



# Off-line comprehensive 2-dimensional hydrophilic interaction $\times$ reversed phase liquid chromatography analysis of procyanidins

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## ARTICLE INFO

### Article history:

Received 27 February 2009

Received in revised form 20 June 2009

Accepted 26 June 2009

Available online 1 July 2009

### Keywords:

Comprehensive 2-dimensional liquid chromatography

Procyanidins

Hydrophilic interaction liquid chromatography (HILIC)

Reversed phase liquid chromatography (RP-LC)

Cocoa

Apple

## ABSTRACT

The development of an off-line comprehensive 2-dimensional liquid chromatography (2-D-LC) method for the analysis of procyanidins is reported. In the first dimension, oligomeric procyanidins were separated according to molecular weight by hydrophilic interaction chromatography (HILIC), while reversed phase LC was employed in the second dimension to separate oligomers based on hydrophobicity. Fluorescence, UV and electrospray ionisation mass spectrometry (ESI-MS) were employed for identification purposes. The combination of these orthogonal separation methods is shown to represent a significant improvement compared to 1-dimensional methods for the analysis of complex high molecular weight procyanidin fractions, by simultaneously providing isomeric and molecular weight information. The low correlation ( $r^2 < 0.2100$ ) between the two LC modes afforded a practical peak capacity in excess of 2300 for the optimal off-line method. The applicability of the method is demonstrated for the analysis of phenolic extracts of apple and cocoa.

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## 1. Introduction

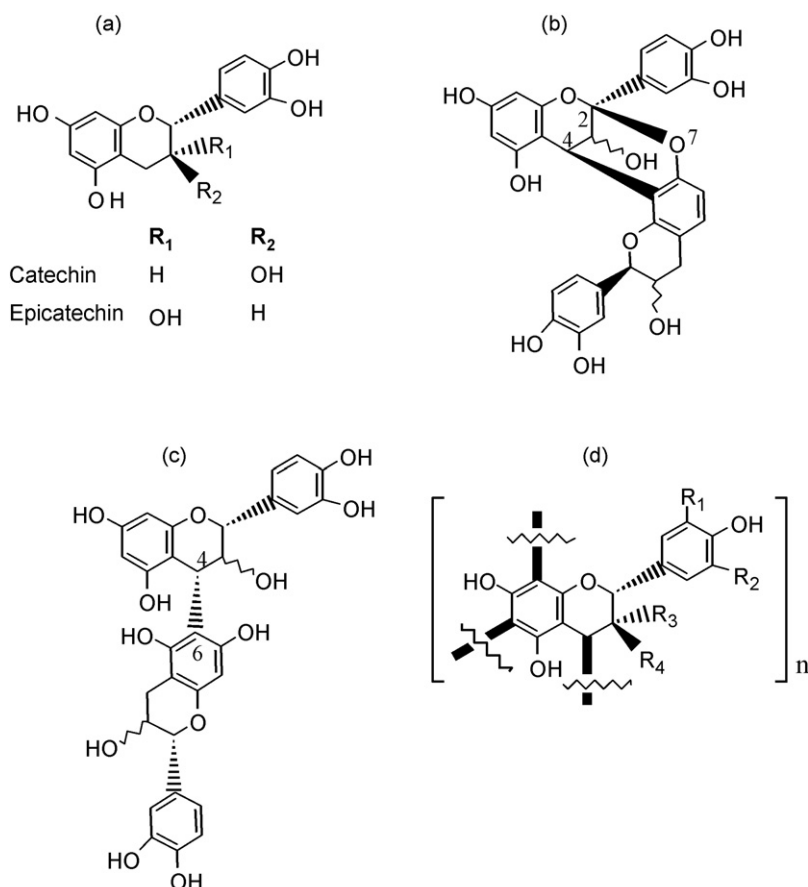
Proanthocyanidins are a group of polyphenolic compounds found widespread throughout the plant kingdom [1], and comprise oligomeric or polymeric phenols composed of flavan-3-ol monomeric units joined through interflavanoid linkages [2]. Monomeric units are most frequently linked through C4  $\rightarrow$  C6 or C4  $\rightarrow$  C8 bonds (B-type), or, less commonly, a second interflavanoid bond could result from oxidative coupling of C2  $\rightarrow$  O7 to form A-type oligomers (Fig. 1) [3–9]. Proanthocyanidins are further subdivided into procyanidins, prodelphinidins and propelargonidins [5]. Procyanidins form the largest class of proanthocyanidins [3] and are made up exclusively of catechin and epicatechin flavan-3-ol units. Prodelphinidins contain gallocatechin and epigallocatechin units, while propelargonidins comprise afzelechin and epiafzelechin building blocks. In addition, monomeric units consisting of gallic acid esters of (epi)catechin and (epi)gallocatechin ((epi)catechin/(epi)gallocatechin gallate) are also found in nature.

Proanthocyanidins are the second most abundant natural phenolic compounds following lignin and form an essential part of the human diet [4,10], as they are present in common foods such as apples, berries, cocoa, grapes, wine, etc. These compounds have

been studied extensively in recent years in the fields of health, nutrition and medicine [1,3,11]. This is due to the growing body of evidence suggesting that they exhibit a diversity of physiological effects such as anti-oxidant, anti-microbial, anti-allergy, anti-inflammatory, anti-carcinogenic and anti-hypertensive activities [1–3,5–8,10–16].

In spite of considerable advances, the separation of proanthocyanidins remains a challenge owing to the complexity of their structures, particularly their degree of polymerisation (DP), a key feature which determines their physico-chemical properties [12,14,15,17]. Improved separation methods for these compounds are required for, amongst others, *in vivo* studies of their physiological activities [12,15]. Reversed phase liquid chromatography (RP-LC) coupled with fluorescence and/or MS detection is currently the most powerful method for the analysis of these compounds, and resolution of isomers up to tetramers has been reported [1,7]. However, the resolution of more structurally diverse higher oligomers has not yet been achieved [11]: these compounds co-elute as a large unresolved “bump” in RP separations [1,7,13]. Promising separation of procyanidins based on molecular weight (MW) has been achieved by normal phase LC (NP-LC), although such methods do not provide resolution of isomers [7,17,18]. Quite recently, the successful application of hydrophilic interaction liquid chromatography (HILIC) for similar MW separation of procyanidins has been reported [4,12]. HILIC is a variant of NP-LC where polar stationary phases are used in combination with aqueous mobile phases

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**Fig. 1.** Structures of (a) proanthocyanidin monomeric units, (b) an A-type procyanidin dimer, (c) a C4 → C6 linked B-type procyanidin dimer, and (d) general polymerisation pattern for proanthocyanidins.

to separate analytes according to polarity. The retention mechanism is thought to involve partitioning of analytes between the mobile phase and a water-layer immobilised on the stationary phase [19,20].

Conventional LC separations offer insufficient resolving power when faced with the analysis of complex procyanidin fractions. For instance, taking into account the four possible types of interflavan bonds, it is theoretically possible to obtain 48 dimeric isomers of catechin and/or epicatechin, while this number increases with the degree of polymerisation [21]. Although not all isomers may be found in a given sample, a large number of isomers have been observed in various natural products. Clearly, the resolution of such large numbers of isomers is not possible with standard 1-dimensional LC methods, which are characterised by maximal theoretical peak capacities not exceeding 200.

Comprehensive 2-D LC techniques offer a powerful approach to increase peak capacity and are ideally suited for the analysis of complex samples, provided the selected separation modes are orthogonal (i.e., based on different separation mechanisms) [22–30]. From this perspective, the combination of HILIC and RP-LC holds significant promise for the comprehensive 2-D LC separations of procyanidins. Several 2-D LC methods have been developed for the analysis of phenolic compounds in a variety of samples [31–39]. However, to the best of our knowledge, no comprehensive 2-dimensional LC method suitable for the analysis of proanthocyanidins has previously been reported. In this paper, an off-line 2-D LC method that allows the separation of procyanidins, as well as additional natural phenolic compounds, is reported. The utility of the developed method is demonstrated for the analysis of cocoa (*Cacao Theobroma*) and apple phenolic extracts.

## 2. Experimental

### 2.1. Reagents and materials

Cocoa beans and apples (Red Starking variety) were purchased from a local supermarket. Standards of (–)-epicatechin and (±)-catechin as well as HPLC grade methanol, acetonitrile, isopropyl alcohol, formic acid and acetone were purchased from Sigma Aldrich (Steinheim, Germany). HPLC grade hexane and acetic acid were purchased from Burdick & Jackson (Muskegon, USA) and Riedel-de Haën (Seelze, Germany), respectively. Dichloromethane (analytical grade) was purchased from ROMIL (Waterbeach Cambridge, England) and ethyl acetate from AnalaR (Midrand, South Africa). Deionised water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA). All solutions were filtered through 0.45 μm HVLP membrane filters (Millipore) and degassed in an ultrasonic bath (Branson Model 3510, Danbury, USA) before use.

### 2.2. Instrumentation

#### 2.2.1. UPLC-PDA-fluorescence analyses

Analyses were performed on an Acquity UPLC system equipped with a binary pump, autosampler, column oven, photodiode array (PDA) detector (500 nL flow cell, 10 mm path length) and controlled by Waters Empower software (Waters, Milford, MA, USA). The mixing chamber on the UPLC was replaced with a low dead volume union, while the pump was connected to the injection valve using 0.1 mm i.d. tubing (Waters) to reduce the system dead volume. An HP 1046A Programmable Fluorescence detector (5 μL flow cham-

ber, Agilent, Waldbronn, Germany) was connected to the UPLC system downstream of the PDA detector. Fluorescence data were acquired using the DAX 8.0 data acquisition software (Van Mierlo software, Amsterdam, The Netherlands).

### 2.2.2. UPLC–MS analyses

LC–MS analyses were performed on a Waters Acquity UPLC system equipped with a binary solvent manager and autosampler using HILIC and RP-LC conditions as outlined below. The UPLC system was interfaced through an electrospray ionisation (ESI) ion source to a Waters Ultima API quadrupole time-of-flight (Q-TOF) mass spectrometer. The mass spectrometer was operated in a negative ionisation mode with a capillary voltage of  $-3.7$  kV and a cone voltage of  $35$  V. The source temperature was  $100$  °C. Masses were scanned from  $285$  to  $2025$  amu and data were collected and processed using MassLynx v.4.0 software (Waters). The instrument was calibrated using a NaF solution. Since no lock-spray function was available on this instrument,  $m/z$  ratios are reported to one decimal place.

For HILIC analyses, a desolvation temperature of  $300$  °C was applied. The desolvation and cone gas flows (both  $N_2$ ) were  $300$  L/h and  $50$  L/h, respectively. For RP-LC analyses, the desolvation temperature was  $350$  °C, and desolvation and cone gas flows (both  $N_2$ ) were  $350$  L/h and  $50$  L/h, respectively. The eluent was split  $1:7$  prior to introduction into the ionisation chamber for RP-LC–ESI-MS analyses.

## 2.3. Chromatographic methods

### 2.3.1. Hydrophilic interaction chromatography (HILIC) analyses

Separations were performed on a Nomura Chemical Develosil Diol-100 column ( $250$  mm  $\times$   $1$  mm i.d.,  $5$   $\mu$ m  $d_p$ , Aichi, Japan) using a method adapted from Kelm et al. [4]. The binary mobile phase consisted of (A) acetonitrile and acetic acid ( $99:1$ , v/v) and (B) methanol, water and acetic acid ( $94.05:4.95:1$ , v/v/v). Separations were effected by a series of linear gradients of B into A as follows:  $4$ – $40\%$  B ( $0$ – $45$  min),  $40\%$  B isocratic ( $45$ – $50$  min),  $40$ – $4\%$  B ( $50$ – $55$  min). The column was re-equilibrated for  $15$  min. The flow rate was  $0.050$  mL/min. Fluorescence detection was performed with excitation at  $276$  nm and emission at  $316$  nm with a photomultiplier (PMT) gain of  $5$ . Separations were performed at ambient temperature. For HILIC-PDA-fluorescence analyses,  $10$   $\mu$ L was injected in the ‘partial loop with needle overfill’ mode using acetonitrile/HOAc ( $99:1$ , v/v) as the weak needle wash solvent. For HILIC–ESI-MS analyses,  $5$   $\mu$ L was injected under the same conditions.

### 2.3.2. Reversed phase liquid chromatography analyses

The binary mobile phase consisted of acetonitrile (A) and  $0.1\%$  formic acid in water (v/v) (B). Injections were performed in the partial loop with needle overfill mode using  $0.1\%$  formic acid as the weak needle wash. The column temperature was  $50$  °C, and UV chromatograms were recorded at  $210$ ,  $280$ ,  $320$  and  $370$  nm. Fluorescence detection was performed as for HILIC analyses.

Two gradient methods were developed. For fast RP-LC-PDA-fluorescence analyses, separation was performed using an Agilent Zorbax SB-C18 column ( $50$  mm  $\times$   $4.6$  mm i.d.,  $1.8$   $\mu$ m) protected with a Phenomenex C18 guard column ( $4$  mm  $\times$   $3$  mm i.d., Torrance, USA). A linear gradient was performed as follows:  $2\%$  A ( $0$ – $0.03$  min),  $2$ – $18\%$  A ( $0.03$ – $1.20$  min),  $18$ – $25\%$  A ( $1.20$ – $2.00$  min),  $25$ – $100\%$  A ( $2.00$ – $2.30$  min),  $100\%$  A until  $2.50$  min before returning to the initial conditions at  $2.80$  min. The column was re-equilibrated for  $1.20$  min before the next analysis. The flow rate was set to  $1.8$  mL/min and  $3$   $\mu$ L was injected.

For high efficiency RP-LC-PDA-fluorescence analyses, separations were performed using two Zorbax SB-C18 columns (each  $50$  mm  $\times$   $4.6$  mm i.d.,  $1.8$   $\mu$ m) coupled in series and protected with

a guard column as above. A linear gradient was performed as follows:  $2\%$  A ( $0$ – $0.25$  min),  $2$ – $18\%$  A ( $0.25$ – $10.70$  min),  $18$ – $25\%$  A ( $10.70$ – $18.00$  min),  $25$ – $100\%$  A ( $18.00$ – $20.70$  min),  $100\%$  A until  $22.50$  min before returning to the initial conditions at  $25.20$  min. The column was re-equilibrated for  $4.80$  min before the next analysis. The flow rate was set to  $0.8$  mL/min and  $2$   $\mu$ L was injected.

UPLC–ESI-MS analyses were performed using identical conditions as outlined above for the high efficiency RP-LC gradient analysis, with injections performed in the partial loop mode using water as the weak needle wash. The injection volume was  $4$  and  $6$   $\mu$ L, respectively, for the apple and cocoa samples.

## 2.4. Off-line 2-dimensional (HILIC $\times$ RP-LC) analyses

One-minute fractions (corresponding to  $50$   $\mu$ L each) of the HILIC separations were automatically collected between  $2$  and  $52$  min using a programmable BIO-RAD Model 2110 fraction collector (Corston, UK). The collected fractions were transferred to  $1.5$  mL vials containing  $250$   $\mu$ L inserts and kept under  $N_2$  until analysed by gradient RP as detailed above. All fractions were analysed within  $2$  days of collection. Raw fluorescence data for the RP analyses were exported into STATISTICA 8 (Statsoft Inc., US) to create 2-D contour plots.

## 2.5. Sample preparation

### 2.5.1. Cocoa beans

The sample was prepared using a modified method to that reported by Hammerstone et al. [11]. Dried cocoa beans were ground in liquid nitrogen using a mortar and pestle. About  $2.0$  g of the ground material was extracted three times with  $9$  mL hexane to defat the sample. The remaining solid material was dried under a gentle stream of  $N_2$  to give a final mass of  $1.39$  g. The dried sample was extracted three times with  $3$  mL  $70\%$  acetone in water (v/v), followed by three times  $3$  mL  $70\%$  methanol in water (v/v). The extracts were combined and centrifuged for  $5$  min at  $5000 \times g$ . The organic solvents were removed by rotary evaporation under reduced pressure at  $40$  °C to give a final volume of  $4$  mL. The remaining aqueous extract was filtered through a  $0.45$   $\mu$ m hydrophilic PVDF filter membrane (Millipore). The sample was kept under nitrogen at  $4$  °C and was used for all the RP-LC analyses. For HILIC analyses, the sample was freeze dried and redissolved in  $70\%$  acetonitrile and  $30\%$  methanol (v/v).

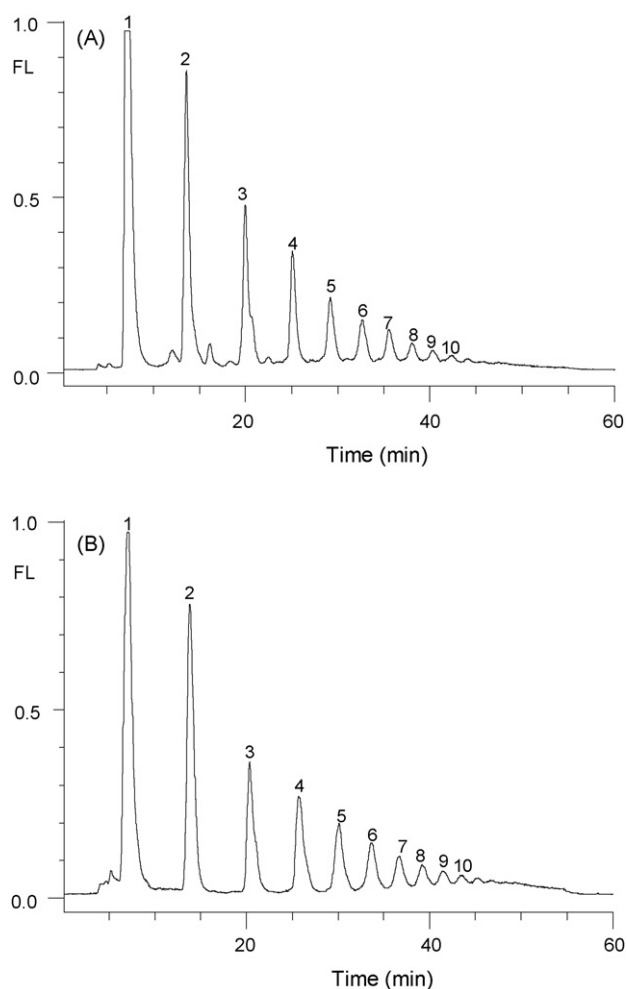
### 2.5.2. Apple

The apple sample was prepared using a modified method to that of Lazarus et al. [7]. Half an apple was ground in liquid nitrogen using a mortar and pestle. About  $10.0$  g of the finely ground material was extracted two times with  $20$  mL  $70\%$  acetone in water (v/v). The suspension was centrifuged for  $10$  min at  $5000 \times g$  after every extraction and the supernatants were combined in a round bottom flask. Acetone was removed by rotary evaporation under reduced pressure at  $40$  °C, to give a final volume of  $\sim 10$  mL. The remaining aqueous extract was filtered through a  $0.45$   $\mu$ m PVDF filter membrane. The sample was kept under nitrogen at  $4$  °C and was used for all the RP-LC analyses. For HILIC analyses, the sample was freeze dried and redissolved in acetonitrile, methanol and water in volume ratios of  $70:29:1$  (v/v/v), respectively.

## 3. Results and discussion

### 3.1. Optimisation of HILIC separation of procyanidins

Fluorescence detection of procyanidins was employed due to the improved selectivity and sensitivity offered by this technique compared to UV detection [1,4,7,40,41]. Initially, four methods



**Fig. 2.** Optimised HILIC analysis of the cocoa (A) and apple (B) procyanidins. Labels 1–10 indicate the DP of procyanidins as identified by HILIC–ESI–MS. Detection: fluorescence; injection volumes: 1  $\mu$ L and 5  $\mu$ L for cocoa and apple samples, respectively.

adapted from literature [4,8,15,17] were evaluated on four different stationary phases, namely BETASIL diol-100 (Thermo Electron corporation, 250 mm  $\times$  4.6 mm i.d., 5  $\mu$ m  $d_p$ ), Spherisorb NH<sub>2</sub> (Waters, 250 mm  $\times$  4.6 mm i.d., 5  $\mu$ m  $d_p$ ), Luna CN (Phenomenex, 250 mm  $\times$  4.6 mm i.d., 5  $\mu$ m  $d_p$ ) and Develosil Diol-100 (Nomura Chemicals, 250 mm  $\times$  1 mm i.d., 5  $\mu$ m  $d_p$ ) columns. The goal was to select the column and mobile phase combination that provided the most efficient separation of procyanidins according to DP. Mobile phases based on hexane/acetone [8] and hexane/methanol/ethyl acetate [15] did not provide adequate separation on any of the stationary phases and were not investigated further. Acceptable separation was obtained using the methods of Kelm et al. [4] and Rigaud et al. [17] on both diol and Spherisorb NH<sub>2</sub> columns. A mobile phase composition of acetonitrile, methanol, acetic acid and water [4] in combination with the Develosil Diol phase was selected due to improved resolution of especially higher molecular weight procyanidins, as well as the compatibility of these solvents with RP solvents to be used in the second dimension.

Method optimisation was performed using cocoa extracts, and subsequently applied to apple samples. Individual fluorescence chromatograms for these samples are shown in Fig. 2. The method proved to be sufficiently repeatable, as evidenced by the average %RSD for retention times between days (4 days) below 2.9%.

**Table 1**

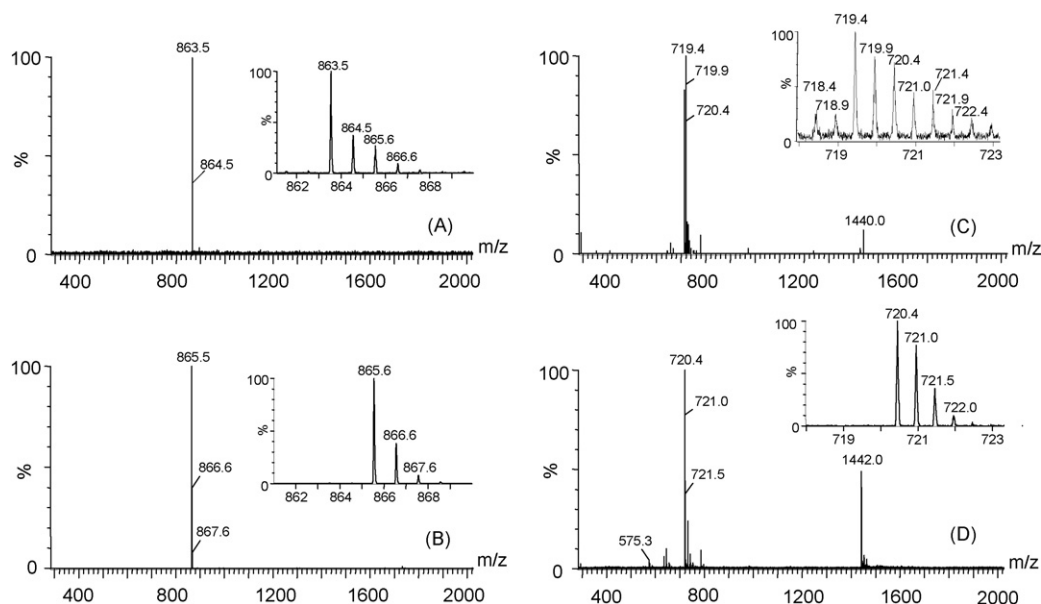
Summary of the predominant ions detected by HILIC–ESI–MS analysis of procyanidins in cocoa and apple extracts. Base peak ions are highlighted in bold.

DP	$[M-H]^-$	$[2M-H]^-$	$[M-2H]^{2-}/2$	$[M-3H]^{3-}/3$
1	<b>289.1</b>	579.3		
2	<b>575.3</b> <b>577.3</b>	1155.7		
3	<b>863.5</b> <b>865.5</b>			
4	<b>1151.7</b> <b>1153.7</b>		576.3	
5	1439.9 1441.9		<b>719.4</b> <b>720.4</b>	
6	1729.9		<b>864.5</b>	
7			<b>1009.1</b>	
8			<b>1152.7</b>	
9			<b>1297.3</b>	
10			1441.9	<b>960.6</b>
11			1586.0	<b>1056.6</b>
12			<b>1730.1</b>	

### 3.2. HILIC–ESI–MS analysis of cocoa and apple procyanidins

The optimised HILIC method was coupled to electrospray ionisation mass spectrometry (ESI–MS) in order to identify procyanidins. ESI is the most common atmospheric ionisation technique used for the study of procyanidins, and has been found to be efficient in both positive and negative modes, depending on analysis conditions. In this study, better ionisation was achieved in the negative ionisation mode (only singly charged ions for DP 1–7 could be detected in the positive ion mode).

Identification was based on mass spectra, including the presence of singly and multiply charged ions, and by comparison of retention times with fluorescence data and literature values. The singly charged molecular ions,  $[M-H]^-$ , were the most abundant ions for monomers until tetramers at  $m/z$  289.1, 577.3/575.3 (B/A-type dimers), 865.5 and 1153.7, respectively. For pentamers and hexamers, the doubly charged ions at  $m/z$  720.4 and 864.5 were the predominant ions. In addition, doubly charged ions were the most abundant ions for heptamers through nonamers as well as for dodecamers, while triply charged species were the most intense ions for decamers and undecamers. A summary of the dominant ions corresponding to different procyanidin oligomers as detected in cocoa and apple samples is presented in Table 1. These data are in accordance with literature values [7,11]. An example of the mass spectrum obtained for trimeric and pentameric procyanidins in the cocoa extract highlighting the principal ions detected is presented in Fig. 3. From the extracted ion chromatograms (EICs) acquired from analysis of the cocoa extract and shown in Fig. 4, it is evident that the procyanidins are indeed separated according to molecular weight using the optimised HILIC method. Also evident from this figure, is that the A-type oligomeric procyanidins, detected at  $m/z$   $[M_{(B-type)}-2]$  (Figs. 3 and 4(B)) are slightly less retained under HILIC conditions, as would be expected due to their lower polarity. Careful examination of mass spectral data indicates the presence of procyanidins up to hexamers containing 1 A-type linkage (data not shown). However, these A-type oligomers are present at much lower levels than their B-type equivalents, and with the exception of a single A-type dimer, are not clearly evident from the fluorescence data. Also of interest is that the major peak corresponding to the B-type trimers displays a significant shoulder at higher retention in both HILIC–fluorescence and HILIC–MS analyses. This might presumably be ascribed to two partially separated trimeric isomers. Since resolution in HILIC decreases with increasing DP, higher MW isomers are not equally resolved.



**Fig. 3.** Negative ESI mass spectra obtained from the HILIC-MS analysis of oligomeric procyanidins in the cocoa extract: A-type (A) and B-type (B) trimers; and pentamers with a single A-type (C) and only B-type (D) linkages.

Two additional compounds were detected at  $m/z$  707.4 and 737.4. The former compound partially co-eluted with dimeric procyanidins, while the latter was resolved between dimers and trimers. Mass spectral data and retention times are in agreement with A-type dimeric glycosides reported in cocoa extracts [11,42]. Porter et al. [42] identified these compounds as O- $\beta$ -D-galactopyranosyl-epicatechin-(2 $\alpha$   $\rightarrow$  7,4 $\alpha$   $\rightarrow$  8)-epicatechin and O-L-arabinopyranosyl-epicatechin-(2 $\alpha$   $\rightarrow$  7,4 $\alpha$   $\rightarrow$  8)-epicatechin. It is interesting to note that none of the corresponding B-type glycosides were observed. In addition, similar hexose derivative(s) of epicatechin/catechin were also observed at  $m/z$  451 [11], as were hexose and pentose derivatives procyanidin trimers through heptamers. Retention of the higher MW procyanidin-glycosides is in accordance with the procyanidin dimer-glycosides, i.e., slightly higher than the corresponding procyanidin aglycones. Although further work is required to determine the exact structures of these oligomeric procyanidin-glycosides, it seems likely that they are structurally related to the A-type dimeric molecules determined by Porter et al. [42]. To the best of our knowledge this is the first time

that higher molecular weight procyanidin-glycosides are reported in cocoa extracts.

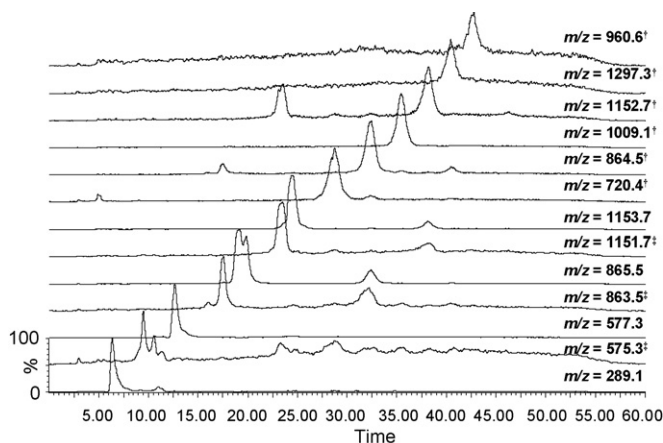
### 3.3. Optimisation of RP-LC separation of procyanidins

In initial studies, three different RP stationary phases and columns formats were evaluated: CN (Phenomenex Luna, 50  $\times$  4.6 mm, 5  $\mu$ m  $d_p$ ), Acquity BEH C18 (Waters, 50 mm  $\times$  2.1 mm, 1.7  $\mu$ m  $d_p$ ) and Zorbax SB-C18 (Agilent, 50 mm  $\times$  4.6 mm, 1.8  $\mu$ m  $d_p$ ). Similar separations were achieved on the Zorbax and UPLC columns, although the former column had a much higher loadability due to the larger internal diameter (i.d.). The CN column offered similar high loadability, although the lower efficiency associated with the larger particle size of this column did not provide satisfactory separation. Therefore, the Zorbax column was selected for further optimisation as this column offered the advantages of fast, efficient separations coupled to higher loadability, which is especially beneficial from a sensitivity point of view in a comprehensive 2-D-LC analysis.

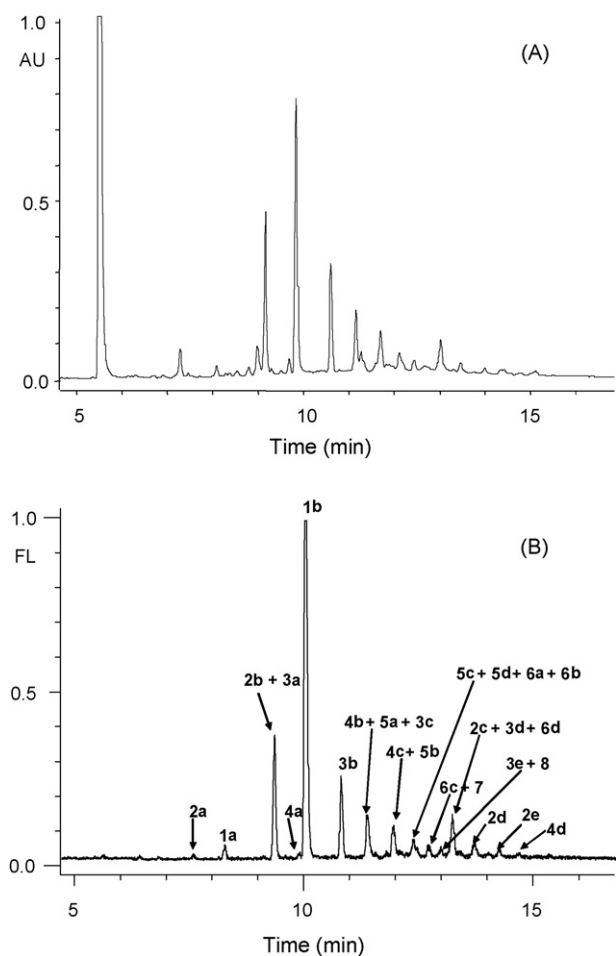
Two approaches were investigated: one employing a fast gradient operating close to maximum pressure for the column (600 bar); and a slower gradient performed on two coupled columns operated at close to optimal linear velocity for procyanidins for maximum efficiency. A column temperature of 50  $^{\circ}$ C was employed to reduce the mobile phase viscosity and allow faster mass transfer of solutes [43], and the gradient was adjusted so as to resolve as many compounds as possible within an acceptable time. Both RP-LC separations provide separation of procyanidin isomers according to hydrophobicity (as confirmed by RP-LC-ESI-MS analyses, see further), and numerous peaks are detected. Typical UV and fluorescence chromatograms for the high efficiency RP-LC analysis of a cocoa extract are shown in Fig. 5. Despite the high chromatographic efficiency offered by the use of two coupled 1.8  $\mu$ m columns, it is also evident from this figure that complete resolution of especially the higher MW isomers is not possible.

### 3.4. RP-LC-ESI-MS analysis of procyanidins

The optimised RP-LC method was coupled to ESI-MS in order to identify the major procyanidin isomers. RP-LC-ESI-MS allowed



**Fig. 4.** Extracted ion chromatograms for individual oligomers obtained by HILIC-ESI-MS analysis of a cocoa extract. Base peak ions were extracted for each DP. (†) Indicates multiply charged species  $[M-2H]^{2-}$ . (‡) Indicates oligomers containing one A-type linkage (for further discussion, refer to text).



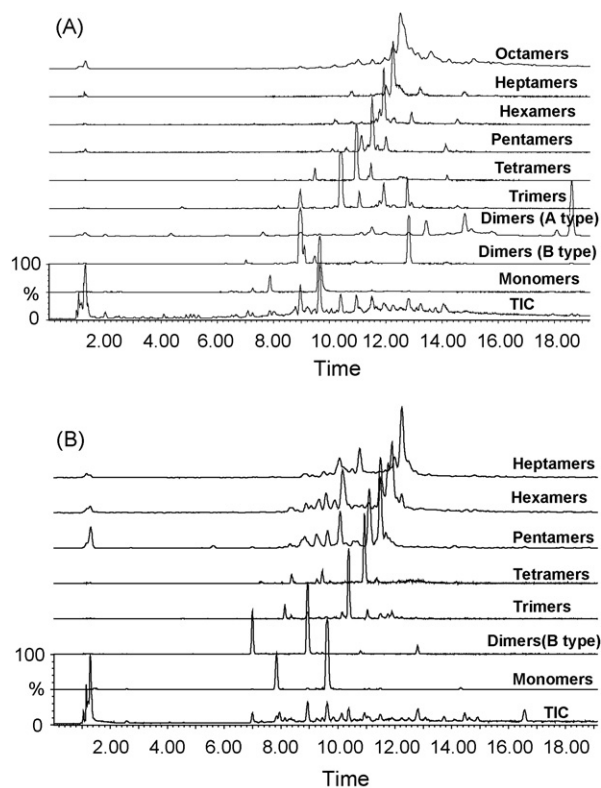
**Fig. 5.** UV chromatogram recorded at 280 nm (A), and fluorescence chromatogram (B) obtained for the optimised high efficiency RP-LC analysis of cocoa procyanidins. Labels indicated the DP of procyanidin isomers as identified by RP-LC-ESI-MS. Columns:  $2 \times 50$  mm Zorbax SB-C18 ( $1.8 \mu\text{m}$   $d_p$ ) columns coupled in series; flow rate: 0.8 mL/min; injection volume:  $3 \mu\text{L}$ ; temperature:  $50^\circ\text{C}$ .

identification of isomeric monomers to octamers as highlighted in Fig. 5. As anticipated, the elution order in RP-LC is not related to the molecular mass [3,13].

Catechin and epicatechin were identified based on mass spectral data and by comparison of retention times with authentic standards. Tentative identification of additional oligomers is based on mass spectral data and correlation of retention times with fluorescence data, due to lack of suitable standards. For the same reason, exact structure elucidation for individual isomers could not be established.

Cocoa and apple procyanidin oligomers were found to consist exclusively of catechin and/or epicatechin monomeric units. Three dimeric-, four trimeric- and tetrameric-, five pentameric-, four hexameric-, and one each heptameric- and octameric procyanidin isomers were tentatively identified in cocoa. In contrast, five dimeric-, seven trimeric-, six tetrameric-, pentameric- and hexameric- as well as one heptameric procyanidin isomer(s) were tentatively identified in the apple extract. Although higher order oligomeric isomers could not be detected by fluorescence, oligomers up to dodecamers were observed in MS traces for both cocoa and apple samples. Extracted ion chromatograms highlighting the most important procyanidin oligomers present in cocoa and apple extracts are shown in Fig. 6.

Both A- and B-type procyanidins oligomers were detected in cocoa extracts: four B-type and four A-type dimeric isomers were



**Fig. 6.** Total ion- and extracted ion chromatograms for the procyanidin oligomers detected by RP-LC-ESI-MS analyses of cocoa (A) and apple (B) extracts. Peak labels indicate the degree of polymerisation of individual isomers and correspond to Figs. 8 and 10. Injection volumes: 4 and  $6 \mu\text{L}$  for cocoa and apple samples, respectively. Other conditions as specified in Fig. 5.

observed, the latter present at much lower levels. In general, A-type oligomers are characterised by higher retention under RP-LC conditions, as expected. Similar to HILIC-ESI-MS, RP-LC-MS data also indicated the presence of procyanidin oligomers up to hexamers containing 1 A-type linkage, although most of these compounds were present at trace levels and not observed in fluorescence chromatograms. As previously observed by Shoji et al. [8], only singly linked (B-type) oligomers were found to be present in the apple extract.

No oligomeric phenolics composed exclusively of prodelfinidin or mixed procyanidin/prodelfinidin monomeric units were detected in cocoa and apple. Neither were propelargonidin monomeric units and oligomers or copolymers made up of (epi)afzelechin units detected.

However, UV data recorder at 320 and 370 nm indicated presence of a number of additional non-procyanidin phenolic compounds present in cocoa, and especially apple extracts. By careful correlation of RP-ESI-MS data with the appropriate UV chromatograms and relevant literature reports [11,44,45], some compounds previously reported in these samples were tentatively identified (Table 2). These include cinnamic acid derivatives, flavonol-glycosides and dihydrochalcones. These compounds all displayed low retention in HILIC mode (results not shown), and were generally well-retained under RP-LC conditions. A typical UV chromatogram recorded at 320 nm and extracted ion chromatograms for the compounds listed in Table 2 are presented in Fig. 7.

Some of these compounds have not previously been reported in these samples, but were tentatively identified as isomers of previously reported compounds, based on similar fragmentation patterns and absorbance spectra. Isomers have been assigned based

**Table 2**  
Summary of additional phenolic compounds tentatively identified in cocoa and apple extracts by RP-LC-ESI-MS.

RP-LC retention time	[M-H] <sup>-</sup>	Compound	Sample
7.13	337.2	3- <i>p</i> -Coumaroylquinic acid	Apple
7.95	353.2	5-Caffeoylquinic acid	Apple [44]
9.33	337.2	5- <i>p</i> -Coumaroylquinic acid	Apple
9.46	353.2	4-Caffeoylquinic acid	Apple
9.85	337.2	4- <i>p</i> -Coumaroylquinic acid	Apple [44]
11.07	358.2	Clovamide	Cocoa [11]
12.49	609.4	Quercetin-3-O-rhamnoglucoside	Apple [44]
12.82	463.2	Quercetin-3-O-galactoside	Apple [44,45], cocoa [11]
13.07	463.2	Quercetin-3-O-glucoside	Apple [44,45], cocoa [11]
13.73	433.2	Unknown quercetin conjugate	Apple
14.08	433.2	Quercetin-3-O-arabinoside	Apple, cocoa
14.46	433.2	Quercetin-3-O-xyloside	Apple
14.62	567.4	Phloretin-2'-O-xyloglucoside	Apple [45]
14.72	433.2	Unknown quercetin conjugate	Apple [45]
14.88	326.2	Dideoxyclovamide	Cocoa [11]
14.91	447.2	Quercetin-3-O-rhamnoside	Apple [44,45]
16.57	435.3	phloretin-2'-O-glucoside (Phloridzin)	Apple [44,45]
19.78	301.1	Quercetin	Apple [44], cocoa [11]

on their relative retention times in accordance with literature reports [45–47]. This specifically applies to ions with  $m/z$  337.2, 353.2, 433.2 and 463.2. For example, all compounds with  $m/z$  433.2 and 463.2 gave a fragment ion at  $m/z$  301.1, which corresponds to a quercetin aglycone. Their relative retention was used in peak assignment: for example, galactoside elutes before glucoside, and arabinoside before xyloside. For *p*-coumaroylquinic and caffeoylquinic acids, the isomers elute in the order of 3', 5' and 4' substitution [46,47]. Clovamide and dideoxyclovamide were assigned according to the elution order described by Sanbongi et al. [48].

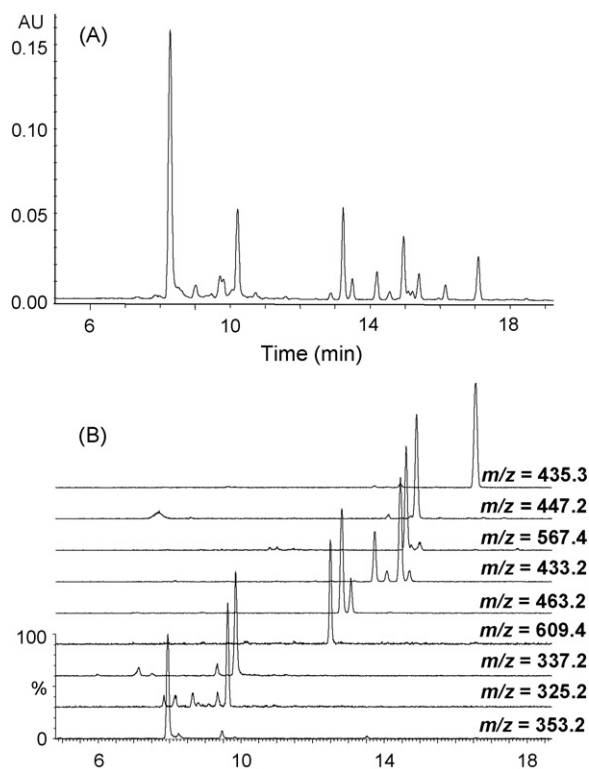
### 3.5. Off-line comprehensive HILIC × RP-LC separation of procyanidins

When designing a comprehensive liquid chromatographic system, orthogonality and solvent compatibility, amongst others, are the main aspects that require careful consideration [49]. This is simply because the first dimension eluent is the second dimension injection solvent [49]. Because HILIC provides different selectivity to RP-LC, the combination of the two modes of separation potentially offers a high degree of orthogonality, as has been demonstrated in several studies [50–52]. Despite the fact that the mobile phases used in HILIC are miscible with RP solvents (in contrast to NP- and RP-LC), interfacing the two separations remains challenging due to the fact that the primary eluent in one mode is a strong solvent in the other. This plays a significant role in on-column focusing of analytes and as a result limits the injection volume if band broadening due to injection is to be avoided in the second dimension.

Both HILIC and RP-LC analyses were optimised independently to maximise resolution in each dimension before their off-line coupling. A 1 mm i.d. column was selected in the first dimension to minimise on-column dilution and avoid the need to evaporate the fractions before analysing them in the second dimension. This could potentially lead to degradation of thermally labile anti-oxidant compounds.

Sample extracts were freeze dried and redissolved in 70/30 (v/v) acetonitrile/methanol to allow higher loadability on the HILIC column and ultimately better sensitivity of the 2-D separation. For the freeze dried apple extracts, 1% water was added to avoid precipitation of the higher molecular weight procyanidins. Maximally 10  $\mu$ L of these samples could be injected on the HILIC column without noticeable peak distortion.

For RP-LC analyses, various injection volumes utilising HILIC mobile phases as sample solvent were evaluated to maximise loadability, while keeping band broadening to an acceptable level. 3 and 2  $\mu$ L were found to be the maximum allowable injection volumes of these samples for the short and long RP-LC gradients, respectively. For the off-line coupling of HILIC and RP-LC methods, 1 min HILIC fractions, corresponding to 50  $\mu$ L, were collected. This is sufficient to provide a minimum of 2 fractions per first dimension peak as required to avoid artificial band broadening due to a too low sampling rate of 1st dimension peaks. This is also made easier by the relatively broad peaks eluting from the HILIC column. Taking into consideration dilution occurring on the first dimension column, this sampling rate effectively implies that 0.4–0.6  $\mu$ L of the extracts were injected onto the second dimension column. The effi-



**Fig. 7.** (A) UV chromatogram recorded at 320 nm for the RP-LC analysis of an apple extract, and (B) Extracted ion chromatograms obtained for the RP-LC-ESI-MS analysis of an apple extract, illustrating the detection of compounds listed in Table 2. Experimental conditions as specified in Fig. 5.

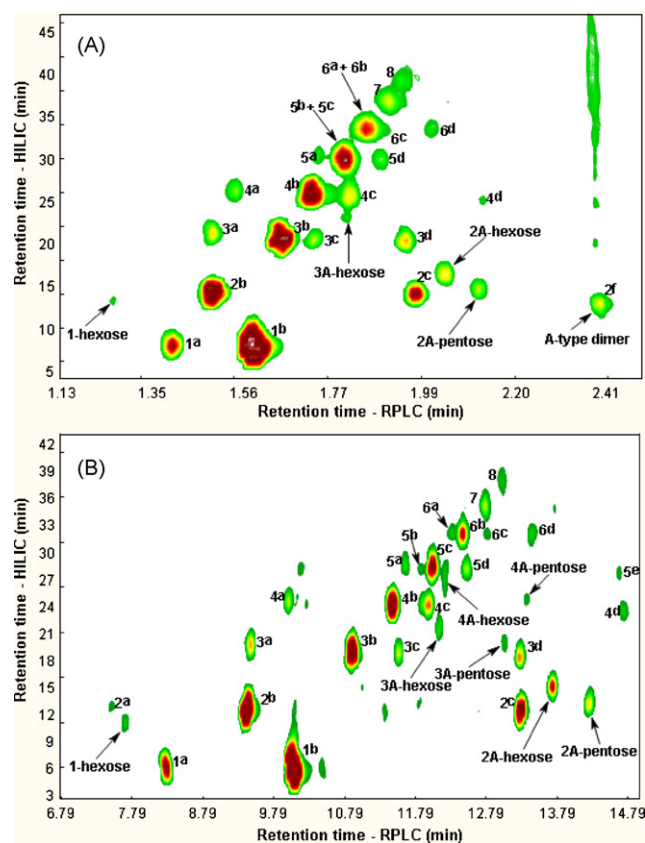
ciency of the 1.8  $\mu\text{m}$  phase combined with fluorescence detection provides sufficient sensitivity under these conditions. The collected HILIC fractions were immediately transferred to low volume vials (200  $\mu\text{L}$ ) and kept under  $\text{N}_2$  to minimise the risk of artefact formation.

2-dimensional contour plots for the HILIC  $\times$  RP-LC analyses of cocoa procyanidins using the short and long RP-LC gradients with fluorescence detection are shown in Fig. 8. Note that throughout the paper contour plots are represented with the second dimensional separation on the  $x$ -axis, contrary to common practice. This is done to facilitate visual interpretation of contour plots with references to RP-LC-MS analysis (see Fig. 9).

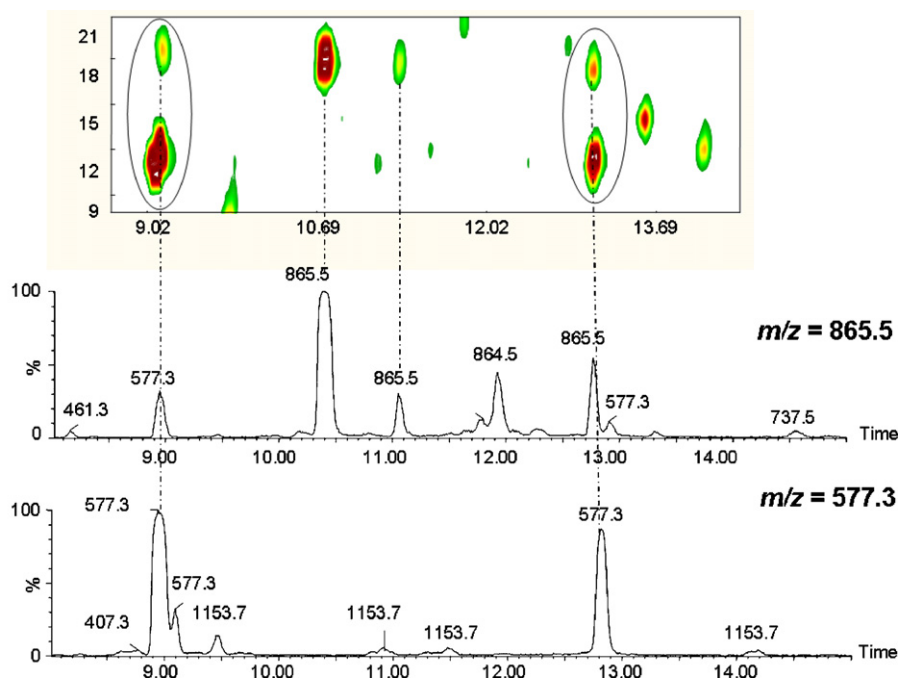
The resolving power offered by the comprehensive 2-D-LC system is clearly evident in Fig. 8: a combination of molecular weight information ( $y$ -axis) as well as the isomeric distribution ( $x$ -axis) of procyanidins is obtained in a single contour plot. The 2-D separation therefore provides additional information which could not be obtained from any single 1-dimensional separations. In fact, these contour plots display structured patterns reminiscent of comprehensive gas chromatography ( $\text{GC} \times \text{GC}$ ), where DP for diverse structures is easily deduced by the  $y$ -intercept of a particular peak. Especially higher MW isomers are much better resolved in the 2-D space than is possible using even a highly efficient 1-D RP-LC separation. Due to extensive overlap of these isomers, (see for example dimers and trimers in Figs. 6 and 9), RP-LC-MS alone could not be used to unambiguously identify higher MW procyanidins.

Comparing Fig. 8A and B it is clear that for procyanidins of  $\text{DP} \geq 4$ , extensive overlap of isomers is evident for the shorter RP-LC gradient, highlighting the significant loss of resolution associated with a reduction in the second dimension analysis time. This has obvious implications for the on-line comprehensive 2-D LC analysis of these samples.

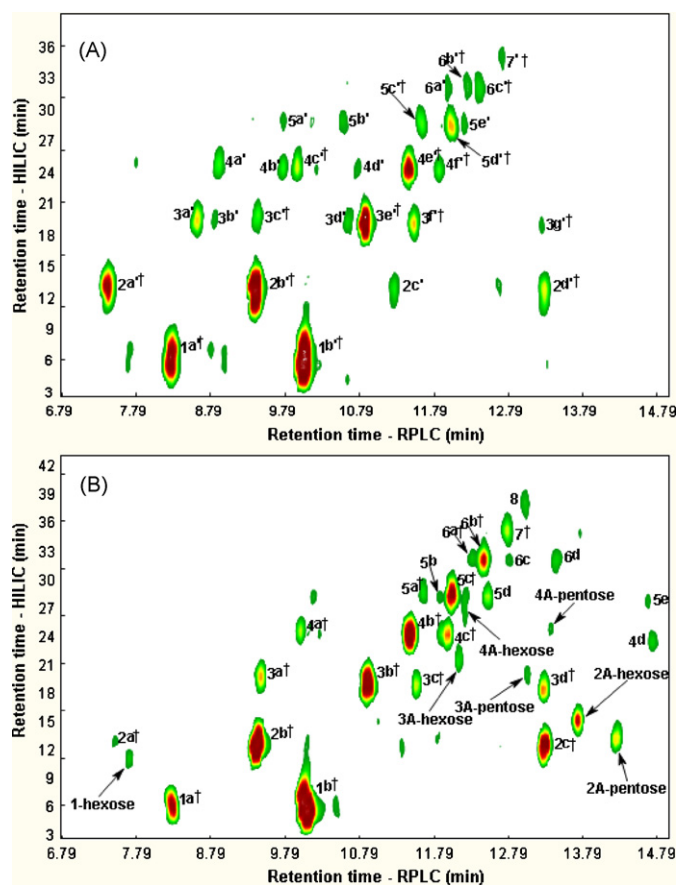
Peaks in the 2-D contour plots were identified by comparison of retention times in both dimensions with 1-dimensional fluorescence and LC-ESI-MS data obtained for both HILIC and RP separations. This is illustrated in Fig. 9, where extracted ion chromatograms for the RP-LC-MS analysis of B-type dimeric and trimeric procyanidins are aligned with the relevant part of the



**Fig. 8.** Fluorescence contour plots obtained for the HILIC  $\times$  RP-LC analysis of cocoa procyanidins with the short (A) and long (B) second dimension RP-LC gradients. Numbers correspond to procyanidin oligomers detected for each degree of polymerisation.



**Fig. 9.** Illustration of the identification of dimeric and trimeric procyanidins by alignment of off-line RP-LC-MS extracted ion chromatograms with the relevant section of the fluorescence contour plot.



**Fig. 10.** Comparison of fluorescence contour plots obtained for the HILIC  $\times$  RP-LC analysis of apple (A) and cocoa (B) procyanidins. Numbers correspond to the degree of polymerisation of procyanidin isomers as identified by ESI-MS. Compounds common to both samples are marked with †.

contour plot. Further confirmation is then achieved by alignment of extracted ion chromatograms obtained by HILIC-MS with the y-axis. Such alignment of MS and fluorescence data with two different separation methods significantly decreases the chances of false identification.

An additional benefit of the comprehensive HILIC  $\times$  RP-LC analysis is that the obtained contour plots greatly facilitate the assessment of procyanidin content of different samples by means of visual comparison. This is illustrated in Fig. 10, where contour plots for cocoa and apple extracts are compared. Most strikingly, a larger number of procyanidin isomers of DP 2–5 are detected in the apple sample. Compounds common to both samples are readily identified by their presence in the same spot in both plots. It is also clear that a number of oligomeric isomers unique to apple are observed in the left of the contour plots, corresponding to lower retention times in RP-LC. This can be attributed to the higher levels of catechin in apple compared to cocoa (peaks labelled 1<sup>a</sup>/1<sup>a'</sup> and 1<sup>b</sup>/1<sup>b'</sup> in Fig. 10). This suggests the presence of more isomers of higher DP containing catechin as constituent in the apple sample, and explains their presence at lower RP retention times (catechin is less retained under RP conditions than epicatechin). This illustrates that, even though the exact chemical structure of oligomeric procyanidins cannot be determined by means of mass spectrometry alone, the combination of MS data with chromatographic retention data in two dimensions provides additional information useful for identification purposes. Also evident from Fig. 10 is the absence of procyanidin-glycosides in the apple sample, which is related to the lack of A-type procyanidins in this sample.

**Table 3**

Summary of the parameters used to calculate the practical peak capacity of the off-line HILIC  $\times$  RP-LC methods.

Parameter	Value Cocoa (4 min)	Cocoa (30 min)	Apple (30 min)
<sup>a</sup> HILIC peak capacity, $n_1$	32	32	27
<sup>a</sup> RP peak capacity, $n_2$	54	137	112
<sup>b</sup> Correlation coefficient, $r^2$	0.0876	0.2092	0.1651
<sup>c</sup> Theoretical peak capacity, $n_T$	1728	4384	3024
<sup>b</sup> Practical peak capacity, $n_p$	1475	3512	2493
<sup>d</sup> Practical peak capacity, $n_p$	1237	3137	2334

<sup>a</sup> Calculated according to Neue [54].

<sup>b</sup> Calculated according to Liu et al. [53].

<sup>c</sup>  $n_T = n_1 \times n_2$ .

<sup>d</sup> Calculated according to Li et al. [58].

In order to quantitatively evaluate the performance of the 2-D HILIC  $\times$  RP-LC method, it is informative to determine the practical peak capacity of the 2-D separation in comparison to either 1-D separation. It is known that the maximum theoretical peak capacity of a 2-dimensional separation is equal to the product of the peak capacities of each of the two dimensions [27,32,53]. However, to benefit from this enhanced resolving power, the separations used in each of the dimensions should be totally uncorrelated (i.e., based on different separation mechanisms) [27,32,53].

Orthogonality may be estimated using the correlation coefficients ( $r^2$ ) obtained from the range-scaled retention times in each dimension [53]. A summary of the relevant data for procyanidins identified in cocoa and apple samples is presented in Table 3. The low  $r^2$  values of 0.2092 and 0.1651 signify an exceptionally small degree of correlation between HILIC and RP dimensions, as is indeed evident from Fig. 8. Differences in  $r^2$  values between cocoa and apple may be ascribed to the presence of a higher percentage of high MW procyanidin isomers in the apple extract displaying low RP-LC retention, as discussed above. This leads to a more even distribution of peaks across the separation space.

Correlation coefficients may be used to calculate the practical peak capacity of the 2-D separation according to the method of Liu et al. [53]. This method essentially uses the correlation coefficient to estimate the effective area of the total 2-dimensional space (i.e.,  $n_1 \times n_2$ ) being utilised. High orthogonality implies effective usage of this space, and should therefore provide a practical peak capacity value close to the theoretical value obtained by the multiplication of peak capacities in each dimension.

Practical peak capacities of 3512 and 2493 (calculated according to [53]) obtained for HILIC  $\times$  RP-LC separations compare relatively well with the theoretical maximum values of 4384 and 3024 for cocoa and apple, respectively. A summary of the experimental values used to calculate peak capacities is presented in Table 3.

One shortcoming of precedent method of calculating effective peak capacity is the failure to take into account the effect of under-sampling of 1st dimension peaks. Especially in on-line comprehensive 2-D LC separations, excessive modulation times are often used due to practical constraints. Long modulation times, relative to the peak widths in the first dimension, lead to a significant decrease in the apparent 1st dimension peak capacity, and therefore of the 2-D system [54]. This aspect may quantitatively be evaluated using the peak broadening factor,  $\beta$ , according to [55]:

$$\beta = \sqrt{1 + 0.214 \left( \frac{t_s}{\sigma_1} \right)^2} \quad (1)$$

where  $t_s$  is the sampling time and  $\sigma_1$  the standard deviation of peaks eluting from the first dimension column. Peak widths in the HILIC separation varied between 1.1–3.9 min and 1.5–5.5 min for the cocoa and apple samples, respectively (average peak widths 2.3 and

3.0 min, respectively). For a sampling time of 1 min as used on the off-line HILIC  $\times$  RP-LC analyses,  $\beta$ -values varying between 1.1–1.9 and 1.1–1.6 are calculated according to Eq. (1) (average  $\beta$ s are 1.4 and 1.3).

According to Davis et al. [55], and in accordance with the work of other authors [56,57], the sampling rate used in comprehensive 2-D separations should obey the inequality  $t_s/\sigma \leq 2$ . This requisite translates into a  $\beta$ -value of 1.4. From this it may be concluded that the effect of under-sampling only plays a minor role by contributing to the effective band broadening of the later-eluting procyanidins: only for  $DP \geq 7$  does  $\beta$  increase above 1.4.

Recently, Li et al. [58] further developed this approach to estimate the practical 2-D peak capacity according to:

$$n'_{2-D} = \frac{n_1 n_2}{\sqrt{1 + 3.35(2t_c n_1 / t_g)^2}} \quad (2)$$

where  $n'_{2-D}$  is the effective 2-D peak capacity,  $n_1$  and  $n_2$  the peak capacities in first and second dimensions, respectively,  $2t_c$  the second dimension cycle time and  $t_g$  the first dimension gradient time. The fraction collection time, 1 min, is substituted for the second dimension cycle time ( $2t_c$ ) since in our case off-line 2-D analyses were performed. Using 60 min for  $t_g$  and 137 and 112 for second dimension peak capacities for cocoa and apple samples, respectively, this provides effective peak capacities of 3137 and 2334 for the off-line HILIC  $\times$  RP-LC analyses of cocoa and apple samples (Table 3). These values are indeed commensurate with those obtained using the method of Liu et al. for the same analyses.

Considering that two different models, one focussing on orthogonality and second on under-sampling, provide similar results, we suggest that the practical peak capacities shown in Table 3 represent an accurate reflection of the resolving power of the off-line HILIC  $\times$  RP-LC method. This is indeed corroborated by the contour plots in Figs. 8 and 10.

Clearly, the use of an off-line 2-D-LC approach places fewer restrictions on the 1st dimension sampling time: in an on-line system, the sampling rate is equal to the 2nd dimension analysis time, or at the best, half the second dimension analysis time [59]. The practical limitations encountered in achieving fast second dimension analyses therefore often result in under-sampling of first dimension peaks. In contrast, for off-line comprehensive analyses, very high sampling rates are in theory possible, the limiting factor in fact being the maximum total analysis time considered feasible. Moreover, since the off-line approach places no restrictions on analysis times, and especially flow rates, in each dimension, the resolving power of each of the two dimensions is maximally utilised. The use of relatively long gradients and optimal flow rates are therefore responsible for the exceptionally high practical peak capacities achieved here.

The effective peak capacity of the 2-dimensional separation also clearly illustrates the considerable benefit of off-line comprehensive 2-D LC analysis, when compared to the peak capacities measured for each 1-dimensional separation. A comparison of comprehensive analyses using short and long RP-LC gradients in the second dimension (Table 3), shows that the practical peak capacity measured using the short gradient is less than half that achieved for the long gradient. The total time required for each of these analyses is  $\sim 4.5$  and 26.5 h, respectively. Despite these long analysis times, the gain in separation power provided by the off-line comprehensive LC method cannot be matched by any single dimensional LC analysis.

Depending on the complexity of the sample of interest, on-line comprehensive 2-D LC combining similar 1-D separations may present a promising alternative for the fast analysis of procyanidins. It is clear from the precedent discussion that the associated reduction in second dimension analysis times, coupled to the chal-

lenges associated with achieving sufficient sampling rates, would result in much lower resolving power. However, an on-line system would provide the advantages of automation, faster analysis and less risk of analyte loss. Another option is to perform stop-and-go 2-D analysis, where the flow from the first dimension column is stopped while each fraction is analysed in the second dimension [60]. Analysis times for such methods are comparable to off-line comprehensive LC methods, although the approach also offers the advantage of automation and reduced risk of analyte loss. Current research in our laboratory is aimed at investigating these alternative methodologies.

In principle, the same approach should be equally suitable for analysis of non-procyanidin oligomeric phenolics. It should be noted that in the case of procyanidins, identification was simplified by the selectivity of fluorescence detection coupled to off-line MS analysis, whereas compounds lacking fluorescence properties might not be identified with the same ease.

#### 4. Conclusions

An off-line comprehensive 2-dimensional LC method for the analysis of procyanidins has been developed. Oligomeric procyanidins were separated according to molecular weight using HILIC in the first dimension. In the second dimension, procyanidin isomers were separated using reversed phase LC. Contour plots were constructed using selective fluorescence detection.

Mass spectral data indicated that cocoa and apple procyanidins consist exclusively of catechin and epicatechin monomeric units. Oligomeric procyanidins up to dodecamers were detected in both apple and cocoa extracts using HILIC-ESI-MS, while isomers up to octamers were tentatively identified by RP-LC-ESI-MS analysis. In addition, A-type oligomers up to DP 6 and procyanidin-glycosides up to DP 7 were detected in cocoa samples.

The combination of two complementary separation mechanisms proved to be particularly effective due to the significantly improved resolution of oligomeric procyanidin isomers provided by the pre-separation according to degree of polymerisation in the HILIC mode. Visual interpretation of procyanidin content of the analysed samples is facilitated by the structured nature of contour plots, with molecular weight and isomeric information easily ascertained from the 1-D and 2-D axes, respectively. HILIC and RP-LC separations were shown to provide high orthogonality ( $r^2 < 0.2$ ), and the 2-dimensional system is characterised by exceptionally high practical peak capacities. Moreover, for the off-line approach utilised here, it is shown that virtually no additional band broadening results from under-sampling of first dimensional peaks.

The off-line HILIC  $\times$  RP-LC method therefore represents an exceptionally powerful separation strategy for the detailed investigation of complex phenolic fractions. The developed methodology should be equally suitable for the analysis of other complex proanthocyanidin fractions, and could essentially be extended to other phenolic compounds. The on-line coupling of HILIC  $\times$  RP-LC-MS, not attempted in the current study, would further enhance the applicability of the method.

#### Acknowledgements

The National Research Foundation (NRF, South Africa) is acknowledged for financial support. M.A. Stander (Central Analytical Facility, Stellenbosch University) is thanked for assistance with LC-MS analysis.

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