and a solution of cysteine hydrochloride (20 g) and 37% HCl (10 mL) in MeOH (400 mL) was added. The mixture was kept at 65 °C for 20 min under agitation. The reaction was then quenched with cold water (3.2 L).

Cation exchange separation of the cysteinyl derivatives from the depolymerised mixture and fractionation by preparative RP-HPLC

Semipreparative runs on a 6 mL bed volume column loaded with MacroPrep™ High S resin were used to set-up the separation conditions at milligram scale. The preparative separations were performed on a 105 mL bed volume column loaded with the same stationary phase. The eluents were [A]: 20 mM sodium phosphate, pH 2.25 buffer/EtOH (7:3) and [B]: 20 mM sodium phosphate, pH 2.25 buffer/EtOH (13:7), 100 mM NaCl. The column was equilibrated with eluent [A], loaded with the quenched depolymerised mixture (500 mL) and washed with [A] (500 mL, 4.75 bed volumes). The retained flavan-3-ol-derivatives were released with 500 mL (4.75 bed volumes) of eluent [B]. The column was then washed with 7.14 bed volumes (750 mL) of 20 mM sodium phosphate buffer, pH 2.25/EtOH (3:2), 1 M NaCl. The operation was repeated (7 times total) until the whole mixture was consumed. The separation process was monitored by analytical RP-HPLC on a VYDAC™ C_{18} column eluted with a binary system, [C] 0.10% (v/v) aqueous TFA, [D] 0.09% (v/v) TFA in water/CH_3CN (2:3) under isocratic conditions 14% [D] (**5** and **6**), 22% [D] (compound **7**), at a flow rate of 1.5 mL/min and detection at 215 nm, 0.016 absorbance units full scale (aufs). The eluates containing compounds **5**–**7** were pooled (3.5 L), the solvent volume was reduced under vacuum down to 1.8 L, and water was added up to a final volume of 2.6 L. The RP-HPLC profile of the pooled eluates as well as the initial and final washing steps were recorded on the same analytical system under gradient conditions 8–23% [D] over 45 min at a flow rate of 1.5 mL/min with detection at 215nm.

The mixture containing compounds **5**–**7** was fractionated on a preparative RP-HPLC cartridge filled with VYDAC™ C18 stationary phase by a CH_3CN gradient in 0.1% (v/v) aqueous TFA (2–14% CH_3CN over 45 min). The solution (2.6 L) was processed in three portions of ca. 900 mL each. Fractions enriched in each of the three compounds were obtained: fraction **I**, 2–5% CH_3CN, compound **6**; fraction **II**, 5–8% CH_3CN, compound **5**; fraction **III**, 10–13% CH_3CN, compound **7**.

Purification of the *S*-cysteinyl derivatives

The 4β-(*S*-cysteinyl)flavan-3-ols were purified from fractions **I**–**III** by preparative RP-HPLC and identified by mass spectrometry and nuclear magnetic resonance.

4β-(*S*-Cysteinyl)epicatechin 5. Fraction **II** (6.7 L) from reversed-phase fractionation was concentrated (5 L final volume) under vacuum to eliminate most of the CH_3CN, loaded onto the preparative cartridge and eluted using a CH_3CN gradient (0–12% over 60 min) in triethylamine phosphate pH 2.25 buffer, at a flow rate of 100 mL/min, with detection at 230 nm. Compound **5** eluted at 4–7% CH_3CN. Analysis of the fractions was accomplished under isocratic conditions in 0.10% (v/v) aqueous TFA/CH_3CN using the VYDAC™ C_{18} column, solvent system, flow rate and detection described above with isocratic elution at 14% [D]. The pure fractions were pooled, diluted with water (1:1) and re-chromatographed on the same cartridge by a CH_3CN gradient (2 to 11% over 30 min) in 0.10% (v/v) aqueous TFA. After combining the eluates and lyophilization, the

resulting solid (0.9 g) was dissolved in tryethylamine phoshate buffer pH 5.54 and loaded again onto the preparative cartridge previously equilibrated in the same buffer. Compound **5** was eluted by a CH_3CN gradient (0–12% over 90 min). The pure fractions were pooled and desalted by a fast CH_3CN gradient in 0.1% (v/v) aqueous TFA on the same cartridge. 4β-(*S*-cysteinyl)epicatechin **5** (550 mg) was obtained as the trifluoroacetate by lyophilisation. ES–MS positive ions, *m/z* 410.0 $(M+1)^+$, calculated for $C_{18}H_{20}N_1O_8S_1$ $(M+H)^+$ 410.1. ^1H NMR (acetone-d_6 + 3 drops D_2O, 300 MHz): δ 3.98 (1H, d *J* = 2.0 Hz, H-4 3, 4-*trans* configuration); 4.08 (1H, dd *J* = 2.0, 0.9 Hz, H-3); 4.45 (1H, m, S–CH_2–C<u>H</u><); 5.09 (1H, s, H-2 2, 3-*cis* configuration); 5.89 (1<u>H</u>, d *J* = 2.1 Hz, H-8); 6.10 (1H, d, *J* = 2.1 Hz, H-6); 6.77–6.86 (2H, m, H-5′, H-6′); 7.07 (1H, d *J* = 2.1 Hz, H-2′). Purity (>99.5%) was ascertained by RP-HPLC on a μRPC C2/C18, 3 μm column; elution, [C] 0.10% (v/v) aqueous TFA, [D] 0.09% (v/v) TFA in water/CH_3CN (2:3), gradient 8 to 23% [D] over 45 min at a flow rate of 200 μL/min with simultaneous detection at 214, 280 and 320 nm.

4β-(*S*-Cysteinyl)catechin 6

Fraction **I** from reversed-phase fractionation was concentrated as stated for **5**, loaded onto the preparative cartridge and eluted using a CH_3CN gradient (0–12% over 90 min) in triethylamine phosphate pH 2.25 buffer, at a flow rate of 100 mL/min, with detection at 230 nm. Analysis of the fractions was accomplished under isocratic conditions in 0.10% (v/v) aqueous TFA/CH_3CN using the VYDAC^{TM} C_{18} column, solvent system, flow rate and detection described above with isocratic elution at 10% [D]. The best fractions were pooled, diluted, reloaded onto the cartridge and eluted with a CH_3CN gradient (0–12% over 90 min) in triethylamine phosphate pH 5.30 buffer. The purest fractions were pooled, desalted with a steep CH_3CN gradient in 0.1% (v/v) aqueous TFA and lyophilised. Then the preparation was re-chromatographed on a semipreparative Perkin–Elmer C18 cartridge eluted with 6 and 9% CH_3CN in 0.10% (v/v) aqueous TFA under isocratic conditions. After pooling the best fractions and lyophilization, 4β-(*S*-cysteinyl)catechin **6** (74 mg) was obtained as the trifuoroacetate. ES–MS, positive ions, *m/z* 409.9 $(M+1)^+$, calculated for $C_{18}H_{20}N_1O_8S_1$ $(M+H)^+$ 410.1. ^1H NMR (acetone-d_6 + 3 drops D_2O, 300 MHz): δ 4.15 (1H, 2d, *J* = 9.7, 4.5, H-3); 4.28 (1H, d *J* = 4.5, H-4 3, 4-*cis* configuration); 4.4 (1H, m, S–CH_2–C<u>H</u><); 4.80 (1H, d *J* = 9.7 Hz, H-2 2, 3-*trans* configuration); 5.76 (1H, d *J* = 2.1 Hz, H-8); 6.12 (1H, d, *J* = 2.1 Hz, H-6); 6.78 (2H, m, H-5′, H-6′); 6.93 (1H, d *J* = 1.2 Hz, H-2′). Purity (99.9%) was ascertained by RP-HPLC on the system described for compound **5**.

4β-(*S*-Cysteinyl)epicatechin 3-*O*-gallate 7. Fraction **III** from reversed-phase fractionation was concentrated as stated for **5**, loaded onto the preparative cartridge and eluted using a CH_3CN gradient (2–14% over 60 min) in triethylamine phosphate pH 2.25 buffer, at a flow rate of 100 mL/min, with detection at 230 nm. Fractions were analysed under isocratic conditions in 0.10% (v/v) aqueous TFA/CH_3CN using the column, solvent system, flow rate and detection described above with elution at 19% [D]. The best fractions were pooled, diluted, re-loaded onto the cartridge and eluted with a CH_3CN gradient (2–14% over 60 min) in triethylamine phosphate pH 5.46 buffer. The purest fractions were pooled and re-chromatographed with a CH_3CN gradient (9–21% over 30 min) in 0.1% (v/v) aqueous TFA. After lyophilisation, 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate **7** (75 mg) was obtained as the trifluoroacetate. ES–MS, positive ions, *m/z* 561.9 $(M+1)^+$ calculated for $C_{25}H_{24}N_1O_{12}S_1$ $(M+H)^+$ 562.1. ^1H NMR (acetone-d_6 + 3 drops D_2O, 300 MHz): δ 4.15 (1H, d *J* = 2.1 Hz, H-4 3, 4-*trans* configuration); 4.37 (1H, m, S–CH_2–C<u>H</u><); 5.28 (1H, 2m, H-3); 5.39 (1H, bs, H-2 2, 3-*cis* configuration); 6.01 (1H, d *J* = 2.1 Hz, H-8); 6.13 (1H, d, *J* = 2.1 Hz, H-6); 6.78 (1H, d *J* = 8.1 Hz, H-5′); 6.90 (1H, dd *J* = 2.1, 8.1 Hz, H-6′); 6.97 (2H, s, galloyl H); 7.11 (1H, d *J* = 2.1 Hz, H-2′). Purity (>99.5%) was ascertained by RP-HPLC on the system described for compound **5**.

Antiradical activity

The free radical scavenging activity was evaluated by the DPPH method.[22,23] The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with 4.8 mg DPPH in 200 mL of MeOH and the mixture incubated for 1 h at room temperature. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517nm} = 11{,}345 \times C_{DPPH}$ as determined by linear regression. The results were plotted as the degree of absorbance disappearance at 517 nm $[(1-A/A_0)\times 100]$ against μmoles of sample divided by the initial μmoles of DPPH. Each point was acquired in triplicate. A dose-response curve was obtained for every product. The results were expressed as the efficient dose ED_{50} given as μmoles of product able to consume half the amount of free radical divided by μmoles of initial DPPH.

Cell culture

HT29 cells (colorectal adenocarcinoma) were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine and antibiotics: 100 U/mL penicillin and 100 mg/mL streptomycin (Biological Industries). Cells were grown in an isolated 37 °C–5% CO_2 tissue incubator compartment and the medium was changed every 3 days. HT29 cell cultures used in this study were free of mycoplasm infection as shown by the Gen-probe rapid mycoplasm detection system prior to the treatments with the samples.

Proliferation assay

Cell growth was determined using the MTT assay.[49] Cells were seeded into 96-well plates at 2.5×10^3 cells/well density and incubated for 24 h prior to addition of

the samples, which were prepared by serial dilutions from a concentrated solution made up in Dulbecco's phosphate buffered saline (PBS). Control cultures were treated with equal volume of PBS as the treated cultures. After 3 days of culture, the supernatant was aspirated and 100 μL of filtered MTT (0.2 mg/mL in cell culture medium) was added to each well. The plates were incubated at 37 °C–5% CO_2 during 4 h. The supernatant was removed, and the blue MTT formazan precipitated was dissolved in DMSO (100 μL) and optical density (OD) measured at 550 nm on a multi-well reader (Merck ELISA System MIOS®).

For each compound, a minimum of four experiments measuring the growth inhibition was conducted and the mean ± SD of OD data from the replicated wells was calculated for each concentration tested. The inhibitory effect of the products at each concentration was expressed as a percentage [(mean OD treated cells after 72 h of incubation with the product/mean OD of control cells after 72 h of incubation with extra-medium instead of product) × 100]. The IC_{50} or sample concentration causing a 50% reduction in the mean OD value relative to the control at 72 h of incubation, was estimated using GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, UK) curve option: IC_{50} curve — start at 0.

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Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells

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Abstract Novel catechin derivatives obtained from grape procyanidins and L-cysteine scavenge free radicals by hydrogen atom donation, rather than electron transfer, and reduce cell viability in A375 and M21 melanoma cells. In particular, 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate has a free radical scavenging capacity as strong as that of tea (−)-epigallocatechin gallate and causes a significant S-phase cell-cycle arrest in both cell lines at doses higher than 100 μM. The other cysteinyl compounds do not affect normal cell cycle distribution. The gallate derivative also induces apoptosis in melanoma cells more strongly than the other derivatives and the parent (−)-epicatechin do. The gallate compound seems to trigger nuclear condensation and fragmentation, which is confirmed by DNA laddering. Interestingly, they do not induce apoptosis in keratinocytes (HaCaT).

Keywords: Catechins; Antioxidants; Gallate ester; Melanoma; Cell cycle; Apoptosis

1. Introduction

Cutaneous melanoma ranks fifth in incidence among men and sixth among women in the USA. Its incidence is rising at a rate of about 5% per year [1]. Some studies have shown that UV light exerts its biological effects, at least in part, via the generation of reactive oxygen species (ROS) and free radicals, which play a major role in the induction of skin cancer [2–5]. Chemopreventive approaches utilizing non-toxic agents aimed at both minimizing ROS formation and inducing apoptosis in tumour cells seem attractive. Recent studies on skin cancer prevention show that certain polyphenols cause both actions and appear to be efficient inhibitors of UV radiation-induced skin carcinogenesis [2,6–8].

Of these polyphenols, the gallate-containing flavan-3-ols from green tea extract stand out because of their antioxidant capacity and their induction of apoptosis in tumour cells [9,10]. Grape extract, whose properties are similar to tea extract's, is another important source of flavan-3-ols. While the tea extract includes mainly monomeric flavan-3-ols, mostly gallocatechins [11], grape extracts contain polymeric flavan-3-ols [12,13], mostly procyanidins (see Fig. 1). Interestingly, depolymerisation of procyanidins is an appropriate way of obtaining monomeric derivatives with modified properties [14,15]. A previous study in our Laboratories described a new family of flavan-3-ols obtained by depolymerisation of grape procyanidins in the presence of the natural amino acid cysteine [16]. In the present study, we evaluated the electron transfer capacity of the novel flavan-3-ol derivatives, 4β-(*S*-cysteinyl)epicatechin, 4β-(*S*-cysteinyl)catechin and 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (Fig. 2), with the new radical chemosensor HNTTM [17]. The study is significant because of the novel compounds, as electron transfer capacity is directly related to pro-oxidant effects in catechins, such as (−)-epigallocatechin (EGC) and (−)-epigallocatechin-gallate (EGCG) [18,19]. We also studied the response of two melanoma cell lines, A375 and M21, to treatment with the novel flavan-3-ol derivatives. Melanoma cells were significantly less viable in both cell lines at higher doses. Moreover, the gallate-containing compound showed a significant arrest in the S phase of the cell cycle and a significant activation of the apoptosis mechanism in both tumour cell lines, but not in keratinocytes. The apoptotic induction of the gallate-containing compound was confirmed by flow cytometry and DNA laddering, showing apparent nuclear fragmentation.

2. Materials and methods

4β-(*S*-cysteinyl)epicatechin (Cys-EC) **2**, 4β-(*S*-cysteinyl)catechin (Cys-Cat) **3** and 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (Cys-ECG) **4** (Fig. 2) were prepared as described [16] and dissolved in *Dulbecco's* phosphate buffer saline solution (PBS, from Sigma, Steinheim, Germany). (−)-Epicatechin (EC) **1**, staurosporine, ethidium bromide, EDTA, boric acid, isopropanol and α,α,α-Tris(hydroxymethyl)aminomethane were also purchased from Sigma. Ethanol, NaCl, NaOH and $CaCl_2 \cdot 2H_2O$ were from Panreac Química SA (Montcada i Reixac, Spain). Trypsin–EDTA solution C (0.05% trypsin and EDTA 1:5000 in PBS) was from Biological Industries (Beit Haemek, Israel). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMSO (dimethyl sulfoxide), Trypan blue solution 0.4%, propidium iodide (PI) and the non-ionic surfactant Igepal CA-630 were from Sigma Chemical Co (Saint Louis, MO, USA). RNase and agarose MP were from Roche Diagnostics (Mannheim, Germany). FITC-Annexin V kit and binding buffer 4× for apoptosis assay were purchased from Bender MedSystems (MedSystems Diagnostics GmbH, Vienna, Austria). The Realpure DNA extraction kit, which included Proteinase K, was purchased from Durviz s.l. (Paterna, Spain). Blue/Orange Loading dye (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene

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Abbreviations: Cys-Cat, 4β-(*S*-cysteinyl)catechin; Cys-EC, 4β-(*S*-cysteinyl)epicatechin; Cys-ECG, 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; EC, (−)-epicatechin; HNTTM, tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical

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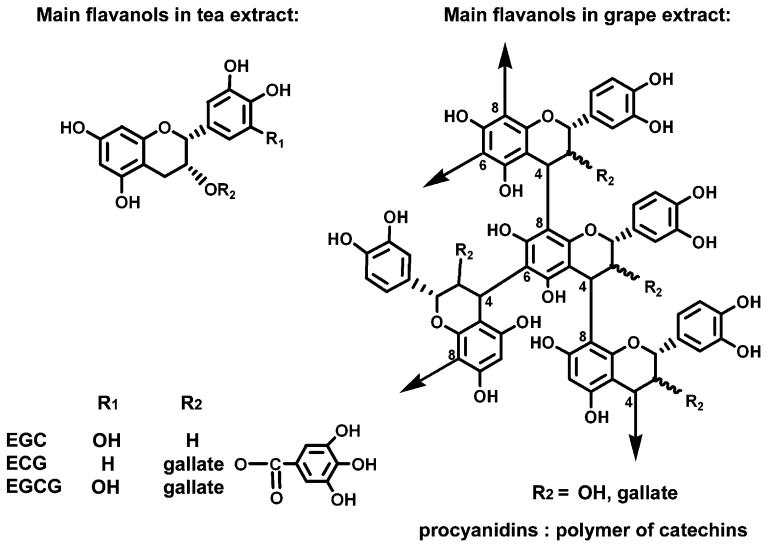

Fig. 1. Molecular structure of the main components in tea extract (monomers of gallocatechins) and grape extract (polymer of catechins).

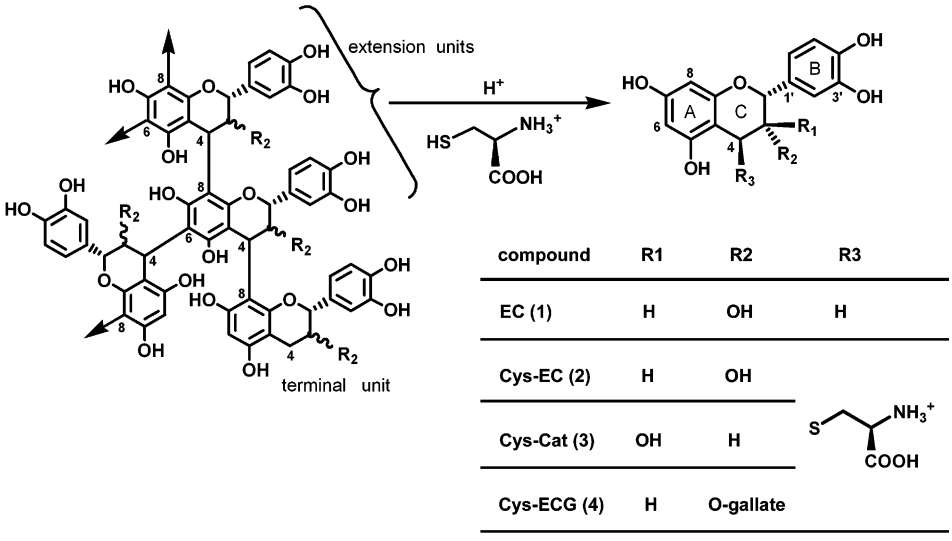

Fig. 2. Molecular structures of (−)-epicatechin and the novel thio-derivatives of flavan-3-ols obtained by depolymerization of grape procyanidins in the presence of cysteine.

cyanol FF, 15% Ficoll 400, 10 mM Tris–HCl, pH 7.5 and 50 mM EDTA, pH 8) and 1 kb DNA ladder were from Promega (Madison, WI, USA).

2.1. Electron transfer assay

EPR measurements were performed on a Varian (Palo Alto, CA) E-109 spectrometer working in the X-band (microwave power, 20 mW; modulation amplitude, 3.2 G). The radical scavengers were tested at various concentrations in chloroform-methanol (2:1). Aliquots (1 mL) were reacted with a solution (1 mL) of HNTTM (tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl) radical (120 μM in chloroform–methanol (2:1)) [17] and the mixture was incubated for 30 min. The initial concentration of the compounds tested ranged from 1 to 30 μM. Each point was acquired in triplicate. A dose–response curve was obtained for each product. The results were expressed as the efficient dose ED_{50} given as μmoles of compound able to consume half the amount of free radical divided by μmoles of initial HNTTM radical. The stoichiometric value (theoretical concentration of antioxidant to reduce 100% of the radical) was obtained by multiplying the ED_{50} by two. The inverse of this value represents the moles of radical reduced by one mole of antioxidant and gives an estimate of the number of electrons involved in the process.

2.2. Cell culture

A375 cell line (human malignant melanoma) was purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco modified Eagle's medium (DMEM) from Sigma. The medium was supplemented with 10% (v/v) heat-inactivated FCS (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine and antibiotics: 100 U/mL penicillin and 100 μg/mL streptomycin (Biological Industries, Beit Haemek, Israel). M21 cell line (human malignant melanoma), generously provided by Prof. A. Mazo (UB, Barcelona, Spain), was cultured in RPMI-1640 medium supplemented as before. The spontaneously immortalised human keratinocyte cell line HaCaT was provided by Advancell (Barcelona, Spain), cultured in DMEM medium from Biowhittaker/Cambrex (Milan, Italy) and supplemented with 10% FCS, 2 mM L-glutamine, 1% HEPES buffer (Invitrogen) and 0.1% antibiotics. Cells were grown in an isolated 37 °C, 5% CO_2 tissue incubator compartment and their medium was changed every 3 days.

2.3. Growth inhibition assay

Cell growth was determined by means of a variation of the MTT assay described by Mosmann [20]. A375 and M21 cells were seeded into 96-well plates at a density of 1.5×10^3 cells/well and 1.2×10^3 cells/well, respectively. Adherent cell lines were incubated for 24 h prior to addition of the compounds. After 3 days culture, the supernatant was aspirated and 100 μL of filtered MTT (0.5 mg/mL in cell culture medium) was added. The cell plates were incubated for 1 h and metabolically active cells reduced the dye to purple formazan. The supernatant was removed, and the dark blue MTT formazan precipitated was dissolved in DMSO (100 μL). Optical density (OD) was measured at 550 nm on a multi-well reader (Merck ELISA System MIOS®).

The IC_{50} or compound concentration causing a 50% reduction in the mean OD value relative to the control was calculated using a GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, UK) curve option: IC_{50}curve – start at 0.

2.4. Cell cycle analysis

Flow cytometry was used to analyse cell cycles and quantify apoptosis. A375 and M21 cells were seeded into 6-well plates at a density of 3.5×10^4 cells/well and incubated for 24 h prior to addition of the compounds. The IC_{50} and $2 \times IC_{50}$ concentration values of each sample were measured after 3 days subculture. Both cell lines were resuspended in ice-cold TBS 1× buffer (1 mL of 10 mM Tris and 150 mM NaCl, pH 7.4). PI (50 μL, 50 μg) and 1 mL Vindelov buffer at pH 7.4, containing 10 mM Tris, 10 mM NaCl, PI (50 μL, 50 μg), Rnasa (1 μL, 10 μg) and Igepal CA-630 (1 μL), were added to each sample. Cells were incubated for 1 h at 4 °C in the dark [21]. Cell cycle distribution was analysed by flow cytometry using the fluorescence-activated cell sorting (FACS) system. DNA histograms were collected with an Epics XL flow cytometer (Coulter Corporation, Miami, FL) and analysed by the Multicycle program (Phoenix Flow Systems, San Diego, CA).

2.5. Assessment of apoptosis

In the assessment of apoptosis, after 3 days subculture of the cell plates in the same way as for the cell cycle treatment, both melanoma cell lines were washed once in ice-cold binding buffer (10 mM HEPES sodium hydroxide pH 7.4, 140 mM sodium chloride, 2.5 mM calcium chloride) and resuspended in the same buffer (95 μL) at a maximum of 0.8×10^6 cells/mL in the presence of FITC-Annexin V binding (3 μL). After 30 min incubation at room temperature in the dark, PI (20 μL, 20 μg) was added [22]. Cells, double-stained with PI and annexin V-FITC, were processed by flow cytometry and laser-scanning cytometry (LSC), which collected green (525 nm) fluorescence for FITC conjugated antibody and red (675 nm) fluorescence for PI, under 488 nm excitation. HaCaT cells (human keratinocytes) were seeded into 6-well plates at 8.5×10^4 cells/well density and treated in the same way as the melanoma cell lines described above.

2.6. DNA fragmentation assay

Cells were treated with compound **4** and stausporine for 120 h. After treatment, cells were scraped off the plates and collected by centrifugation at 400 g for 5 min. Cells were lysed in a lysis buffer (1 mL, Realpure kit) and incubated with Proteinase K (6 μL, 120 μg) at 55 °C for 1 h. RNA was digested by incubation with RNase (7 μL, 70 μg) at 37 °C for 1 h. After treatment, protein precipitation buffer (800 μL, Realpure kit) was added and lysates were vortexed and centrifuged at $16\,000 \times g$ for 10 min. The DNA in the aqueous supernatant was extracted with isopropanol (600 μL) and centrifuged at $14\,000 \times g$ for 3 min. The supernatant was removed and the pellet was rinsed with 70% ethanol (600 μL), dried at room temperature for 15 min and resuspended in DNA hydratation solution (100 μL, Realpure kit).

Hydrated DNA was diluted 1/50 in MilliQ water for DNA quantification by UV spectrophotometer at 260 nm. Loading dye (3 μL) was added to 20 μg of DNA for each treatment, and the samples were resolved over 1% agarose gel (75 min at 80 V), containing ethidium bromide (2 μL) in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA; pH 8.0). The DNA bands were viewed under a UV transilluminator and (Vilber Lourmat, Marne-la-Vallée, France), followed by a video copy processor (Mitsubishi P91 system, Kyoto, Japan).

2.7. Statistics

The Student's *t* test was used for statistical analysis. For each compound, a minimum of four independent experiments with duplicate values to measure growth inhibition and a minimum of three independent experiments for cell cycle analysis, assessment of apoptosis and DNA fragmentation were conducted. Data are given as the mean ± SD.

3. Results and discussion

3.1. Free radical scavenging activity. Hydrogen donation versus electron transfer

We had previously measured the hydrogen donation capacity of **1**–**4** in the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay [16]. The thio-derivatives **2**–**4** donated 4.2, 3.8 and 10.0 hydrogen atoms per molecule, respectively, whereas the underivatised (−)-epicatechin **1** gave 2.8 hydrogen atoms. Using the new radical chemosensor HNTTM [17], we measured the electron transfer capacity of **1**–**4**. The epicatechin and catechin conjugates **2** and **3** transferred only 2.4 and 2.3 electrons per molecule to HNTTM, respectively, and compound **1** had similar results (2.4). The most potent compound, Cys-ECG **4**, transferred 6.7 electrons per molecule, which was still less than the 10.0 hydrogen atoms donated to DPPH (Table 1). The hydrogen donation capacity of **4** was similar to that of EGCG and superior to the vitamin E analogue (Trolox) and another galloylated compound, propyl-gallate. The results prove that the thio-derivatives showed greater scavenging capacity by hydrogen atom donation than by electron transfer (high H/e values, Table 1). The order of efficiency in both scavenging

Table 1
Free radical scavenging power and stoichiometry

Compound	DPPH (hydrogen donation)[a]			HNTTM (electron transfer)			
	ARP ($1/ED_{50}$)	Stoichiometric value	H atoms per molecule[b]	ARP ($1/ED_{50}$)	Stoichiometric value	Electrons per molecule[c]	H/e^- ratio
1	5.5	0.36	2.8	4.8	0.42	2.4	1.2
2	8.3	0.24	4.2	4.8	0.42	2.4	**1.7**
3	7.7	0.26	3.8	4.7	0.43	2.3	1.7
4	20.0	0.10	**10.0**	13.3	0.15	**6.7**	**1.5**
Trolox	3.9	0.52	1.9	5.4	0.37	2.7	0.7
EGCG	21.3	0.09	10.6	11.3	0.17	5.9	**1.8**
Propyl-gallate	9.5	0.21	4.7	6.1	0.33	3.1	**1.5**

Standard deviation ($n = 3$): $\leqslant 0.3$ (ARP), $\leqslant 0.04$ (stoichiometric value), $\leqslant 0.2$ (H or e^- per molecule).
[a]Results for compounds **1**–**4** from Ref. [16].
[b]Moles reduced DPPH per mole antioxidant.
[c]Moles of reduced HNTTM per mole antioxidant.

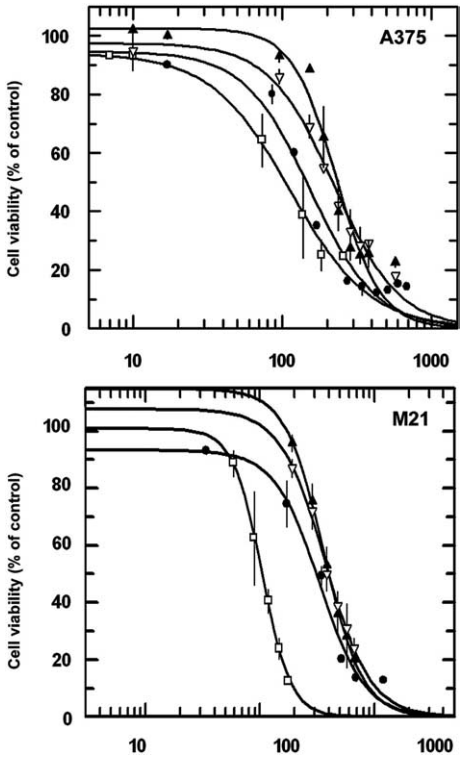

Fig. 3. Proliferation of A375 cells and M21 cells in response to EC (●), Cys-EC (▲), Cys-Cat (▽) and Cys-ECG (□). Cell cultures were treated with increasing doses of these compounds, as indicated on the *x* axis. Mean ± S.D., $n \leqslant 8$.

assays was **4** > **3** ∼ **2** ∼ **1**. This may be of biological significance because the capacity to transfer electrons is regarded sometimes as an undesired effect [23] related to the pro-oxidant action of gallocatechins such as (−)-epigallocatechin (EGC) and (−)-epigallocatechin-gallate (EGCG), and is mainly associated with the pyrogallol group on ring B [18,19]. Potent compounds such as Cys-ECG **4**, which do not include this group, might be safer than equipotent gallocatechins such as EGCG.

3.2. Growth inhibition capacity

Treatment of melanoma cells with the thio-conjugates for 72 h resulted in a reduction in cell viability in a dose-dependent fashion (Fig. 3), with the order of cell growth inhibition capacity being **4** > **1** ∼ **2** ∼ **3** for both A375 and M21 cells, according to the IC_{50} values obtained (Table 2). The cysteinyl conjugates were equally or slightly less efficient than the underivatised (−)-epicatechin, **1**, in inhibiting the growth of melanoma cells. Interestingly, the gallate-containing derivative **4**, which was the most effective compound as a free radical scavenger, was also the most efficient molecule in inhibiting cell growth in both melanoma cell lines.

Table 2
Antiproliferative potency against human melanoma cell lines

Cell line	Compound	*n*[a]	Mean IC_{50} (μM)	S.D.
A375	**1**	4	154	15
	2	4	231	14
	3	4	223	10
	4	4	113	6
M21	**1**	7	332	39
	2	5	363	18
	3	5	365	14
	4	5	104	4

S.D., standard deviation.
[a]*n*, number of experiments performed.

3.3. Cell cycle analysis and apoptosis induction

To examine the effects of the flavan-3-ols **1**-**4** on the cell cycle pattern at concentrations equal to their IC_{50} and twice their IC_{50}, A375 and M21 cells were treated with each compound for 72 h, and then analysed by FACS (Fig. 4). The galloylated cysteinyl compound **4** induced the most significant increase in the S cycle phase, at $2 \times IC_{50}$ over the untreated cells. The rest of the flavanols, **1**-**3**, showed a cell cycle distribution similar to the untreated cells in A375 and M21. The fact that Cys-ECG (**4**) treatment caused a major arrest in S phase in both cell cycles, whereas Cys-EC (**2**) did not cause any significant effect on either A375 or M21 at twice its IC_{50}, demonstrated that the gallate structure was, at least in part, responsible for this effect on the cell cycle (Fig. 4).

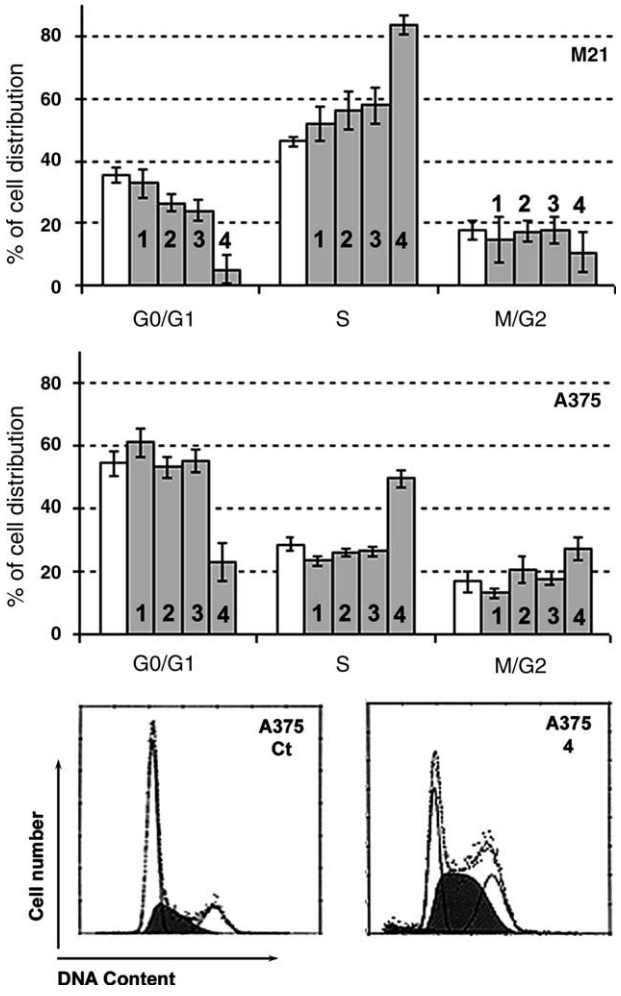

Fig. 4. Influence of the flavanols on M21 and A375 cell cycles after 72 h of treatment. Non-treated cells are indicated by open bars. Doses ($2 \times IC_{50}$) in M21 cells: EC (**1**), 660 μM; Cys-EC (**2**) and Cys-Cat (**3**), 720 μM; Cys-ECG (**4**), 208 μM. Doses ($2 \times IC_{50}$) in A375 cells: **1**, 310 μM; **2** and **3**, 420 μM; **4**, 237 μM. Cell cycle distribution of untreated A375 cells (Ct) and Cys-ECG (**4**) in A375 cells.

Since cell cycle arrest may lead to apoptosis, in the next series of experiments we used FITC-FACS analysis to establish whether necrosis or apoptosis was involved. A375 and M21 cells treated with the compounds **1**-**4** at their IC_{50} slightly increased the number of early ($PI^-/FITC^+$) and late ($PI^+/FITC^+$) apoptotic cells from the control cell figure. With increasing doses ($2 \times IC_{50}$) of compounds **1**–**3**, apoptosis induction doubled (Fig. 5, *right bottom* $PI^-/FITC^+$ *and upper* $PI^+/FITC^+$ *quadrants*). The gallate-containing Cys-ECG (**4**, at $2 \times IC_{50}$) showed an apoptotic effect four times greater than in control cells in both melanoma cell lines (Fig. 5 *right quadrants*, where 21% of cells in A375 and 31% of cells in M21 were considered apoptotic cells). This result corroborates findings of other authors, who described how gallate compounds induced apoptosis effectively, whereas induction was weaker in compounds that lacked a gallate ester [10,24–28]. To discriminate between late apoptotic and necrotic cells after treatment with compound **4**, we investigated PI and annexin V-FITC positive cells using LSC analyses with microscopic observations. A375 cells treated with compound **4** showed 8% of cells in the *right bottom quadrant* ($PI^-/FITC^+$ region). These, viewed by LSC microscope, showed the limited nuclear fragmentation and the typical green appearance, which is a distinctive morphology of early apoptosis caused by the labelling of annexin V by FITC. Late apoptosis/necrosis was present in 13% (*right upper quadrant*, $PI^+/FITC^+$ region) of A375 cells, which showed advanced nuclear fragmentation and limited staining with pycnotic nuclei, which is a definitive sign of the formation of the apoptotic cell. Similarly in M21, compound **4** had 5% of cells in early apoptosis and 26% of cells in late apoptosis/necrosis (Fig. 5).

To explore the selectivity of the effect on tumour cells, we tested compounds **1**-**4** in a non-malignant cell line. HaCaT cells were treated with the compounds at the highest doses used for A375 melanoma, and the apoptosis mechanism was activated in none of them (Fig. 5).

The order of efficiency in both the antiradical and apoptotic induction assays coincided. The gallate-containing compound **4** was the most scavenging agent and compounds **2** and **3** were little more efficient than underivatized **1** as free radical scavengers, with the order of efficiency being $\mathbf{4} > \mathbf{3} \sim \mathbf{2} \sim \mathbf{1}$ (Table 1). Moreover, compound **4** was also the major apoptotic inducer in melanoma cells and compounds **2** and **3** were slightly less efficient than **1**, with the order of induction being $\mathbf{4} > \mathbf{1} \sim \mathbf{2} \sim \mathbf{3}$. The gallate group provides, first, great scavenging power through its three contiguous hydroxyl groups [16,29] and, second, pro-apoptotic action probably through inhibition of kinase activities [30,31]. Although electronic distribution within the gallate moiety might play a role in both cases, the two effects may, in the final analysis, be unrelated.

3.4. Detection of fragmented DNA by agarose gel electrophoresis

The induction of apoptosis will stimulate endonuclease that involves double-strand DNA breaks into oligonucleosome length fragments, resulting in a characteristic ladder of DNA electrophoresis [32]. DNA fragmentation was observed in melanoma cells A375, but not in HaCaT cells at Cys-ECG (**4**) concentrations of 200 μM. Fragmentation is a late event in apoptosis mechanism and DNA fragments (DNA ladders), showing varying sizes between 250 and 2000 bp, were clearly visible after agarose gel electrophoresis (Fig. 6). No specific DNA fragments were detected when control cell cultures were analysed. As a positive control, an apoptotic inducer (staurosporine, 1.6 μg/mL) was incubated in A375 and HaCaT cell cultures 5 h before DNA extraction. Staurosporine treatment

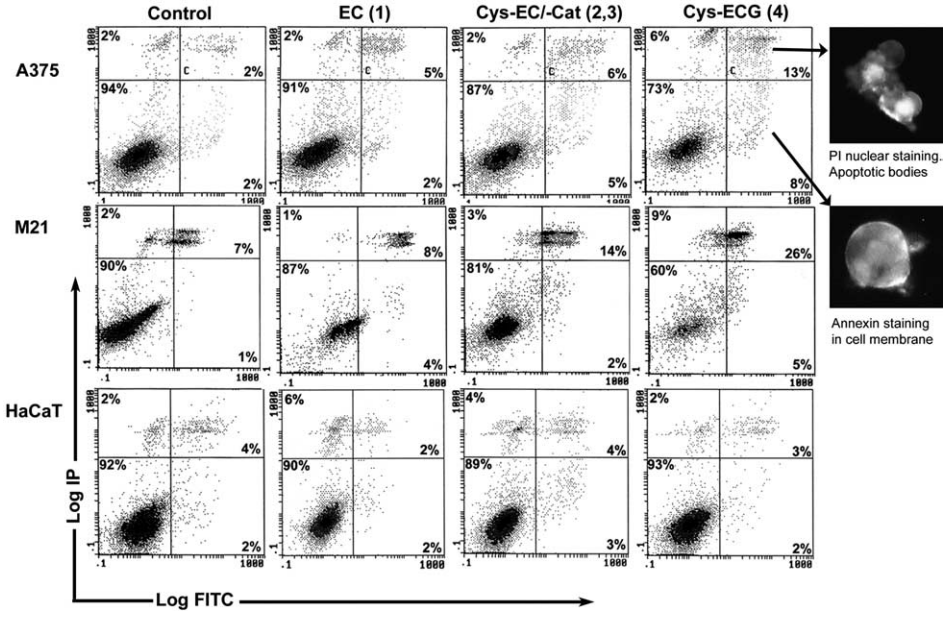

Fig. 5. Flavanols induced apoptosis of A375 and M21 cells, but not of HaCaT cells. Doses in A375 cells: EC (**1**), 310 μM; Cys-EC (**2**) and Cys-Cat (**3**), 420 μM; Cys-ECG (**4**), 237 μM. Doses in M21 cells: **1**, 660 μM; **2** and**3**, 720 μM; **4**, 208 μM. In HaCaT cells: **1**, **2** and **3**, 400 μM; **4**, 200 μM. The percentage of apoptotic cells was determined using PI (represented on the *y* axis) and annexin V-FITC staining (on the *x* axis). After 72 h treatment, early apoptosis ($PI^-/FITC^+$, right bottom quadrant) and late apoptosis ($PI^+/FITC^+$, right upper quadrant) are shown as % of apoptotic cells. The LSC photomicrographs showed single cells as representative of A375 cell population treated with **4**.

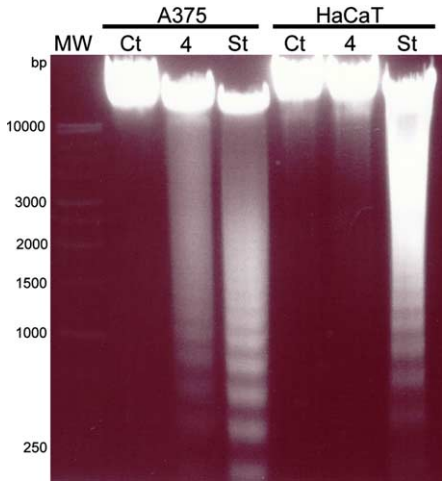

Fig. 6. Compound **4** causes DNA laddering in A375 melanoma cells, but not in HaCaT cells. Staurosporine (ST) is used as a positive control for both cell lines. Cells were incubated with compound **4** (200 μM) or staurosporine (2 μM) for 120 h. Cell DNA was extracted and analysed by agarose gel electrophoresis. Data shown are representative of three independent experiments.

showed specific DNA fragmentation in both cell lines (Fig. 6). At 72 h of treatment, FACS analysis showed less than 25% of cells in apoptotic mechanism (Fig. 5), so apoptotic bodies were not noticeable in DNA fragmentation assay. To increase the rate of apoptotic cells, fragmentation was measured after 120 h of treatment. In this case, the later events in programmed cell death were clearer and the results confirmed those obtained in FACS analysis, showing compound **4** as an apoptotic inducer in melanoma cells but not in keratinocytes.

Most chemotherapeutic drugs currently used in cancer therapy kill cancer cells by indirectly activating checkpoint-mediated apoptosis after creating non-selective damage to DNA or microtubules, which accounts for their toxicity toward normal cells. A promising example that might avoid such damage is lapachone, which acts by activating checkpoints in cancer cells resulting in cell cycle arrest in S phase and selective induction of apoptosis in cancer cells, but not in proliferating normal cells [33]. The new flavanol derivative 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (Cys-ECG, **4**), obtained from grape procyanidins, showed an antioxidant capacity equal to the well-known EGCG and with the advantage that compound **4** enhanced the scavenging capacity by hydrogen atom donation rather than by electron transfer, which is related to the pro-oxidant effects of catechins such as EGC and EGCG [18,19]. Moreover, compound **4** triggers cell cycle arrest in S phase and selective induction of apoptosis in melanoma cells, but not in proliferating keratinocytes. Interestingly, several authors describe EGCG as an inducer of apoptosis in HaCaT cells [34]. All these findings make compound **4** a promising molecule to be considered in new strategies seeking to target cancer cells by directly activating checkpoint regulators of the cell cycle without creating non-selective DNA damage. Furthermore, as it lacks the pyrogallol group on the condensed flavanic structure, this compound might be safer than other potent polyphenols of the gallocatechin type.

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6.3 ANNEX III : ALTRES PUBLICACIONS

En el transcurs d'aquest treball de recerca s'han originat col·laboracions amb d'altres grups d'investigació, donant lloc publicacions conjuntes que es recullen en aquest **Annex III** amb una breu descripció del treball:

Annex III.1

Micellar electrokinetic chromatography estimation of size and composition of procyanidins after thiolysis with cysteine.

JOSÉ M. HERRERO-MARTÍNEZ, CLARA RÀFOLS, MARTÍ ROSÉS, ELISABETH BOSCH, CARLES LOZANO AND JOSEP L. TORRES.

Electrophoresis, 24, 1404-1410 (2003)

Es proposa el mètode de cromatografia electrocinètica micel·lar (MEKC) com una alternativa a l'HPLC a la hora d'estimar la mida i la composició dels compostos flavanolics després de la tiòlisi. En aquest cas, el cru de *Vitis vinifera* despolimeritzat amb cisteïna mostrà una millor separació dels seus monomers en la cromatografia MEKC que en l'HPLC.

Annex III.2

Percutaneous absorption of flavan-3-ol conjugates from plant procyanidins.

ALONSO C., RAMÓN E., LOZANO C., PARRA J.L., TORRES J.L., CODERCH L.

Drugs under Experimental and Clinical Research 30 (1), 1-10 (2004)

S'avaluà l'absorció percutànea dels conjugats emprant metodologia in vitro amb pell humana i de porc. S'obtingué una bona correlació en ambdues pells i, depenent de la conjugació, els compostos penetraren en diferents capes de la pell. Així, la 4β-(S-cisteinil)epicatequina (**7**) mostrà una major tendència a ser localitzada en les capes més externes de la pell mentre que la 4β-(S-aminoetiltio)epicatequina (**4**) mostrà una pronunciada capacitat de penetració.

Annex III.3

Novel epicatechin derivatives with antioxidant activity modulate interleukin-1β release in lipopolysaccharide-stimulated human blood.

MONTSERRAT MITJANS, VERÓNICA MARTÍNEZ, JAIME DEL CAMPO, CELIA ABAJO, CARLES LOZANO, JOSEP LLUÍS TORRES AND MARÍA PILAR VINARDELL.

Bioorganic & Medicinal Chemistry Letters, 14, 5031-5034 (2004)

Els conjugats de epicatequina **4** i **7**, degut a la seva capacitat antioxidant, s'incubaren amb una suspensió d'eritròcits, observant la protecció a l'hemòlisi induïda per radicals. La capacitat de protecció fou més efectiva amb els conjugats **4** i **7** que amb la pròpia epicatequina **1**. A més, s'observà que aquests compostos inhibiren la producció d'interleuquina-1 β en sang, considerada un dels factors que més influencien en la resposta inflamatòria. El compost més efectiu fou el conjugat **4.**

Annex III.4

Immunomodulatory activity of a new family of antioxidants obtained from grape polyphenols.

MONTSERRAT MITJANS, JAIME DEL CAMPO, CELIA ABAJO, VERÓNICA MARTÍNEZ, ARIADNA SELGA, CARLES LOZANO, JOSEP LLUÍS TORRES AND MARÍA PILAR VINARDELL.

Journal of Agricultural and Food Chemistry, 52, 7297-7299 (2004)

Es seguí el treball posterior afegint l'estudi dels conjugats galats **6** i **9**. El compost galat **6** fou el més efectiu en la protecció dels eritròcits en front de l'hemòlisi induïda per radicals. Igualment, s'evaluà la seva influència sobre la resposta inflamatòria, on tots els compostos inhibiren la producció de interleuquina-1 β i interleuquina-6 en sang. El compost més efectiu seguí sent el conjugat **4**, en front dels compostos gal·loitzats..

Annex III.5

Procyanidin fractions from pine (P*inus pinaster*) bark: Radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells.

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Journal of Agricultural and Food Chemistry, 53, 4728-4735 (2005)

A partir del cru de *Pinus pinaster*, es van extreure diverses fraccions amb diferent grau de polimerització però sense la presencia d'èsters de galat en la seva estructura. Es determinà el seu poder antioxidant en solució i en emulsió i es comparà amb les fraccions corresponents al cru de *Vitis vinifera* (que si contenien cert grau de gal·loització). En l'estudi es veié que les fraccions contenint grups galats augmentaren la capacitat antioxidant en solució però no en les emulsions. A més, les fraccions presentaren una moderada capacitat d'inhibir la proliferació cel·lular en una línia de melanoma humà.

Annex III.6

Conjugation of catechins with cysteine generates antioxidant compounds with enhanced neuroprotective activity.

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Phytochemistry, 66 (17), 2032-2037 (2005)

La conjugació de la epicatequina amb grups tiols permeté augmentar la protecció de cèl·lules neuronals en front del glutamat. Es mostrà que la capacitat antioxidant i neuroprotectora no estan relacionades, degut a que el conjugat galat **9** mostrà la mateixa capacitat protectora que el conjugat **7**, sent ambdós més eficients que la epicatequina **1**. Els resultats suggereixen que un mecanisme diferent a la capacitat antiradicalària és el que està involucrat en la neuroprotecció i possiblement estigui més relacionat amb mantenir els nivells de glutatió.