Separation of Flavanol Isomers in Chocolate Products using Multi-Pass Cyclic Ion Mobility Coupled to High-Resolution Mass Spectrometry

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INTRODUCTION

Chocolate is rich in polyphenols, which have been linked to health benefits such as reduction in cardiovascular disease.[1,2] Flavanols are one major class of polyphenols in chocolate and are composed of catechin or epicatechin and their oligomers known as procyanidins. "B-type" procyanidins (B1-B8, Fig. 1) — dimers of catechin/epicatechin — are typically the most abundant oligomeric forms.[2]

Chocolate and cocoa powder
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Because degree of polymerization (DP) can affect bioavailability[2] and activity, procyanidins are often characterized by DP using hydrophilic-interaction chromatography (HILIC), with minimal isomer separation. In contrast, using reversed-phase chromatography (RPLC), procyanidins generally do not elute in order of DP, complicating the oligomeric classification.[3]

Objective: To elucidate procyanidin isomers in selected chocolate and cocoa products using a combination of chromatographic and ion mobility separation.

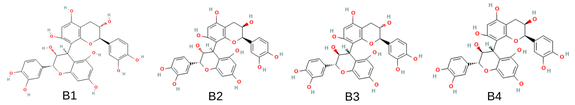

Figure 1. Structures of procyanidins B1-B4

METHODS: SAMPLE PREPARATION

Commercially available dark chocolate products with varying cocoa content (60%, 72%, and 85%) were selected, along with a non-dutch-processed cocoa powder. These products underwent the sample preparation described below (following AOAC Method 2020.05).[4] NIST Reference Material 8403 (cocoa flavanol extract)[5] was reconstituted in diluent and syringe filtered (0.22 µm PTFE) prior to analysis. Authentic standards of procyanidins B1-B4 (Cayman Chemical) were also analyzed for isomer identification.

1. Defatting: ~1g of each chocolate product was mixed with 10 mL of hexanes and vortexed for 1 min. The mixture was sonicated for ~5 min, then centrifuged for 5 min at 1700 × g. The extraction was repeated 2×; the combined supernatants were left to dry under ambient conditions.
2. Extraction: 500 mg of defatted chocolate was extracted with 10 mL of 70:30:1 acetone:water:acetic acid (AWAA) under sonication for 5 min, then centrifuged for 5 min at 1700 × g.
3. Cleanup: The supernatant was passed through an Oasis™ PRiME MCX (6cc, 150 mg) SPE cartridge. The eluent was collected.

All extracts were diluted 10× with AWAA diluent prior to analysis.

References

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2. de Pascual-Teresa, S., et al. Flavanols and Anthocyanins in Cardiovascular Health: A review of Current Evidence, *Int. J. Mol. Sci.*, 11 (2010) 1679-1703
3. Wollgast, J. et al., Analysis of Procyanidins in Chocolate by RP-HPLC with electrospray ionization mass spectrometric and tandem mass spectrometric detection, *J. Chrom. A.*, 926 (2001) 211-220
4. Bussy, U., et al., Determination of Cocoa Flavanols and Procyanidins (by Degree of Polymerization DP1-7) in Cocoa-based Products by HILIC with Fluorescence Detection: Collaborative Study, *J. AOAC International*, 105 (2022) 1060-1068
5. Rimmer, C.A., et al., Production and analysis of RM 8403 Cocoa Flavanol Extract, NIST Special Publication 260-207 (2020)
6. Hellström, J., et al., Isolation and Structure Elucidation of Procyanidin Oligomers from Saskatoon Berrier (Amelanchier alnifolia), *J. Agric. Food Chem.*, 55 (2007) 157-164

METHODS: LC-MS ANALYSIS

LC: ACQUITY™ I-Class Plus with FLR
Column: Atlantis™ Premier BEH™ Z-HILIC (1.7 µm, 2.1 × 100 mm)
MPA: Water + 0.1% formic acid
MPB: Acetonitrile + 0.1% formic acid
Column Temp: 50 °C
Flow rate: 0.4 mL/min
Injection volume: 3 µL
FLR Excitation λ: 230 nm
FLR Emission λ: 321 nm

	%A	%B
Initial	15	85
0.4	15	85
7.0	60	40
13.0	60	40
13.1	15	85
16.0	15	85

Table 1. LC Gradient

Figure 2. The SELECT SERIES Cyclic IMS with an ACQUITY UPLC I-Class Plus System

MS: SELECT SERIES™ Cyclic™ IMS
Acquisition Mode: HD-MS/MS
Polarity: Negative
Capillary Voltage: -0.5 kV
Mass Range: 50-2000 Da
Transfer cell collision energy: 20 – 50 V
MS Analyzer Mode: V Mode (MS Resolution ~60,000 FWHM)

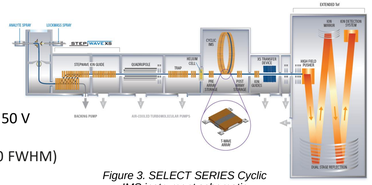

Figure 3. SELECT SERIES Cyclic IMS instrument schematic

Data were processed using MassLynx™ and Driftscope™ software platforms.

RESULTS: CHROMATOGRAPHIC SEPARATION

With RPLC separation, catechin and its dimers/trimers elute across the same retention time range (Fig. 4a). Two HILIC columns were compared in this work: the Torus™ Diol (Fig. 4b)— which is utilized in AOAC Method 2020.05[4]—and Atlantis Premier BEH Z-HILIC (Fig. 4c). The BEH Z-HILIC column provided improved, though incomplete, separation of the dimers and trimers compared to the Torus Diol column.

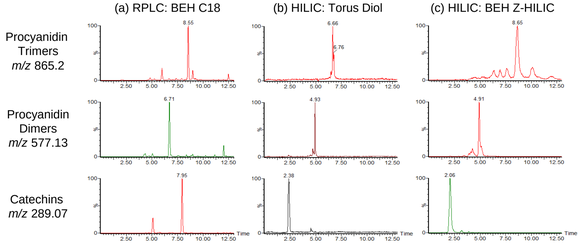

Figure 4. Comparison of the chromatographic separation of catechin and procyanidin dimers and trimers (displayed as XICs) using (a) ACQUITY BEH C18 column (RPLC), (b) Torus Diol HILIC column (following AOAC method 2020.05), and (c) Atlantis Premier BEH Z-HILIC columns. Data are shown for the 85% dark chocolate extract.

RESULTS: ION MOBILITY SEPARATION

The SELECT SERIES Cyclic IMS allows for scalable ion mobility resolution by varying the number of passes ions travel in the IMS cell. Multiple passes were needed to separate isomers of procyanidin dimers (Figure 5). For example, with six passes an additional constituent was clearly resolved from the major peak at 5.2 min (Fig. 5c), which was only partially resolved with four passes (Fig. 5b), and unobservable after a single pass (Fig 5a).

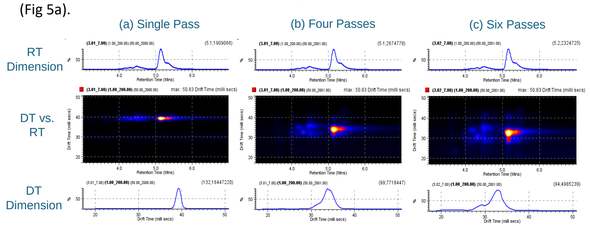

Figure 5. Comparison of the drift separation for quad-selected m/z 577 in NIST SRM 8403 with (a) one, (b) four, and (c) six passes in the mobility cell (RT = retention time; DT = drift time)

A comparison of the mobility separation for all chocolate/cocoa samples is shown in Figure 6, each for six passes in the mobility cell. Results for standards of procyanidins B1-B4 are also included in Fig. 6f.

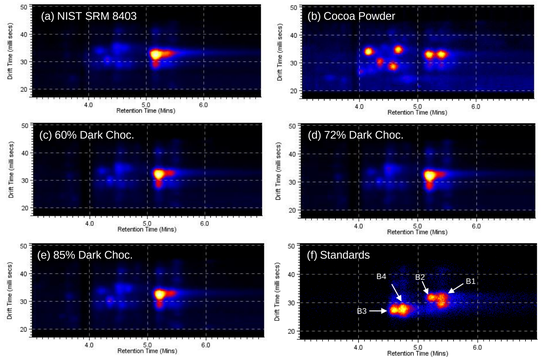

Figure 6. RT vs. DT plots of each chocolate/cocoa powder sample and procyanidin B1-B4 standards acquired with six passes in the mobility cell; quad-selected m/z 577

The chemical fingerprints for the three dark chocolate samples (60%, 72%, and 85%) are qualitatively similar to the NIST SRM8403 flavanol extract. The major peak at 5.2 min is identified at procyanidin B2, through a combination of RT, DT, and spectral matching (not shown). In contrast, the composition of cocoa powder is clearly different than the chocolate samples, with peaks observed for procyanidins B1, B2, and B3, along with other unidentified components.

RESULTS: SPECTRAL COMPARISON

Fragmentation spectra for ion mobility-resolved peaks in the cocoa powder sample are shown in Figure 7. Peaks 7 & 9 that separated from Procyanidin B2 (Peak 6) and B1 (Peak 8), respectively, show lower relative abundance of *m/z* 425 and 451 (Fig. 7 b & c), known fragments of procyanidins.[6] The different fragmentation patterns are likely due to subtle structural differences.

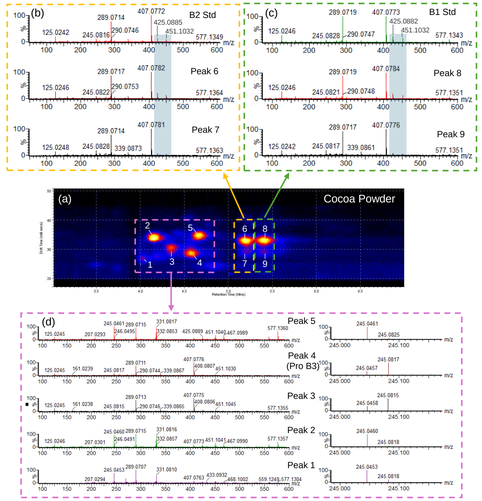

Figure 7. Comparison of fragmentation spectra of mass-selected m/z 577 precursor ions that were resolved in cocoa powder with six passes in the mobility cell

Additional unknown peaks with precursor *m/z* 577.13 were observed in cocoa powder (Peaks 1,2,3,&5; Fig. 7d), each with spectral similarities to known procyanidins (e.g., *m/z* 407.077 and 289.071), but broadly different fragmentation patterns. For example, several unknown compounds feature a fragment mass of *m/z* 331.082 ($C_{17}H_{15}O_7^-$) and different ion ratios of *m/z* 245, with *m/z* 245.081 ($C_{14}H_{13}O_4^-$) being more abundant among known procyanidins and *m/z* 245.045 ($C_{13}H_9O_5^-$) predominant in the unknown peaks (Fig. 7d, right side).

CONCLUSIONS

- **Multi-pass ion mobility revealed isomers of B-type procyanidins in chocolate samples that were not resolved chromatographically.**
- **Several dark chocolate samples were qualitatively similar, whereas additional unidentified components were observed in cocoa powder.**
- **Fragmentation spectra of unknown compounds in cocoa powder displayed fragment ions in common with known procyanidins and unique spectral features. This underscores that accurate spectral interpretation requires adequately resolving constituents in complex mixtures, coupled with high mass resolution to distinguish isobaric species.**

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