# Determination of A-Type and B-Type Procyanidins in Apple, Cocoa and Cinnamon Extracts

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# Overview

**Purpose:** Develop robust methods to separate and quantify the major procyanidins in extracts from polyphenol-rich foods and spices. Use complimentary detectors to identify and measure minor components and impurities and to gain insight into their antioxidant behavior.

**Methods:** Gradient HPLC and UHPLC with photodiode array detection, electrochemical array detection or charged aerosol detection**.**

**Results:** Cinnamon, cocoa and crab apple were found to be rich in 15 procyanidins ranging in size from dimer to pentamer. Numerous minor components were detected at concentrations as low as 0.1% by peak area. The methods were sensitive and precise.

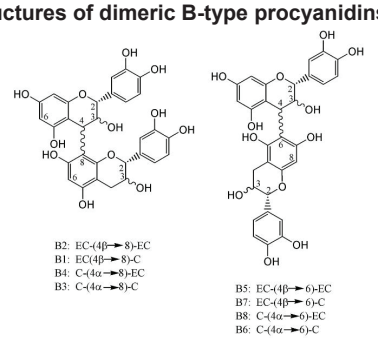
# Introduction

Procyanidins are condensed tannins consisting of polymerized subunits of epicatechin or catechin. They are structurally highly diverse because of the many possible combinations based on number of subunits, type of bonding, and branching. For convenience, procyanidins are typically characterized based on their degree of polymerization, with DP2 representing a dimer, DP3 a trimer, and so on. Procyanidins can be further distinguished by the type of bonding between subunits. For example, in B-type procyanidins (apples, cocoa) the subunits are connected by a single bond, which is predominantly 4β→8 or less often 4β→6. In apples and cocoa, the 4β→8 bond between connected epicatechin units occurs in the dominant components of various oligomers, as represented by the dimer B2, trimer C1, and tetramer D, etc. The dimeric B-type procyanidins occurring in nature are represented by all 8 structures shown in Figure 1. In A-type procyanidins (e.g. cinnamon, cranberries, and peanuts) an additional bond between adjacent subunits occurs that is often between 2β→7. The existence of an A-type bond imposes on the molecule a more rigid, compact structure.

Procyanidins, after lignans, are the second most common class of natural phenolic substances found in nature.[1] Since they occur in many foods,[2] with apples and cocoa being most prominent in the western diet, there is a strong and ever increasing interest in determining their biological properties and significance as dietary antioxidants.[3] Procyanidins have demonstrated several beneficial actions including anti-inflammatory,[4] hypoglycemic,[5] insulin activation,[7,7a] antioxidant,[6,7] hypocholesterolemic[8,9], and anti-allergic[10] properties. Particularly important may be a connection between procyanidin consumption and the lowering of risk of cardiovascular disease.[11-13] To correlate dietary intake of procyanidins with an impact on disease prevention and amelioration, there is a need to develop new as well as improved methodologies to facilitate analytical determination for pharmacological studies and analytical standardization of foods and dietary supplements.

Naturally occurring procyanidin comprise a very large family of individual molecules. This complexity contributes to challenges in both the preparative isolation of the individual oligomeric procyanidins or even specific DPs, as well as their routine analysis. Presented here is an evaluation of two novel HPLC-based analytical approaches for the determination of individual procyanidins in various sample matrices including extracts of crab apple, cocoa and cinnamon, and a commercial dietary supplement from a major commercial producer that is a water extract of cinnamon. The spectro-electro array (SEA) combines electrochemical and spectrophotometric detection.[14] It is both selective and sensitive, and uses an analyte's voltammetric and spectral properties for correct identification and measurement. Charged aerosol detection is a universal approach capable of measuring any non-volatile (and many semi-volatile) analytes and produces similar inter-analyte response independent of chemical structure. Compounds do not have to possess a chromophore or need to be ionized in order to be detected.

**FIGURE 1. Structures of dimeric B-type procyanidins.**



# Methods

### Sample Preparation

Dried extracts of cinnamon, cocoa and crab apple were reconstituted in a preservative solution of methanol containing 0.2% ascorbic acid and 0.02% EDTA.

### Liquid Chromatography

Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation Dual system including:

| | |
|---|---|
| Pump: | UltiMate DGP-3600RS with Solvent Rack |
| Autosampler: | UltiMate WPS-3000TRS |
| UV Detector: | UltiMate DAD-3000RS Diode Array Detector<br>Channel 1: 218 nm  Channel 2: 240 nm<br>Channel 3: 254 nm  Channel 3: 275 nm |
| EC Detector: | Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector with Thermal Organizer |
| EC Parameters: | 16 channel array; -100, then from 0 to +560 mV in 40 mV increments |
| Mobile Phase A: | 20 mM monobasic sodium phosphate, 3% acetonitrile, 0.2% tetrahydrofuran, pH 3.35 |
| Mobile Phase B: | 20 mM monobasic sodium phosphate, 50% acetonitrile, 10% tetrahydrofuran, pH 3.45 |
| Mobile Phase C: | 90% methanol |
| Gradient: | 0-2 min: 2%B/3%C. 30 min: 97%B/3%C. 45 min 97%B/3%C, Curve 7 |
| Analytical Column: | Thermo Scientific™ Acclaim™ 120, C18, 3 × 150 mm, 3 µm |
| Charged Aerosol Detector: | Thermo Scientific™ Dionex™ Corona™ Veo™ RS Charged Aerosol Detector |
| Detector Parameters: | Evap. temp. 35°C; data rate 2 Hz; filter 3.6 sec; PF 1.0; 0.05% formic acid replaced 20 mM monobasic sodium phosphate in mobile phases |
| Flow Rate: | 0.65 mL/min |
| Injection: | 2 µL |

### Data Analysis

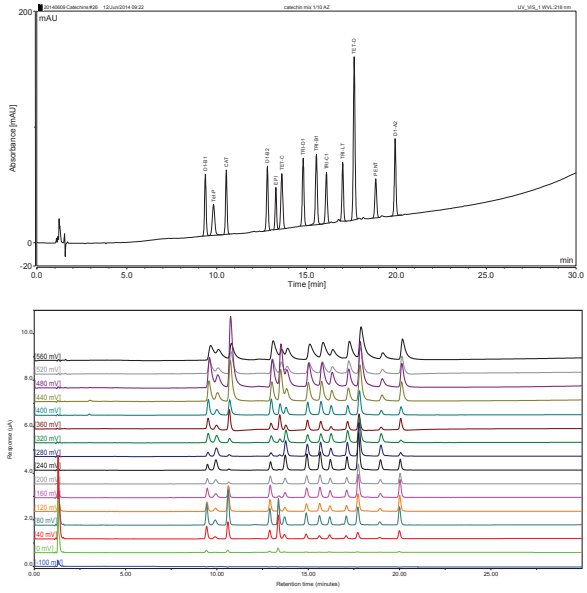
Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System 7.2 and CoulArray software 3.1.

# Results and Discussion

### Spectro-electro Array

A mixed procyanidin standard analyzed by the SEA is shown in Figure 2. Note how the electrochemical array detector captures the differences in electrochemical response exhibited by the dimer B2, monomer EPI and tetramer TET-C (cassiatannin A) across the range of applied potentials (bottom trace, retention time 13-14 min).

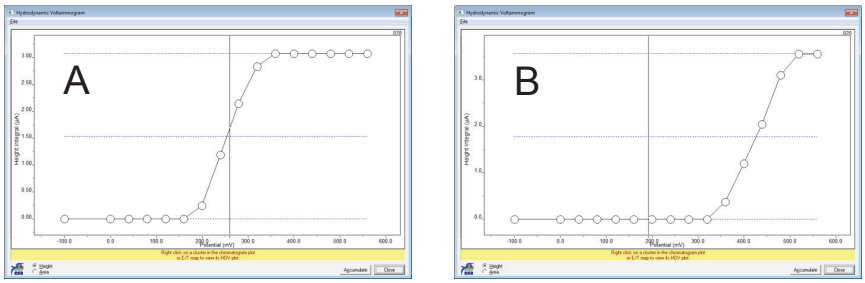
**FIGURE 2. HPLC-SEA chromatograms of a mixed procyanidin standard.**



### SEA Hydrodynamic Voltammograms

One means to characterize electroactive analytes is to compare voltammograms (HDVs), i.e., plots of oxidation current versus applied potential. The SEA quickly furnishes this information as shown below in Figure 3. PENT, the analyte with a first half-wave reduction potential of 200 mV (A) is more easily oxidized than Tri-C1 with a HWP of 400 mV (B). Higher DP procyanidins can exhibit several HWPs as their multiple hydroxy or methoxy substituents are oxidized successively (not shown).

**FIGURE 3. HDVs of PENT (A) and TRI-C1 (B).**




### SEA Performance

The SEA uses two detectors that work in tandem with complimentary strengths. The coulometric electrode array is superbly sensitive for measuring electroactive species such as the procyanidins, whose antioxidant behavior is attributable to their facile oxidation. The photodiode array detector responds nonspecifically at low UV wavelengths to most organic analytes and matrix components. UV achieves some specificity for particular functional groups by monitoring specific wavelengths. When combined with a robust HPLC method using state-of-the-art instrumentation, the method demonstrated here is both sensitive and precise. The limits of quantification are typically 0.2 -1 ng on-column by ECD and 0.5 – 5 ng by UV. A typical response range of over seven orders of magnitude by ECD allows detection of impurities at well below 0.05% of total peak area.[14] Selected precision and calibration data for this method are summarized in Table 1.

**TABLE 1. SEA method, selected* precision and calibration performance data.**

| Analyte | RT (min) | Precision, % RSD[1] | | Linearity | | |
|---|---|---|---|---|---|---|
| | | RT | Amount | Range (ng/μL) | LOD[2] (ng/μL) | $R^{2**}$ |
| D1-B1 | 9.80 | 0.03 | 0.56 | 0.32 - 329 | 10.5 | 0.99969 |
| TET-P | 9.59 | 0.24 | 0.62 | 0.53 - 540 | 4.97 | 0.99935 |
| CAT | 10.99 | 0.03 | 0.51 | 0.32 - 329 | 11.5 | 0.99963 |
| DIM-B2 | 13.30 | 0.02 | 0.43 | 0.32 - 328 | 10.5 | 0.99969 |
| EPI | 13.85 | 0.02 | 0.48 | 0.32 - 328 | 11.6 | 0.99963 |
| TET-C | 13. 70 | 0.12 | 0.51 | 0.50 - 510 | 10.8 | 0.9969 |
| TRI-D1 | 14.96 | 0.11 | 0.34 | 0.47 - 480 | 1.67 | 0.99993 |
| TRI-B1 | 15.72 | 0.1 | 0.23 | 0.45 - 460 | 2.82 | 0.9998 |
| TRI-C1 | 16.62 | 0.01 | 0.54 | 0.32 - 328 | 8.5 | 0.9998 |
| TRI-LT | 17.26 | 0.08 | 0.4 | 0.58 - 590 | 2.44 | 0.99985 |
| TET-D | 18.16 | 0.01 | 0.52 | 0.32 - 328 | 7.6 | 0.99984 |
| TRI-AB | 17.94 | 0.07 | 0.19 | 0.45 - 460 | 8.06 | 0.99827 |
| PENT | 19.31 | 0.01 | 0.49 | 0.32 - 328 | 7.4 | 0.99985 |
| DIM-A2 | 20.47 | 0.01 | 0.5 | 0.32 - 328 | 8.1 | 0.99982 |

[1] for n = 7 replicates; [2] Hubaux-Vos method; *UV data, other data omitted for the sake of brevity; **linear fit with offset

### SEA Examples

Many plants are rich in procyanidins and the SEA method is particularly well suited to measure and characterize procyanidin content, allowing comparison of different materials, processing methods and stability during storage. As an example, a chromatogram of a commercial cinnamon extract is shown in Figure 4. Of particular note here is the ability to detect higher DPs including tetrameric and pentameric procyanidins. In Table 2, results from SEA analysis of extracts from cinnamon, cocoa and crab apple highlight qualitative differences in procyanidin distribution between A-type (cinnamon) and B-type (cocoa, apples); note that these extractions were not evaluated for quantitative completeness.

**FIGURE 4. HPLC-SEA chromatograms of a commercial *Cinnamomum burmannii* extract and A-type procyanidin standard.**

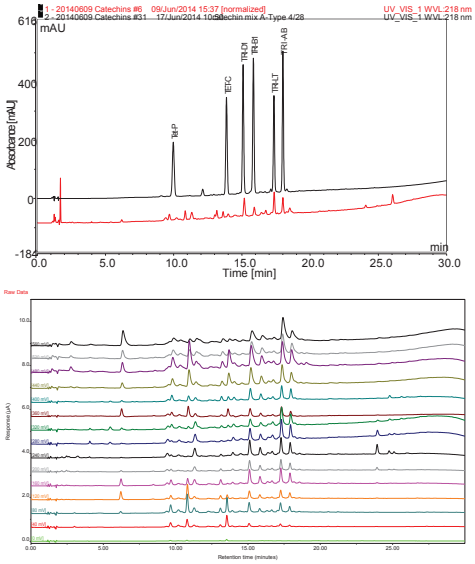



**TABLE 2. Measured procyanidin content of extracts from cinnamon, cocoa, crab apple and a commercial product containing *C. burmannii.***

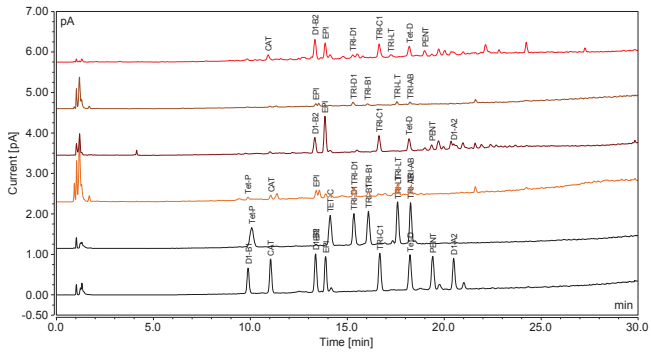
| Analyte | Amount Found, ug/mg | | | |
|---|---|---|---|---|
| | Cinnamon | *C. burmannii* | Cocoa | Crab Apple |
| D1-B1 | * | | 1.51 | 14.5 |
| TET-P | 12.1 | 1.46 | | |
| CAT | * | * | 3.94 | 1.60 |
| DIM-B2 | * | | 49.0 | 68.6 |
| EPI | * | * | 174 | 56.7 |
| TET-C | 11.6 | 1.69 | * | * |
| TRI-D1 | 17.4 | 3.69 | | |
| TRI-B1 | 12.3 | 2.16 | | |
| TRI-C1 | * | | 45.0 | 39.6 |
| TRI-LT | 18.3 | 4.55 | | |
| TET-D | | | 38.5 | 30.9 |
| TRI-AB | 12.3 | 3.02 | | |
| PENT | | | 13.4 | 3.89 |
| DIM-A2 | | | 29.4 | 6.84 |

* Trace amount, not quantified

## Charged Aerosol Detector

The charged aerosol detector is complimentary to the SEA because it's response to all non-volatile analytes is typically uniform for a given mass of analyte. This uniform response is especially useful when one needs to estimate the quantity of unknown minor components or impurities as for which one does not have a calibration standard. Many components in addition to the Type-A and -B standards are seen in the HPLC-Charged Aerosol Detector chromatograms shown in Figure 5.

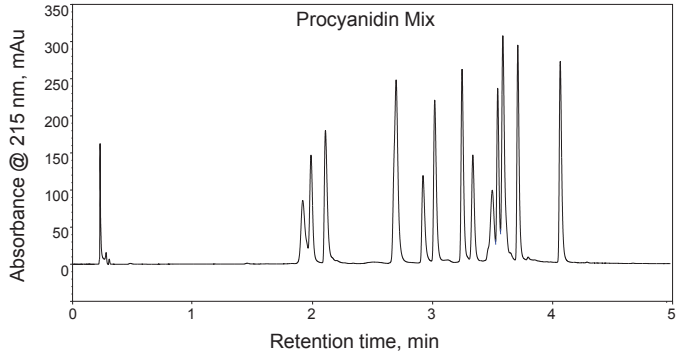
**FIGURE 5. HPLC-Charged Aerosol Detection chromatograms of plant extracts and standards of Type-A and –B procyanidins.**



### Future Direction

Advances in UHPLC instrumentation continue to provide faster and higher resolution separations. An example of a preliminary result obtained on the new Thermo Scientific™ Vanquish™ UHPLC platform with the Thermo Scientific™ Accucore™ Vanquish™ UHPLC column, 1.5 µm, 2.1 x 100 mm, is displayed in Figure 6. Work to optimize faster separations will continue.

**FIGURE 6. Vanquish UHPLC platform speeds analysis while maintaining resolution.**




## Conclusions

- The SEA resolves and quantifies all of the major A- and B-type procyanidins, in the range of DP2 to DP5, found in extracts from cinnamon, cocoa and crab apple.
- This quantitative method is precise (RSD of amounts below 1 %) and sensitive (LODs below ~10pg by electrochemical detection).
- Hydrodynamic voltammograms (HDVs) obtained by SEA furnish information on the antioxidant behavior of procyanidins as well as unidentified species in the extracts.
- Charged aerosol detection is sensitive to non-electroactive and non-chromophoric analytes and is compatible with UHPLC methods that reduce analysis time to < 5 min.

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