Targeted Analyses of Secondary Metabolites in Herbs, Spices, and Beverages Using a Novel Spectro-Electro Array Platform

Paul A. Ullucci, Ian N. Acworth, Christopher Crafts, Bruce A. Bailey, and Marc Plante
Thermo Fisher Scientific, Chelmsford, MA, USA

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Key Words
Polyphenols, Flavonoids, Stilbenes, Chalconoids, Electrochemical Detection, Gradient HPLC, Diode Array Detection, Antioxidant Properties

Introduction

Plant secondary metabolites show great structural diversity and wide variability. No single analytical method is capable of simultaneously separating and detecting all of these compounds. Rather, individual chemistries are used to target specific compounds or groups of compounds that possess similar chemical structures. For example, gradient reversed-phase high-performance liquid chromatography (HPLC) using a C18 column and diode array detection is most often the method of choice for the measurement of polyphenols when present at relatively high abundance. Another approach—the spectro-electro array platform—combines the universality of diode array detection with the selectivity and sensitivity of coulometric electrode array detection.

In this study, a gradient HPLC spectro-electro array platform is used to resolve and quantify specific polyphenols in crude extracts of a variety of natural products, supplements (ginseng, black cohosh, St. John's wort, and ginkgo), beverages (black tea, green tea, wine, beer, whisky, and bourbon), culinary herbs (oregano, rosemary, sage, and thyme), and spices (cloves and nutmeg). The relative abundance and lability (ease of oxidation on the coulometric electrochemical [EC] array) of the individual analytes can be used to estimate the antioxidant capacity of the sample. This is important not only to the consumer who may be taking supplements for their antioxidant content and purported health benefits, but also to the food industry where antioxidant activity may retard oxidative degradation of nutrients.

Goal

To use a spectro-electro array platform for the targeted measurement of secondary metabolites in herbs, spices, and beverages

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system, including:
  - LPG-3400BM Biocompatible Quaternary Micro Pump
  - SR-3000 Solvent Rack without Degasser
  - WPS-3000TBSL UltiMate 3000 Biocompatible Thermostatted Analytical Split-Loop Autosampler
  - DAD-3000RS UltiMate 3000 Rapid Separation Diode Array Detector
- Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector, Model 5600, with CoulArray Thermal Organizer Module
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software version 6.8 (SR9)
- CoulArray software version 3.1

Consumables

- Centrifugal Filters, 0.22 µm
- Sample Tubes, 40 mL

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Reagents and Standards

Standards		
Gallic Acid	Fisher Scientific	P/N AC410860050
4-Hydroxybenzyl Alcohol	Fisher Scientific	P/N 50-700-3921
p-Aminobenzoic Acid	Fisher Scientific	P/N ICN1025690
3,4-Dihydroxybenzoic Acid	Fisher Scientific	P/N ICN15642110
Gentisic Acid	Fisher Scientific	P/N AC165200050
2-Hydroxybenzyl Alcohol	Fisher Scientific	P/N 50-014-36177
Chlorogenic Acid	Fisher Scientific	P/N ICN15061801
4-Hydroxyphenylacetic Acid	Fisher Scientific	P/N AC121710250
p-Hydroxybenzoic Acid	Fisher Scientific	P/N ICN10257780
Catechin Hydrate	Fisher Scientific	P/N 50-749-8352
Vanillic Acid	Fisher Scientific	P/N AAA1207414
4-Hydroxybenzaldehyde	Fisher Scientific	P/N AC16277-0500
Syringic Acid	Fisher Scientific	P/N AC13289-0100
Caffeic Acid	Fisher Scientific	P/N ICN10479705
Vanillin	Fisher Scientific	P/N AC140821000
Syringaldehyde	Fisher Scientific	P/N 50-701-9419
Umbelliferone	Fisher Scientific	P/N AC12111
p-Coumaric Acid	Fisher Scientific	P/N ICN10257610
3,4-Dimethoxybenzoic Acid	Fisher Scientific	P/N AC11545-0250
Sinapic Acid	Fisher Scientific	P/N 50-121-8328
Salicylic Acid	Fisher Scientific	P/N AC14770
Ferulic Acid	Fisher Scientific	P/N AC15636
Ellagic Acid Dihydrate	Fisher Scientific	P/N AC11774
Coumarin	Fisher Scientific	P/N AC11053
Rutin	Fisher Scientific	P/N AC13239
Ethyl Vanillin Bourbonal	Fisher Scientific	P/N ICN15795980
4-Hydroxycoumarin	Fisher Scientific	P/N AC12110
Hesperidin	Fisher Scientific	P/N AC12346
Naringin	Fisher Scientific	P/N AC20691
Rosemarinic Acid	Fisher Scientific	P/N ICN15979210
Fisetin	Fisher Scientific	P/N 50-749-1075
Myricetin	Fisher Scientific	P/N 50-328-725
trans-Resveratrol	Fisher Scientific	P/N 50777-94
Luteolin	Fisher Scientific	P/N 50-148-702
cis-Resveratrol	Fisher Scientific	P/N NC9905571
Quercetin Dihydrate	Fisher Scientific	P/N ICN15200310
Kaempferol	Fisher Scientific	P/N ICN15514310
Isorhamnetin	Fisher Scientific	P/N 50-908-546
Eugenol	Fisher Scientific	P/N AC11911
Isoxanthohumol	ChromaDex®	P/N ASB-00009638
Chrysin	Fisher Scientific	P/N AC11032
Carvacrol	Fisher Scientific	P/N 50-014-24614
Thymol	Fisher Scientific	P/N AC15033
Carnosol	ChromaDex	P/N ASB-00003199
Xanthohumol	ChromaDex	P/N ASB-00024010
Carnosic Acid	ChromaDex	P/N ASB-0000319

Reagents		
Acetonitrile	Fisher Scientific	P/N A9981
Methanol	Fisher Scientific	P/N A-456-1
Sodium Phosphate Monobasic	Fisher Scientific	P/N ICN19485083
Tetrahydrofuran (THF)	Fisher Scientific	P/N T425-1
Phosphoric Acid	Fisher Scientific	P/N A260-500
Ascorbic Acid	Fisher Scientific	P/N AC105021000
Ethylenediamintetraacetic Acid (EDTA)	Fisher Scientific	P/N S311-100

Conditions	
Column:	Thermo Scientific™ Acclaim™ 120, C18, 3 µm Analytical (3.0 × 150 mm) P/N 063691
Mobile Phase A:	20 mM Sodium Phosphate Monobasic, 3% Acetonitrile, 0.2% Tetrahydrofuran, pH 3.35
Mobile Phase B:	20 mM Sodium Phosphate Monobasic, 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 (concave)
Flow Rate:	0.65 mL/min
Inj. Volume:	20 µL
Detection:	UV; Channel 1, 218 nm; Channel 2, 240 nm; Channel 3, 254 nm; Channel 4, 275 nm
EC Detector Parameters:	16 Channel Array from 0 to +900 mV in 60 mV increments

Standard Preparation

Depending on solubility, prepare stock standards in ethanol, methanol, or methanol/water solutions at 1000 µg/mL or 100 µg/mL. For a 1 M sodium phosphate monobasic buffer, dissolve 120.0 g of sodium phosphate monobasic in 1 L of 18 MΩ water, then filter with a 0.22 µm centrifugal filter. For Mobile Phase A, combine 40 mL of the 1 M sodium phosphate monobasic, 60 mL of acetonitrile, 4.0 mL of THF; add water to bring the volume to 2 L; then adjust the pH to 3.35 with 25% phosphoric acid. For Mobile Phase B, combine 20 mL of the 1 M sodium phosphate monobasic, 500 mL of acetonitrile, 100 mL of THF; add water to bring the volume to 1 L; then adjust the pH to 3.45 with concentrated phosphoric acid. Prepare working standards at 0.2, 0.5, and 1.0 µg/mL in 5% methanol containing 0.2% ascorbic acid and 0.02% EDTA.

Sample Preparation

Prepare supplements, culinary herbs, and spices for analysis by extracting 100 mg of the material with 20 mL of methanol. Sonicate the samples for 30 min, then centrifuge to obtain a clear solution. Dilute the solution 5x with a preservative solution (10% methanol containing 0.2% ascorbic acid with 0.02% EDTA) for injection into the HPLC system. Dilute wine samples 50x with the preservative solution. Prepare tea by steeping 0.5 g of tea with 75 mL of boiling water for 15 min. Then dilute this solution 10x with the preservative solution. Analyze beverage samples directly without further processing.

Results and Discussion

There continues to be considerable interest in the potential health benefits of phenolic and polyphenolic compounds present in a number of botanical supplements, foods, and beverages. For example, rosemary, thyme, sage, and wine are purported to have medicinal value. Many of these compounds have antioxidant properties and, as shown in numerous animal studies, may be protective against inflammation, cancer, and cardiovascular disease.[1] Although most of these compounds can be measured by HPLC with UV or diode array detection, their UV spectra are often indistinguishable. In complex samples such as botanical supplements, foods, and beverages, analyte coelutions are common, making identification and quantitation of many compounds difficult.

HPLC with coulometric electrode array detection uses multiple sensors that can be optimized to overcome the issue of chromatographic coelution. Easily oxidized compounds can be selectively detected upstream at low-potential sensors, while compounds that require a higher potential to oxidize respond downstream at higher-potential sensors. This approach extends the number of analytes that can be simultaneously measured and provides qualitative information. In addition to improved selectivity, coulometric electrode array detection is typically more sensitive and has a wider linear range than UV detection. However, EC detection is not universal.

The combination of UV and EC detection in the spectro-electro array platform extends the range of compounds that can be detected simultaneously. The theory behind this platform and its analytical merits are not covered here but are discussed by Ullucci et al.[2]

The targeted analytes measured for this study are presented in Table 1. Table 2 shows the amount of these analytes in a variety of beverages and the dried herb oregano. Analyte levels are in good agreement with previous publications.[3–5] Few of these compounds were found in extracts of ginseng, black cohosh, and ginkgo.

Table 1. Analyte identity: UV1–UV6 are analytes that have strong UV but weak EC response.

Peak	Compound	Peak	Compound
1	Gallic Acid	23	Rutin
2	4-Hydroxybenzyl Alcohol	24	Ethyl Vanillin Bourbonal
3	p-Aminobenzoic Acid	UV3	Methoxybenzaldehyde
4	3,4-Dihydroxybenzoic Acid	25	4-Hydroxycoumarin
5	Gentisic Acid	26	Hesperidin
6	2-Hydroxybenzyl Alcohol	27	Naringin
7	4-Hydroxybenzoic Acid	28	Rosemarinic Acid
8	Chlorogenic Acid	29	Fisetin
9	p-Hydroxyphenylacetic Acid	30	Myricetin
10	Catechin Hydrate	31	*trans*-Resveratrol
11	Vanillic Acid	UV4	Cinnamic Acid
12	4-Hydroxybenzaldehyde	32	Luteolin
13	Syringic Acid	33	*cis*-Resveratrol
14	Caffeic Acid	34	Quercetin Dihydrate
15	Vanillin	UV5	Apigenin
16	Syringaldehyde	35	Kaempferol
17	Umbelliferone	36	Isorhamnetin
18	p-Coumaric Acid	37	Eugenol
UV1	3,4-Dimethoxybenzoic Acid	38	Isoxanthohumol
19	Salicylic Acid	UV6	Chrysin
20	Sinapic Acid	39	Carvacrol
21	Ferulic Acid	40	Thymol
22	Ellagic Acid Dihydrate	41	Carnosol
UV2	Coumarin	42	Xanthohumol
		43	Carnosic Acid

The purported active phytochemicals in these supplements are not phenols and polyphenols but include triterpene glycosides, such as 27-deoxyactein, actein, and cimiracemoside (black cohosh); the triterpene saponin ginsenosides (ginseng); the sesquiterpenoid bilobalide; and diterpenoid ginkgolides (gingko) instead. These phytochemicals often lack strong chromophores, thus limiting the use of UV detection, and many cannot be measured by EC detection. However, as these compounds are not volatile, they can be readily measured with the Thermo Scientific™ Dionex™ Corona™ Charged Aerosol Detector.

Table 2. Abundance of different analytes in a variety of beverages and oregano.

Compound	Green Tea (mg/g)	Black Tea (mg/g)	Wine* (mg/L)	Scottish Whisky (mg/L)	American Bourbon (mg/L)	Oregano (mg/g)
4-Hydroxybenaldehyde	—	—	—	—	0.05	0.02
4-Hydroxybenzoic Acid	—	—	—	—	0.17	0.04
4-Hydroxybenzyl Alcohol	—	—	—	0.06	0.06	0.05
4-Hydroxycoumarin	—	—	—	0.04	—	0.98
Caffeic Acid	—	—	8.0	—	—	0.05
Carnosol	—	—	—	0.28	0.34	—
Catechin	3.73	3.0	37.0	—	—	0.76
Carvacrol	—	—	—	—	—	2.79
Chlorogenic Acid	—	—	—	—	—	0.25
Dihydroxybenzoic Acid	—	—	—	0.11	0.34	0.07
Ellagic Acid	—	—	52.0	—	—	—
Epicatechin	50.8	9.3	19.0	—	—	—
Epicatechin Gallate	65.3	40.6	—	—	—	—
Epigallocatechin	49.2	2.5	—	—	—	—
Epigallocatechin Gallate	180.0	31.3	—	—	—	—
Ethyl Vanillin Bourbonal	—	—	—	0.05	0.12	—
Eugenol	—	—	—	0.05	0.05	—
Ferulic Acid	—	—	1.0	0.03	0.20	0.10
Fisetin	—	—	—	—	0.06	0.04
Gallic Acid	—	—	57.0	0.11	0.10	1.05
Gallocatechin	18.8	3.2	—	—	—	—
Gallocatechin Gallate	5.9	7.0	—	—	—	—
Hesperidin	—	—	—	—	0.12	0.04
Isorhamnetin	—	—	—	—	—	0.10
Kaempferol	—	—	—	0.04	—	0.04
Luteolin	—	—	—	0.07	1.02	0.23
Myricetin	—	—	11.0	—	—	0.11
Naringin	—	—	—	—	0.48	0.35
p-Coumaric Acid	—	—	8.5	—	0.03	0.03
Quercetin Dihydrate	—	—	—	—	—	—
Rosemarinic Acid	—	—	—	—	0.10	1.98
Salicylic Acid	—	—	—	—	0.86	0.23
Sinapic Acid	—	—	2.0	—	3.64	0.17
Syringaldehyde	—	—	—	2.27	—	—
Syringic Acid	—	—	19.2	—	—	0.05
Thymol	—	—	—	—	—	0.13
Umbelliferone	—	—	—	0.20	0.53	0.06
Vanillic Acid	—	—	6.3	0.15	1.49	0.03
Vanillin	—	—	—	0.65	—	—

*Cabernet Sauvignon, 2008, from Argentina

Examples of samples differing in complexity are presented in Figures 1–3, which compare UV with EC detection capabilities. St. John's wort (*Hypericum perforatum*) is reported to be effective in the treatment of moderate depression in a number of clinical trials.[6] The two major polyphenols in St. John's wort, hypericin and pseudohypericin, are easily measured using both UV and EC detection (Figure 1, ~18 min). Nutmeg is particularly abundant in eugenol (Figure 2, Peak 37). Although this methoxyphenol is a hepatotoxin, it also has antimicrobial and anticarcinogenic properties.[7,8] Carnosic acid, a potent antioxidant and anticarcinogen, is particularly abundant in rosemary (Figure 3, Peak 43), typically making up 1.5–2.5% of the dried leaf.[9,10] All examples show that EC detection is much more sensitive than UV detection.

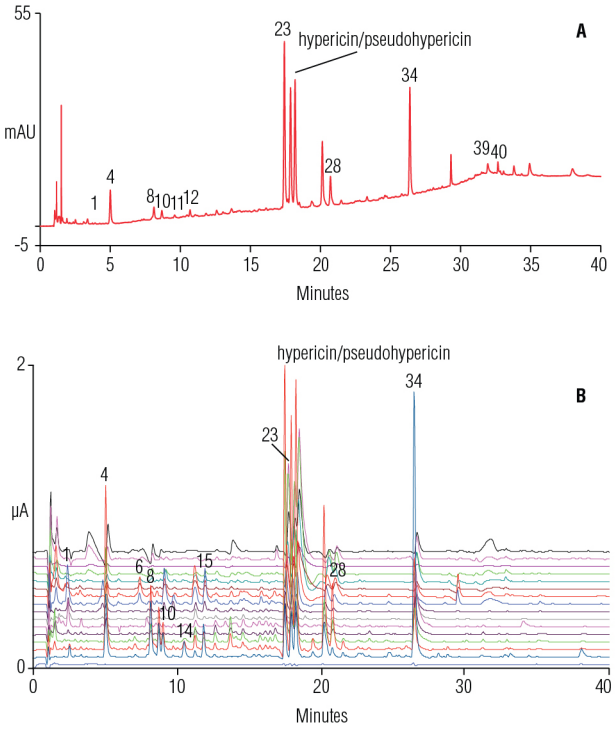

Figure 1. St. John's wort analyzed using UV detection at 254 nm (A). St. John's wort analyzed using EC detection (B).

Analyte oxidation across the electrode array can be used as an indicator of compound lability, with the more easily oxidized compounds reacting at the earlier (upstream) electrodes and the more stable compounds reacting at the later (downstream) electrodes. Analyte lability is also a reflection of antioxidant activity—the more easily oxidized the compound, the more potent it is as an antioxidant. Data from the CoulArray Coulometric Array Detector can be used in two ways. One, the summation of all analyte peaks in the chromatogram gives an indication of the total antioxidant capacity of the sample. Two, the summation of analyte response on each channel enables the total antioxidant capacity of the sample to be categorized by contribution of each class of antioxidant (a rank ordering of antioxidant contribution to the total antioxidant capacity of the sample). The antioxidant capacity of a sample obtained from the CoulArray Coulometric Array Detector is equivalent to data obtained using an oxygen radical antioxidant capacity assay.[11,12]

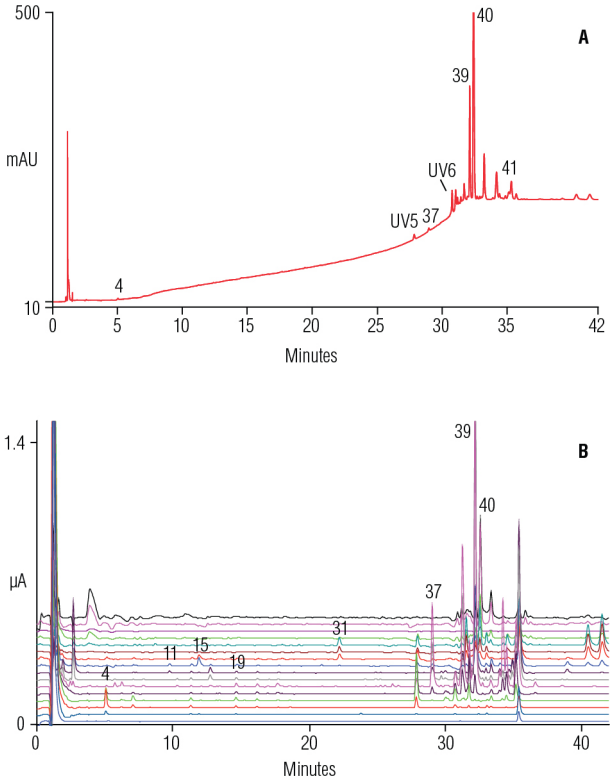

Figure 2. Nutmeg analyzed using UV detection at 210 nm (A). Nutmeg analyzed using EC detection (B).

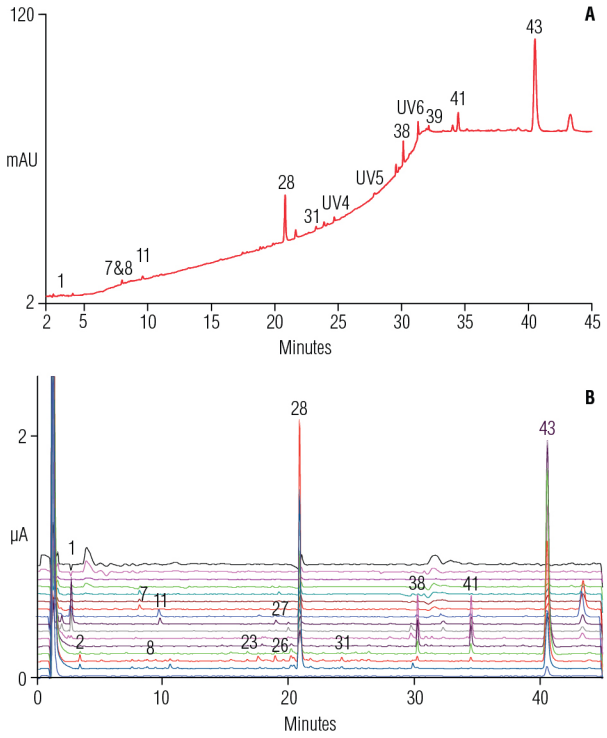

Figure 3. Rosemary analyzed using UV detection at 218 nm (A). Rosemary analyzed using EC detection (B).

Conclusion

- A multianalyte-targeted technique uses a spectro-electro array to resolve and quantify phenols, phenolic acids, and polyphenols in a variety of samples, including botanicals and beverages.
- The CoulArray Coulometric Array Detector uses unique three-dimensional (3D) voltammetric resolution to enable compound separation superior to that of traditional spectrometric techniques.
- The sensitivity of EC detection surpasses that of UV detection,[13] therefore allowing more complete characterization of trace levels of compounds in the samples.
- Because this approach makes it possible to sum the EC response of each analyte across the EC array, it can also be used to measure the antioxidant capacity of the sample, as well as the contribution of individual groups of antioxidants to that total capacity.
- This approach enables the separation and quantitation of 49 different phytochemicals commonly found in a number of herbs, spices, and beverages.

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