Journal of Chromatography A, 1216 (2009) 4831–4840

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Method performance and multi-laboratory assessment of a normal phase high pressure liquid chromatography–fluorescence detection method for the quantitation of flavanols and procyanidins in cocoa and chocolate containing samples

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ARTICLE INFO

*Article history:*
Received 6 January 2009
Received in revised form 30 March 2009
Accepted 3 April 2009
Available online 9 April 2009

*Keywords:*
Flavanols
Procyanidins
Cocoa
Multi-laboratory validation
Quantitative
HPLC
FLD
NIST
Chocolate
Diol

ABSTRACT

The quantitative parameters and method performance for a normal-phase HPLC separation of flavanols and procyanidins in chocolate and cocoa-containing food products were optimized and assessed. Single laboratory method performance was examined over three months using three separate secondary standards. $RSD_{(r)}$ ranged from 1.9%, 4.5% to 9.0% for cocoa powder, liquor and chocolate samples containing 74.39, 15.47 and 1.87 mg/g flavanols and procyanidins, respectively. Accuracy was determined by comparison to the NIST Standard Reference Material 2384. Inter-lab assessment indicated that variability was quite low for seven different cocoa-containing samples, with a $RSD_{(R)}$ of less than 10% for the range of samples analyzed.



## 1. Introduction

Interest in flavanols and procyanidins, especially those found in cocoa, has increased substantially in the past decade due to the purported health benefits of these naturally occurring compounds [1–6]. Flavanols are a subclass of flavonoids. They possess the general flavonoid, C6–C3–C6 carbon backbone and in particular, the distinguishing functional group consisting of a hydroxyl group at position 3 on the center ring (oxidation of the C-ring) (Fig. 1). The procyanidins – one specific class of proanthocyanidins – are the oligomeric counterparts of the flavanols and are comprised exclusively of the monomers epicatechin and catechin. The procyanidins, most commonly have single carbon–carbon linkages – referred to as B-type procyanidins linkages – between the monomeric subunits. Doubly linked procyanidins exist as well and are referred to as A-types (Fig. 1). The molecular weight or size of a procyanidin oligomer is expressed as their degree of polymerization (DP) and are commonly referred to as dimers, trimers, tetramers, etc. [7].

Often, due to the complexity in their structural diversity, analytical methodology for proanthocyanidins and procyanidins has been chromatographic separation based upon DP as opposed to the individual compounds and specific isomers [8,9]. This approach has permitted the measurement of the large structural diversity of these compounds in a categorical manner (e.g. oligomeric size). Specifically for cocoa and cocoa-containing foods, flavanols and procyanidins have been quantified up to a pre-defined molecular weight cut-off, namely DP 1–10 [10–13].

Developing a robust, reliable and transferable measurement tool for flavanols and procyanidins is necessary for a more homogeneous content assessment of foods, which enables as well as supports necessary research to establish links between these food

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doi:10.1016/j.chroma.2009.04.006

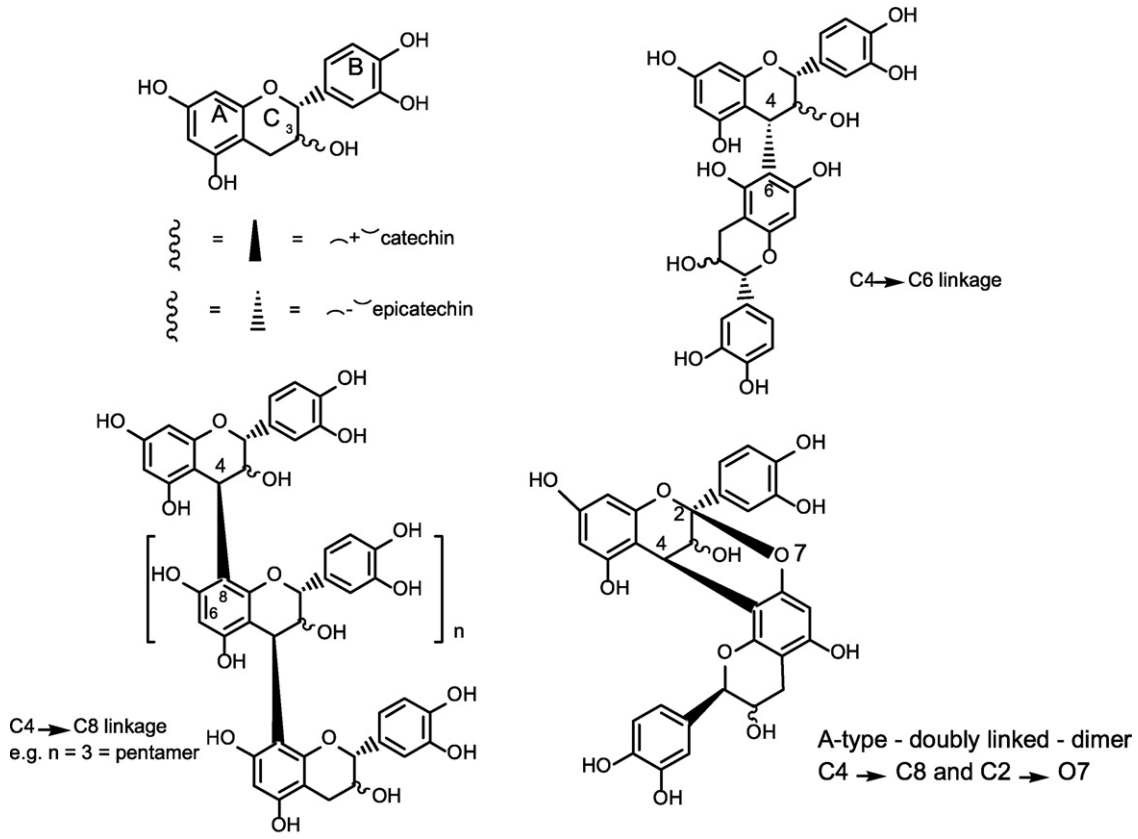


**Fig. 1.** Examples of common structural features of procyanidins: A–C rings on the flavanol monomer pentamer and two dimers are shown. Note linkage differences of B-type (4–6 and 4–8) and A-type dimers.

components and their purported health benefits [3,14,15]. With this in mind, we report the further development – into a quantitative and applicable measurement tool – a previously published qualitative separation [16]. The methodology detailed herein employs a simple binary gradient and a more environmentally friendly chromatographic system than previous methodologies that have analyzed based on DP [10,17].

One common limitation in the analysis of these components has been the low resolution for the higher oligomeric species – making quantification challenging. The chromatographic conditions reported herein were modified from the Kelm et al. [16] publication – enhancing baseline separation between oligomeric fractions. Detection conditions were altered as well to provide greater signal intensity (excitation and emission set to 230 and 321 nm, respectively) [18]. Due to an increased signal intensity (by a factor of 25, in some instances), a gain gradient was also implemented, as part of the detection protocol, in order to have all fractions within the linear range of the detector within one HPLC run. This enhanced signal intensity, and higher resolution also enabled the increase in precision of the content measurement for material containing low levels of cocoa flavanols and procyanidins. Additional methodological parameters examined and optimized in this work include: alternate column manufacturers; recovery experiments using a highly alkalized cocoa powder; determination of the accuracy of measurement by comparison to the reference baking chocolate NIST SRM 2384; determination of the methodological precision through the analysis of content data collected over a 3-month time-period; and finally, an assessment – between three operators using three instruments (in two laboratories) – of the complete measurement system (sample preparation and quantitation) for seven cocoa-containing products.

## 2. Experimental

### 2.1. Reagents and supplies

HPLC-grade hexane, acetone, and glacial acetic acid were obtained from Fisher Scientific (Fairlawn, NJ, USA). HPLC-grade methanol and acetonitrile were purchased from Sigma–Aldrich (Milwaukee, MI, USA). Water was prepared using a Millipore-Milli-Q purification system (Millipore, New Bedford, MA, USA). Extract-Clean SPE-Complete Kit was purchased from Alltech (Deerfield, IL, USA) and solid phase extraction (SPE) cartridge Strata SCX, the precolumn SecurityGuard cartridge and SecurityGuard cartridge are products from Phenomenex (Torrance, CA, USA). The part numbers for the silica and cyano (CN) precolumns are AJ0-4348 and AJ0-4305, respectively. No commercially available standards are available for the procyanidins with DP greater than 2. Therefore a re-constructed standard, prepared in house, was employed for calibration of analytical instrumentation and quantitative method development work. Using preparative methodology as described in previously published work, individual oligomeric fractions were isolated and purified (DP 1–10) [16]. Analyte identity and purity was confirmed through previously employed and accepted methodologies namely LC–MS masses [12,17], UV–Vis, LC/FLD [16] and thiolysis work [19].

### 2.2. Sources of food samples

NIST standard reference material SRM 2384 was purchased from NIST, Gaithersburg, MD, USA. Jet Black cocoa powder (a highly alkalized cocoa powder) was purchased from Blommer Chocolate Company, East Greenville, PA, USA. The dark chocolate (DC), cocoa-

containing granola bars, cocoa powders and chocolate liquors were obtained from Mars, Inc. (Hackettstown, NJ, USA).

### 2.3. Sample preparation

#### 2.3.1. Removal of lipid fraction from cocoa powder/chocolate samples

Samples containing more than 10% fat (w/w) content were defatted prior to analysis. Removal of the lipid fraction from chocolate, chocolate liquor and cocoa powder samples consisted of weighing out approximately 10–20 g of a sample into a labeled 50 mL disposable centrifuge tube which were filled to the 45 mL mark with hexane and then tightly capped. Sample tubes were then vortexed for at least 1 min to facilitate complete dispersion of the matrix and dissolution of the lipids. Tube(s) were then placed into a sonicator at 50 °C for 5 min and then centrifuged (Sorvall HBB-6, Kendro Products Newtown, CT) for 5 min at 2567 × *g*. The hexane layer was subsequently decanted. This lipid extraction was repeated twice more (for a total of three times). The remaining defatted sample was then allowed to air dry to evaporate any remaining hexane prior to extraction of the flavanols and procyanidins.

#### 2.3.2. Extraction of flavanols and procyanidins for all solid samples

To extract the flavanols and procyanidins, solid samples (defatted chocolates, cocoa liquors and cocoa powders) were weighed in amounts of 0.5 or 1.00 ± 0.02 g and were placed into a 15 mL disposable centrifuge tube. The solid material was covered with an acetone-based extraction solution (acetone:water:acetic acid; 70:29.5:0.5; v/v/v) via a bottle top dispenser. Five milliliters of this acetone solution was used for all samples except for the granola bars, where 10 mL of this solution was used. The cocoa-containing granola bars did not require defatting as they contained less than 10% fat. Instead these samples were covered with liquid nitrogen, ground to a fine powder, and directly covered with the acetone-based extraction solution. All samples were first hand-shaken, then vortexed for 2 min to facilitate dispersion of sample. This is especially important for the granola bars which are made with a binder syrup, making complete dissolution of the sample difficult to achieve without proper sample handling. Sample tubes were then placed into a 50 °C sonicator bath for a maximum of 5 min and vortexed again for 1 min. Tubes were then centrifuged for 5 min at 2567 × *g*, and the supernatant solution (flavanol and procyanidin acetone extract solution) was collected after centrifugation.

In preparation for analysis, the collected supernatent was passed through the SPE cartridge, Strata SCX (55 μm, 70 Å, 500 mg/3 mL. Conditioning of the SPE packing bed was performed with 5 mL of de-ionized water on a vacuum manifold (24-position, Phenomenex, Torrance, CA). The vacuum was set to 0.2 bar, ensuring all excess water was removed from cartridge. The packing bed of the cartridges was not allowed to dry at any time prior to loading the sample. The supernatant solution (∼2 mL) was then loaded onto the SPE cartridge. A matched-sized syringe plunger was utilized to push the liquid through the SPE bed. Approximately half of the sample was discharged (∼1 mL) through the cartridge and then a 0.45 μm PTFE filter placed at the end of the SPE cartridge. The remaining acetone extract solution was passed through both the SPE cartridge and filter, and the filtered sample collected in a HPLC vial for subsequent chromatographic analysis.

#### 2.3.3. Blank matrix and spiked materials

Preparation of a blank matrix material was generated in order to assess recovery of this extraction process. Jet black cocoa powder (100 g) was dispersed in 200 mL water. A pH measurement of this solution yielded a pH 10.45. The pH was assessed using Orion pH meter (Version 2.0, Thermo Electron Company). The solution was acidified, brought to a pH 5.0 using glacial acetic acid (50:50, v/v), and the acidified material was then freeze-dried, yielding a brown cocoa powder, being referred to as acidified jet black cocoa powder (AJB). HPLC analysis of this material (using the sample preparation technique described earlier in this section and the analytical method detailed in the next section) confirmed no detectable cocoa flavanols and procyanidins.

A spiked cocoa powder material (SAJB) was generated by taking the AJB cocoa powder (1.455 g) and adding in a cocoa flavanols and procyanidins reference standard material (450 mg). Solid materials were covered with 5 mL of a 10% ethanol in water solution, and stirred (with a stir bar) to ensure homogeneity of the resulting spiked material. An additional 15 mL of water was added, the mixture stirred for 15 min, and then freeze-dried. The resulting spiked, acidified jet black (SAJB) cocoa powder contained 32.9 mg/g of flavanols and procyanidins, DP 1–10 (on a defatted basis).

The recovery experiments consisted of a comparison of three materials: (1) a dark chocolate control (DC), (2) a dark chocolate with AJB and cocoa butter (DC-AJB), (3) a dark chocolate with SAJB and cocoa butter (DC-SAJB). Samples were generated by taking approximately 8.3 g of DC, 3.0 g of either AJB or SAJB and 0.67 g of cocoa butter. The cocoa powders were mixed with the cocoa butter and dark chocolate using a Rev2 tempering unit (ChocoVision Corporation, Poughkeepsie, NY). To ease the handling of the final, blended materials, the tempering bowl was chilled – solidifying the chocolate materials – prior to transferring to vessels used in the extraction steps thereby minimizing loss resulting from residue remaining in the tempering bowl.

### 2.4. Instrumentation and chromatographic conditions

#### 2.4.1. Chromatographic conditions for normal phase separation

The separation of cocoa flavanols and procyanidins was performed on an Agilent 1100 Series HPLC system equipped with an autosampler, solvent degasser, quaternary pump, column heater, photodiode array and fluorescence detectors. The column used was the Develosil Diol 100 Å (250 × 4.6 mm, 5 μm particle size) purchased from Phenomenex (Torrance, CA, USA). The chromatographic mobile phase was a binary gradient (solvents A and B) and consisted of an acidic acetonitrile ((A), $CH_3CN$:HOAc, 98:2; v/v) and acidic aqueous methanol ((B),$CH_3OH$: $H_2O$:HOAc, 95:3:2; v/v/v). The starting mobile phase condition was 7% B was held, isocratically, for 3 min. Subsequently, solvent B was increased to 37.6% over 57 min and then to 100% B over the next 3 min. The conditions were held at 100% B for 7 min prior to returning to 7% B (starting condition) over 6 min. Post run time was 10 min.

Resolution ($R_s \geq 1.5$) for flavanols (DP 1) and procyandins (DP 2–10) was achieved within a total run time of 76 min. The UV detection was set to 280 nm. Fluorescence detection was conducted with an excitation wavelength of 230 nm and an emission wavelength of 321 nm. Other FLD conditions included a photomultiplier tube (PMT) gradient. For the samples and conditions described herein, the photomultiplier tube gain was set to 7 from 0 to 8 min, set to 9 from 8.1 to 15.0 and finally set to 10 from 15.1 to 76 min. (Gain gradient should be fine-tuned per column and/or chromatographic system accounting for small systematic differences in retention times and resolution.) The column temperature was held at 35 °C throughout the run. The flow rate was 1 mL/min and the typical injection volume was 5 μL. A cyano (4 × 3.0 mm) SecurityGuard cartridge was obtained from Phenomenex (Torrance, CA) and employed to protect the column. The guard column was installed with a SecurityGuard Guard Cartridge Kit, also obtained from Phenomenex. The autosampler was set to and held at 5 °C.

The HPLC system used in the Arkansas Children's Nutrition Center was an Agilent 1100 HPLC consisting of binary pump, a quaternary pump, a solvent degasser, an autosampler, a thermostat

column compartment, a diode array detector, and a fluorescence detector (Agilent Technologies, Wilmington, DE). Separation was carried out on a different Phenomenex Develosil column (100 Diol-5, 250 × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, CA, USA). Chromatographic conditions and spectrophotometeric detection parameters were the same as those described above.

*2.4.2. Column conditioning*

Prior to employing a column for quantitative work, the column was preconditioned overnight using 15% solvent B at a flow rate of 0.1 mL/min. Subsequently, a series of high and low standard concentrations were run as unknowns until area values obtained were consistent with the expected stock solution concentrations. The typical number of samples run to achieve equilibration ranged from 15 to 20, with the exact number run being column dependent.

*2.4.3. Alternate columns*

Additional diol stationary phase columns from several alternate manufacturing sources were examined, namely: (1) Cosmosil Packed column 5 Diol 120-II from Nacalai Tesque Inc. (250 × 4.6 mm, 5 μm particle size) 120 Å (donated), (2) Lichrospher 100 Diol (250 × 4.6 mm, 5 μm particle size) purchased from Labhut, Inc., UK – Cronus HPLC columns, (3) Lichrosorb Diol (250 × 4.6 mm, 5 μm particle size) purchased from Labhut, Inc. – Cronus HPLC columns, (4) Inertsil WP300 Diol (250 × 4.6 mm, 5 μm particle size) donated by GL Sciences Inc. However, for all quantitative work, examination of method performance and multi-lab assessment were conducted with the 100 Å Develosil Diol column.

*2.4.4. Chromatographic conditions for reversed phase*

Chromatographic analysis for the flavanol monomers, epicatechin and catechin, was performed on Agilent 1100 Series HPLC (Hewlett-Packard) equipped with an UV detector. Reversed-phase separations were performed using a Phenomenex C18 (2) Luna column (4.6 × 250 mm, 5 μm particle size) maintained at 30 °C and using a 5 μL injection volume. The mobile phases consisted of (A) acetonitrile/ethyl acetate (7:1, v/v, respectively) and (B) 0.05% acetic acid in water. The starting mobile phase condition was set at 5% A, which was held isocratically for 5 min. Subsequently, solvent A was increased to 18% over 40 min. At 46 min, solvent A was reduced to 0%, and held in this state for an additional 4 min. At 51 min solvent A was returned to the starting condition of 5% A and held there for an additional 4 min (total run time 55 min). Between runs there was a 5-min post-run (maintaining the use of 5% A) to re-equilibrate the column before the next sample injections.

*2.5. Quantitation*

*2.5.1. Preparation of standard solutions*

A parent stock solution was made up employing 300.49 mg of reconstructed standard [16] which was dissolved in a 25 mL volumetric flask with acetone:water:acetic acid (70:29.5:0.5; v/v/v) creating a stock solution of concentration 12 mg/mL of total flavanols and procyanidins. Further stock solutions were made through serial dilutions of the initial stock solution ranging from 0.5 to 6.0 mg/mL. The stock solutions were stored at −80 °C. Stock solutions placed in HPLC vials were prepared as needed which were stored in freezer at −20 °C for easy access prior to use. The percentages of the individual oligomeric fractions in the reconstructed standard were as follows: 21.7% monomers, 13.0% dimers, 11.5% trimers, 11.2% tetramers, 10.2% pentamers, 10.0% hexamers, 5.8% heptamers, 6.0% octamers, 7.0% nonamers and 3.6% decamers.

*2.5.2. Calibration curves, LOD and LOQ*

An external standard method was used for quantitation. For each analyte, peak areas from HPLC chromatograms were plotted against the concentrations of stock solutions of known concentrations. Calibration curves for each analyte were generated using quantitation functions from HP Chem station software. Equations generated via linear regression were used to establish concentrations. Limits of detection (LOD) were calculated by obtaining the average height (signal to noise) at the appropriate retention time for each DP fraction on blank runs ($n = 20$). This average height was converted to an area (employing calibration curves), and then a concentration calculated (using the established linear response curve). The LOD was determined by multiplying the concentration by 3, the limit of quantitation (LOQ) was determined by multiplying the LOD concentration obtained by 10 (Table 1).

## 3. Results and discussion

*3.1. Method development and established conditions*

A measurement system – from sample preparation to detection – for determining the flavanol and procyanidin content of cocoa and chocolate products has been optimized and assessed. The methodology consists of a separation based on degree of polymerization (e.g. molecular weight) employing a diol column for the stationary phase. Although there exists polymeric procyanidins (with DP >10), they were not included in the quantification aspect of this study. The mobile phase consists of a binary gradient of aqueous acidic methanol into acidic acetonitrile. The selection of running a gradient of methanol into acetonitrile was based on matching physical properties of solvents used in normal-phase, silica-based column methods [20–22].

Chromatographic conditions were modified from previous qualitative work to achieve the desired separation and resolution of the complex mixture of oligomeric fractions DP 1–10 (e.g. Fig. 2A). Specifically, two noteworthy modifications were made to the previously developed run conditions, including the introduction of an isocratic period in the early part of the run (held at 7% B for 3 min) followed by a slowed increase in the addition of the methanolic mobile phase (B) into the acetonitrile phase (A) (i.e. an increase of 30.6% B over 54 min versus an increase to 40% B over 35 min) [16]. These modifications were made to ensure adequate separation between all oligomeric fractions. A compromise between the length of the chromatographic run and resolution ($R_S$) was chosen. The resolution that is typically sought for quantitative measurements for complex matrices such as foods and botanical samples is 1.5, indicating baseline and usable separation of compounds [23]. The calculated resolutions for the oligomeric fractions ranges from 1.5 to 4.4 and are listed in Table 1. The $R_S$ values for all oligomers are equal to or greater than this 1.5 threshold value. Maintaining a column temperature of 35 °C throughout analysis was found to assist with obtaining good resolution and deviations of ±5 °C did not significantly impact separations.

Fluorescence was the main detection system used for quantitation since it is known to be more selective and provides a stronger signal than UV absorption detection for procyanidins [11,17,24]. Both changing the solvent system, as well as the detection conditions increased the intensity of the signal observed for the flavanols and procyanidins in the cocoa-containing samples [16,25,26]. In Fig. 3, the difference in response factors (area/concentration) for the oligomeric fractions (DP 1–10) on the diol column using two different solvent systems is shown. The solvent systems compared were the binary gradient described herein and the ternary gradient (including methylene chloride) employed in previous methodologies. [10,12]. The excitation and emission wavelengths – in this experiment – were set to 276 nm and 317 nm, respectively [18]. A notable outcome of this comparison was that the elimination of the methylene chloride resulted in a significant increase in signal intensity of ∼3–10-fold depending upon the DP.

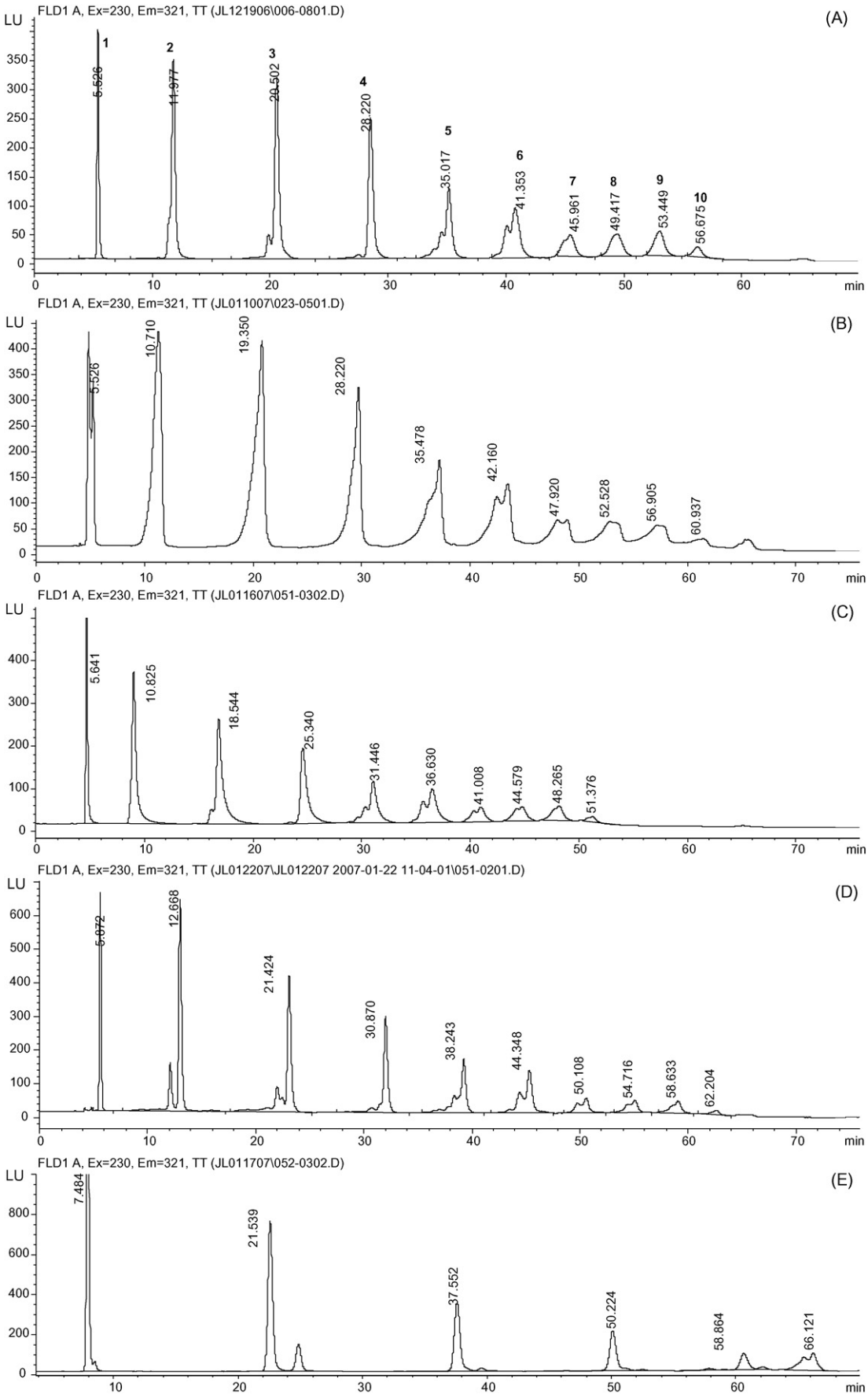


**Fig. 2.** Comparison of diol columns from different manufacturers: (A) Develosil Diol, (B) Lichrosorb, (C) Inertsil, (D) Lichrospher, (E) Cosmosil. All traces were collected using FLD at 230/321 nm using a standard solution at 2.0 mg/mL of cocoa flavanols and procyanidins.

**Table 1**
Performance characteristics for chromatographic conditions described in the text: including calibration range, retention time (RT), variation, resolution, detection mass to charge ratio, for DP 1–10 under chromatographic conditions discussed in the manuscript.

| Oligomeric fraction (DP) | Resolution of peaks ($R$)[a] | Gain (PMT setting) | Retention time (min ± SD)[b] | $RSD_{(r)}$ (RT)[c] | $m/z$ | Calibration range (mg/mL) | LOD/LOQ[d] |
|---|---|---|---|---|---|---|---|
| Monomers (1) | – | 7 | 5.41 ± 0.05 | 0 | 288.9 | 0.005–0.87 | 0.06/0.19 |
| Dimers (2) | 4.0 | 9 | 11.77 ± 0.03 | 0.5 | 577.0 | 0.003–0.52 | 0.07/0.24 |
| Trimers (3) | 4.4 | 10 | 20.56 ± 0.15 | 0.3 | 865.2 | 0.003–0.46 | 0.07/0.23 |
| Tetramers (4) | 3.9 | 10 | 28.53 ± 0.08 | 1.5 | 1153.3 | 0.006–0.45 | 0.08/0.25 |
| Pentamers (5) | 2.3 | 10 | 35.16 ± 0.10 | 0.8 | 1441.3 | 0.003–0.41 | 0.08/0.25 |
| Hexamers (6) | 1.7 | 10 | 40.82 ± 0.40 | 0.7 | 1729.3 | 0.003–0.40 | 0.08/0.27 |
| Heptamers (7) | 1.6 | 10 | 45.46 ± 0.07 | 0.7 | 2018.5 | 0.001–0.23 | 0.09/0.31 |
| Octamers (8) | 1.5 | 10 | 49.46 ± 0.50 | 0.6 | 1152.4 | 0.002–0.24 | 0.08/0.31 |
| Nonamers (9) | 1.5 | 10 | 53.02 ± 0.08 | 0.5 | 1297.4 | 0.002–0.28 | 0.08/0.28 |
| Decamers (10) | 1.6 | 10 | 56.26 ± 0.03 | 0.4 | 1441.2 | 0.001–0.11 | 0.10/0.33 |

[a] $R = \frac{(P_2 - P_1)}{(1/2)(W_1 + W_2)}$ where $P_n$ = retention time and $W_n$ = peak width.
[b] Data collected with the one column $n = 7$ (S/N 0312427).
[c] Data collected with one column over a month time frame ($n = 4$).
[d] Five microliters injection volume.

When the excitation and emission wavelengths were changed to 230 and 321 nm, respectively, a 5-fold increase was observed [12,13,16,17,27]. To remain within the detection limits of the detector and to be able to calibrate all oligomeric fractions (DP 1–10) in one chromatographic run, a photomultiplier tube gain gradient was implemented; the details are listed in Table 1. The PMT gain was reduced in the region for the monomeric and dimeric species, with the gain set to 7 and 9, respectively, and then returned to the default value 10 for DP 3–10.

This enhanced signal and resolution enabled increasing the precision for samples with low concentrations of flavanols and procyanidins. Measuring the same dark chocolate (secondary standard) with previous methodology [17] over a period of 3 months gave an average value cocoa flavanol and procyanidin content of 2.35 ± 0.42 mg/g whereas the same sample measured with the methodology described herein gave 1.81 ± 0.08 mg/g, giving a reduction of over 70% in the relative standard deviation ($RSD_{(r)}$).

### 3.1.1. Alternate columns

To ensure that this separation behavior was not unique to one column manufacturer, several columns with the diol bonded phase from alternate manufacturing sources were examined under the same mobile phase conditions described above. The columns, specifically, Develosil Diol, Lichrosorb Diol, Inertsil WP300 Diol, Lichrospher, and Cosmosil, were chosen based on availability, similarity in column length, inner dimension, as well as particle size. Fig. 2A–E provides a comparative visual, indicating that several alternative diol column sources were possible for this separation work. There existed minor differences between these columns, including differences in peak shape and retention times, as well as speciation within the peak representing the DP; however, separation according to DP was achieved on four out of the five columns examined. Under the described mobile phase conditions, Cosmosil Diol gave a larger resolution for DP 1–6; however, peaks associated with DP 7–10 were not observed, even when the run time was extended. Attempts to wash off the remaining oligomers, with various solvents, proved unsuccessful. This enhanced resolution might prove useful for foods containing extremely complex mixtures of proanthocyanidins (e.g. grape seed extract) [9]. Since the Develosil Diol column gave approximate symmetrical peak shape and consistent resolution according to DP with minor inter-fraction speciation, it was used for further examination of quantitative experiments and method performance assessments. Additionally, the column-to-column variation for the Develosil Diol from the same source was monitored and was within acceptable limits.

In this methodology, the peak representing DP does not consist of only one molecular species, but of molecules with the same

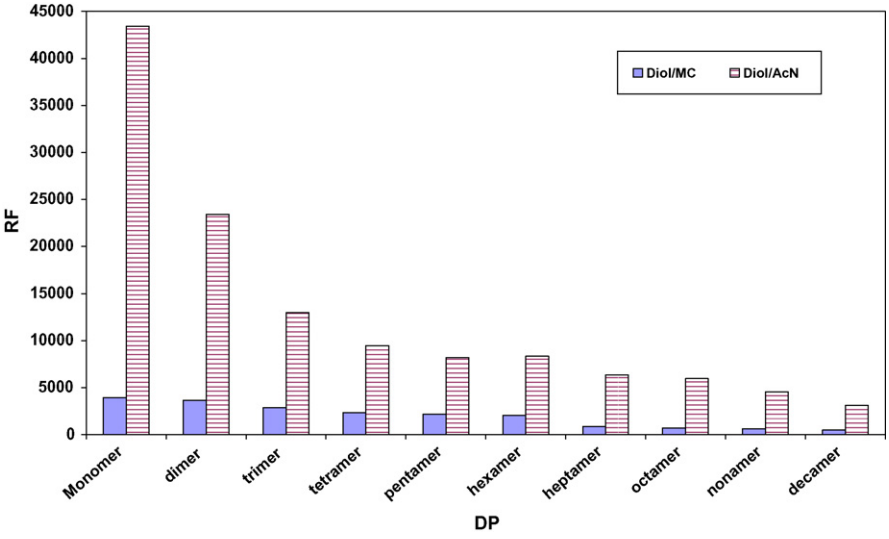


**Fig. 3.** Comparison of the response factors (area/concentration) for oligomeric fraction DP 1–10 on the diol column using the ternary solvent system using methylene chloride and with the binary solvent system using acetonitrile. The excitation and emission wavelengths – in this experiment – were set to 276 and 317 nm, respectively.

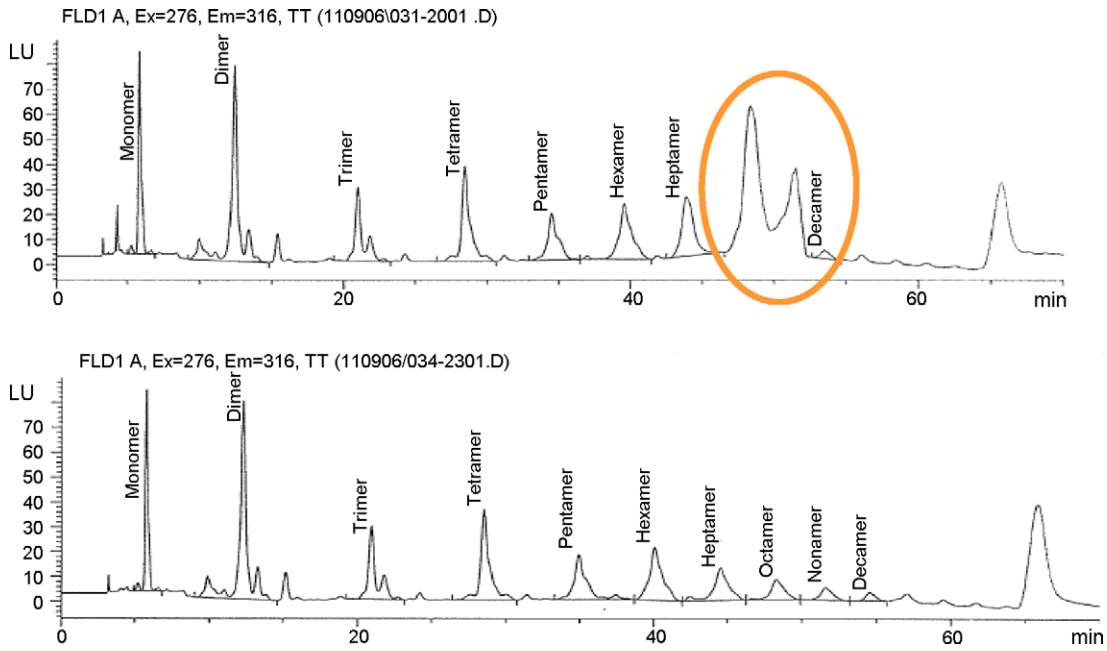


**Fig. 4.** HPLC traces demonstrating the before (top) and after (bottom) treatment with the cation exchange SPE cartridge on the sample liquor sample. Unknown interferences are encircled. The SPE cartridge selectively eliminated chromatographic interferences found in certain cocoa products (namely liquors and some dark chocolates). Neither the profile nor the measured content of flavanols and procyanidins was affected by the use of the SPE cartridge.

molecular mass and different connectivities (Fig. 1). Hence, there exists some minor speciation within a DP due to the natural structural differences (e.g. stereo- or regio-isomers showing up as small shoulders or split peaks). This can also be seen in Fig. 4 (bottom). The integration protocol that was employed in this work was therefore a combination of valley-to-valley and the total baseline methods of integration. This was chosen for consistency. One example of the minor speciation observed can be seen for hexamer (DP 6) in Fig. 2A. The baseline approach was used for the molecular species with DP 6, yet between different DP, the valley-to-valley approach was employed. This was possible due the achieved $R_s$ of 1.5 discussed in an earlier section. Considering that this measurement methodology aims to quantify based on DP, we did not want the speciation – within a DP – to affect the integration parameters.

### 3.1.2. Precolumns

Another methodological modification that was considered as part of this quantitative method development was an examination of precolumns. In order to safeguard and extend the life of the column, several precolumns were investigated. Since precolumns packed with a diol stationary phase were difficult to acquire, cyano and silica phases were examined. The CN surface was chosen due to concerns of oxidation of phenolic groups on the silica surface. Using the CN-based precolumn proved to extend the life of the diol column by a factor of nearly 10 at the time of writing this report. An increase in the back pressure (from ~40 to ~80 bar) was used to indicate that the precolumn needed to be replaced.

### 3.1.3. Standards, confirmation of identity and calibration

*3.1.3.1. Confirmation of identity.* No commercially available standard reference materials were available for the procyanidins with DP greater than 2. Therefore, a reconstructed standard prepared in-house was employed for the calibration of analytical instrumentation and quantitative method development work. Using preparative methodology as described in previously published work, individual fractions were isolated and purified (DP 1–10) from cocoa, and then recombined to develop a reference standard for the analysis of cocoa and cocoa-containing materials [16]. Analyte (DP) identity and purity were confirmed through previously employed and accepted methodologies namely UV–Vis, LC/FLD [16], thiolysis work [19] and LC–MS masses [12,17]. The peaks for monomer through heptamers were identified as $[M-H]^-$ ions on mass spectrometer. Octamers, nonamers and decamers appeared as doubly charged ions ($[M-2H]^{2-}$) at $m/z$ 1153, 1297, and 1441, respectively. Masses obtained for fractions (according to DP) are indicated in Table 1 and were consistent with previously published identification parameters [12]. The average degree of polymerization obtained from the thiolysis experiments again were consistent with what was anticipated [13,16,19].

*3.1.3.2. Calibration conditions.* Quantification was accomplished with the external standard (ESTD) approach. An individual calibration curve was constructed for each flavanol and procyanidin fraction. The total flavanol and procyanidin for each sample was reported as the sum of the concentrations determined for each individual oligomeric fraction (DP 1–10) [17].

The response from the FLD plotted against the series of concentrations listed gave linear correlations for all the oligomeric fractions, with correlation coefficients ranging from 0.9963 to 0.9996. This linear behavior was observed reproducibly over the course of these investigations. The calibration employed was a linear regression (not a quadratic fit as with previous methodologies) with 6–7 concentration points. The calibration concentrations are given in Table 1. The HP Chem station software was set to include, but not force zero as part of the fit. The LOD and LOQ's are listed in Table 1. The total flavanol and procyanidin content for each sample is reported as the sum of the concentrations determined for each individual oligomeric fraction (DP 1–10) [17].

### 3.1.4. Sample preparation

*3.1.4.1. SPE cartridges.* The solvent conditions and choices for the most efficient extraction of flavanols and procyanidins from various cocoa-containing solid matrices have been previously assessed [28] and have been described in Section 2 of this report. These were not modified since they have been shown to give maximum extraction of these components [17]. However for certain

sample types, an interfering peak was observed. To eliminate this chromatographic interference, an additional clean up step, namely, employing a strong cation exchange solid phase extraction cartridge was implemented. The SPE cartridge selectively and consistently eliminated chromatographic interferences commonly found in processed cocoa products, namely liquors and some dark chocolates (shown in Fig. 4 is a liquor sample). Flavanol and procyanidin content was not affected (no loss) by the use of the SPE cartridge. Multiple SPE cartridges were investigated using a standard method development kit (Alltech Associates), however, only the strong cation exchange surface selectively removed the interference. These unknown interferences were only observed in specific sample types.

Attempts at identifying interfering species by LC–MS were unsuccessful; however, it is reasonable to speculate that the interference may have been an oxidized species (i.e. a quinone). Quinone species are known to react with the nitrogen present in amino acids [29]. It is possible that under the acidic conditions of the sample preparation, an amine containing species – generated in an amine–quinone addition reaction (e.g. Michael addition) – would be protonated and eliminated with the cation-exchange cartridge. After successful and reproducible elimination of these common interferences with the SPE cartridge, no further work was conducted to identify the interfering species.

*3.1.4.2. Recovery experiments.* A series of samples were generated to assess recovery of flavanols and procyanidins during the sample preparation procedure. Typically, the pH of a purchased jet black cocoa powder (a highly alkalized cocoa powder) dispersed in water approaches the range of 10–11. Therefore, in order to generate a spiked cocoa powder and prevent the destruction of the pH-sensitive flavanols and procyanidins, a large sample of jet black cocoa powder was mixed in water and acidified to a pH of 5–6. Chromatographic analysis of acidified jet black material (AJB) confirmed that no peaks at retention times consistent with analytes DP 1–10 were observed. An aliquot of this acidified powder was then spiked with a known amount of reference standard material [16]. Analysis of the spiked, acidified jet black cocoa powder (SAJB) gave an average recovery of 91.1% ($n = 3$).

Additional recovery experiments for different matrices were conducted. In addition to the SAJB cocoa powder described above, a dark chocolate sample, a dark chocolate with added jet black cocoa powder and cocoa butter (DC-AJB), and a dark chocolate with added spiked jet black cocoa powder (DC-SAJB) and cocoa butter were prepared. The addition of cocoa butter was to assist with the blending of the jet black cocoa powders with the dark chocolate matrices, as well as keep the % lipids constant in the all of the samples.

Flavanol and procyanidin content measured in the dark chocolate sample was 4.47 mg/g on a defatted basis. Considering that sample test sample preparation (i.e. addition of AJB and cocoa butter to the dark chocolate) diluted the flavanol and procyanidin content by 30%, the expected content was calculated to be 3.09 mg/g (defatted basis). The flavanol and procyanidin content of the DC–AJB was determined $3.30 \pm 0.30$ mg/g ($n = 3$) (on a defatted basis). For DC–SAJB, the two sources of flavanols and procyanidins were the DC itself (3.09 mg/g), and the added SAJB (10.87 mg/g). Based on this, the expected concentration was 13.92 mg/g (on defatted basis). The measured content for this material was $13.0 \pm 0.5$ mg/g ($n = 3$), indicating an average recovery of 93.4% of cocoa flavanols and procyanidins from this test matrix.

### 3.2. Method performance

#### 3.2.1. Single laboratory – performance characteristics

The examination of retention time variation indicated that there was a high degree of reproducibility on one column. Retention time variations examined in a continuous sequence have been reported in Table 1. The standard deviations were small and ranged from 0.03 to 0.50 for $n = 7$. The $RSD_{(r)}$ for RT over a period of a month ranged from 0% to 1.5%.

Longer term method performance (precision) was assessed in a single laboratory, primarily through the use of secondary standards, including cocoa powder, chocolate liquor, and dark chocolate. A total of 600 samples and standards were analyzed over a 3-month time-period without any observable degradation in chromatographic resolution on the diol column. The mean total flavanol and procyanidin contents (mg/g in defatted samples) were 74.39, 15.47 and 1.87 mg/g for the cocoa powder, chocolate liquor and dark chocolate samples, respectively. Statistical data from the secondary standards showed percent relative standard deviations ($\%RSD_{(r)}$) of 1.9%, 4.5% and 9.0% for the cocoa powder, chocolate liquor and dark chocolate samples, respectively. The trend increase in %RSD values tracks the flavanol and procyanidin content of the sample, that is, there is a decrease in chromatographic sensitivity (higher $RSD_{(r)}$) with a decrease in the total flavanol and procyanidin content of a sample.

Since flavanol monomer reference materials (both epicatechin and catechin) are commercially available, and as C18 methods are commonly employed for measuring these simpler flavanol components, the total flavanol monomer content of the cocoa-based samples was examined and compared using the diol method (DP 1) described herein and a C18 reversed-phase technology [30]. For this comparison, total monomer content has been reported as the sum of epicatechin and catechin. For seven separate measurements, the diol methodology generated a monomer content of $2.76 \pm 0.06$ mg/g and the C18 methodology gave a total of $2.75 \pm 0.03$ mg/g, indicating consistency and reproducibility between two very different methodological approaches.

#### 3.2.2. Inter-laboratory assessment

The reproducibility of the diol methodology for the analysis of cocoa flavanols and procyanidins was studied through a three instrument and three analyst (although two laboratory) protocol design. Seven representative sample types were prepared for analysis. For each sample type, a larger composite was prepared in order to ensure homogeneity and divided into five equal fractions (three were analyzed and two were stored at −80 °C for future reference). The samples were chosen to be representative of typical concentrations for common sample types and within the range of the calibration curves assessed. Samples included three different dark chocolates, one cocoa liquor, one cocoa-containing granola bar and two different cocoa powders. The samples were prepared and coded by an analyst that did not partake in the measurements of these samples. Samples were then distributed to the analysts (internal and collaborating investigators) involved in performing the measurements.

The results from the individual analysts for each sample have been reported in Table 2. These values represent the total flavanol and procyanidin content of each product, represented as the sum of the calculated concentration of each individual flavanol and procyanidin fraction (DP 1–10); all sample values have been reported on a defatted basis.

Sample A was a dark chocolate and had a total content of $4.61 \pm 0.22$ mg/g; sample B (dark chocolate) yielded $11.64 \pm 0.60$ mg/g for total flavanol content. Other values obtained were: sample C (dark chocolate) $1.55 \pm 0.15$ mg/g, sample D (granola bar) $4.31 \pm 0.23$ mg/g, sample E (cocoa liquor) $23.56 \pm 1.4$ mg/g, sample F (cocoa powder) $7.40 \pm 0.24$ mg/g, sample G (cocoa powder) gave $43.1 \pm 3.6$ mg/g. The values obtained for the samples labeled A–G were found to be in the anticipated ranges. The inter-lab measurements for the seven samples measured gave $RSD_{(R)}$ values ranging from 3.2% to 9.8% (reported in Table 2). Considering

**Table 2**
Results of the seven samples measured in the intra- and inter-laboratory assessment experiment. Total flavanol and procyanidin content (DP 1–10) expressed as mg/g (on a defatted basis). Date are expressed as the mean for $n = 2$. Three instruments (labeled 1–3) and three analysts (labeled 1–3) in two locations (location 1 and 2) participated in the inter-laboratory assessment.

| Sample | Location 1, analyst 1, instrument 1 ($n = 2$) | Location 1, analyst 2, instrument 2 ($n = 2$) | Location 2, analyst 3 ,instrument 3 ($n = 2$) | AVG | STD ($n = 6$) | $RSD_{(R)}$ |
|---|---|---|---|---|---|---|
| Dark chocolate A | 4.81 | 4.43 | 4.43 | 4.61 | 0.22 | 4.6 |
| Dark chocolate B | 10.81 | 11.93 | 11.64 | 11.36 | 0.60 | 5.1 |
| Dark chocolate C | 1.47 | 1.34 | 1.65 | 1.55 | 0.15 | 9.8 |
| Granola bar D | 4.48 | 4.59 | 4.15 | 4.31 | 0.23 | 5.4 |
| Cocoa liquor E | 24.93 | 24.11 | 22.21 | 23.56 | 1.40 | 5.9 |
| Cocoa powder F | 7.6 | 7.65 | 7.21 | 7.40 | 0.24 | 3.2 |
| Cocoa powder G | 47.5 | 44.84 | 40.05 | 43.7 | 3.77 | 8.6 |

that different Develosil Diol columns, different users and different instrumentation were employed and the complexity of the system (matrix and components being measured), these low $RSD_{(R)}$ values support and demonstrate the robustness and transferability of this methodology.

Variation in flavanol and procyanidin content in different sample types is expected for many reasons considering their biosynthetic origin stems from adaptation to environmental factors and that processing parameters can affect the content of these labile components. Variability is inherently high and establishing content even on the same food type can be challenging, making accuracy a difficult characteristic to inspect. However, to gain further insight into the accuracy of the measurement system, the NIST Standard Reference Material 2384 was employed as one of the samples in the inter-lab assessment experiment. The sample labeled dark chocolate B was SRM 2384. The total flavanol and procyanidin content was (based on intra-lab value) $11.36 \pm 0.6$ mg/g ($n = 8$, defatted value). This value was consistent with the $10.30 \pm 1.1$ mg/g reported on the certificate of analysis as a reference concentration for this material (analysis reported to be based on a normal phase silica based HPLC methodology). Even though vastly different chromatographic systems were employed, the diol methodology provided results for the flavanol and procyanidin content of the NIST SRM within acceptable limits, demonstrating the accuracy of the measurement system described in detail in this report.

Furthermore, the total monomeric content reported on the certificate of analysis as a certified concentration was $1.49 \pm 0.22$ mg/g (obtained via C18 methodology). The value obtained employing the current diol methodology – from the total area for the peak representing a DP 1 – was $1.43 \pm 0.20$ ($n = 8$).

## 4. Conclusion

A HPLC methodology for the separation and quantitation of flavanols and procyanidins in cocoa and chocolate-containing products was developed, optimized and assessed. The method is based on bonded diol stationary phase technology with a mobile phase consisting of a binary gradient of acidic acetonitrile ($CH_3CN$:HOAc, 98:2; v/v) and acidic aqueous methanol ($CH_3OH$: $H_2O$:HOAc, 95:3:2; v/v/v). Flavanols and procyanidins were separated according to the degree of polymerization ranging from 1 to 10. The flavanols and their oligomeric counterparts were monitored with fluorescence detection using excitation and emission set to 230 and 321 nm, respectively. These conditions provided a better resolution for higher oligomers, as well as greater signal intensity allowing for easier quantification and thereby a higher degree of repeatability and reproducibility relative to other methodologies for this complex class of compounds. To have all fractions (DP 1–10) within the linear range of the detector, a gain gradient was employed. An external standard calibration approach was utilized for quantitation. The transferability and repeatability of this measurement tool was assessed by monitoring data over a period of 3 months relative to secondary standards ($RSD_{(r)}$ ranged from 1.9%, 4.5% to 9.0% for samples containing 74.39, 15.47 and 1.87 mg/g, respectively), as well as by having three analysts perform the analysis on identical samples producing results within acceptable $RSD_{(R)}$ (3.2–9.8% for seven different sample types ranging approximately from 1 to 50 mg/g). The analysis of the NIST SRM 2384 provides strong evidence for the accuracy of this measurement system. Considering the complexity of the measurement, the method performance and multi-laboratory assessments demonstrate that this methodology is of acceptable reproducibility, accuracy and transferability for the determination of flavanols and procyanidins (DP 1–10) in cocoa-containing products. A more routine use of such a method would allow for the improved characterization (based on DP) and more reliable measurement for the content assessment of these food components. Additionally, this methodology could serve as one tool to support and enable research efforts to establish links between food components and human health.

## Acknowledgements

The authors would also like to acknowledge Nacalai Tesque Inc. and GL Sciences Inc. for providing diol phase columns Cosmosil and Inertsil WP300, respectively.

## References

[1] C. Heiss, P. Kleinbongard, A. Dejam, S. Perre, H. Schroeter, H. Sies, M. Kelm, J. Am. Coll. Card 46 (2005) 1276.
[2] D. Rein, T.G. Paglieroni, T. Wun, D.A. Pearson, H.H. Schmitz, R. Gosselin, C.L. Keen, Am. J. Clin. Nutr. 72 (2000) 30.
[3] N.D. Fisher, M. Hughes, M. Gerhard-Herman, N.K. Hollenberg, J. Hypertens. 21 (2003) 2281.
[4] R.L. Prior, L. Gu, Phytochemistry 66 (2005) 2264.
[5] H. Schroeter, C. Heiss, J. Balzer, P. Kleinbongard, C.L. Keen, N.K. Hollenberg, H. Sies, C. Kwik-Uribe, H.H. Schmitz, M. Kelm, Proc. Natl. Acad. Sci. 103 (2006) 1024–1029.
[6] M.B. Engler, M.M. Engler, C.Y. Chen, M.J. Malloy, A. Browne, E.Y. Chui, H.-K. Kwak, P. Milbury, S.M. Paul, J. Blumberg, M.L. Mietus-Snyder, J. Am. Coll. Nutr. 23 (2004) 197.
[7] L.J. Porter, in: J.B. Harborne (Ed.), The Flavanoids, Chapman and Hall Ltd., New York, 1988, p. 23.
[8] C. Santos-Buelga, G. Williamson, in: C. Santos-Buelga, G. Williamson (Eds.), Methods in Polyphenol Analysis, The Royal Society of Chemistry, Cambridge, 2003, p. 267.
[9] A.L. Waterhouse, S. Ignelzi, J.R. Shirley, Am. J. Enol. Vitic. 51 (2000) 383.
[10] J. Rigaud, M.T. Escribano-Bailon, C. Prieur, J.M. Souquet, V.J. Cheynier, J. Chromatogr. A 654 (1993) 179.
[11] S.A. Lazarus, G.E. Adamson, J.F. Hammerstone, H.H. Schmitz, J. Agric. Food Chem. 47 (1999) 3693.
[12] J.F. Hammerstone, A.S. Lazarus, A.E. Mitchell, R.B. Rucker, H.H. Schmitz, J. Agric. Food Chem. 47 (1999) 490.
[13] L. Gu, M. Kelm, J.F. Hammerstone, G. Beecher, D. Cunningham, S. Vannozzi, R.L. Prior, J. Agric. Food Chem. 50 (2002) 4852.
[14] D. Rein, S. Lotito, C.G. Fraga, H.H. Schmitz, C.L. Keen, J. Nutr. 130 (2000) 2120S.
[15] M.B. Engler, M.M. Engler, C.Y. Chen, M.J. Malloy, A. Browne, E.Y. Chiu, H.K. Kwak, P. Milbury, S.M. Paul, J. Blumberg, M.L. Mietus-Snyder, J. Am. Coll. Nutr. 23 (2004) 197.
[16] M.A. Kelm, J.C. Johnson, R.J. Robbins, J.F. Hammerstone, H.H. Schmitz, J. Agric. Food Chem. 54 (2006) 1571.
[17] G.E. Adamson, S.A. Lazarus, A.E. Mitchell, R.L. Prior, G. Cao, P.H. Jacobs, B.G. Kremers, J.F. Hammerstone, R. Rucker, K.A. Ritter, H.H. Schimtz, J. Agric. Food Chem. 47 (1999) 4184.

[18] L. Gu, A. Hager, R.J. Robbins, R.L. Prior, FASEB 22 (2008) 5.
[19] L. Gu, M.A. Kelm, J.F. Hammerstone, G. Beecher, J. Holden, D. Haytowitz, R.L. Prior, J. Agric. Food Chem. 51 (2003) 7513.
[20] L.R. Snyder, J.L. Glajch, J.J. Kirkland, Practical HPLC Method Development, John Wiley & Sons, 1988.
[21] L.R. Snyder, J. Chromatogr. Sci. 16 (1978) 223.
[22] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, 1979.
[23] http://www.aoac.org/dietsupp6/Dietary-Supplement-web-site/slv_guidelines.pdf.
[24] S.A. Lazarus, J.F. Hammerstone, G.E. Adamson, H.H. Schmitz, in: J.N. Abelson, M.I. Simon (Eds.), Methods in Enzymology, Academic Press, Berkeley, California, 2001, p. 46.
[25] J.H. Krech, S.L. Rose-Pehrsson, Appl. Spec. 50 (1996) 1528.
[26] S.K. Saha, S.K. Dogra, J. Lumin. 75 (1997) 117.
[27] W.L. Mattice, in: R.W. Hemingway, P.E. Laks (Eds.), Plant Polyphenols, Plenum Press, New York, 1992, p. 447.
[28] C. Counet, S. Collin, J Agric. Food Chem. 51 (2003) 6816.
[29] S. Bittner, Amino Acids 30 (2006) 205.
[30] L. Gu, S. House, E.X. Wu, B. Ou, R.L. Prior, J Agric. Food Chem. 54 (2006) 4057.