Application Note 1064

Product Authentication and Adulteration Determination Using a Novel Spectro-Electro Array Platform

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Key Words
Secondary Metabolites, Polyphenols, Terpenoids, Electrochemical Detection, Gradient HPLC, Diode Array Detection

Introduction

Plants contain an extraordinarily diverse range of secondary metabolites, including polyphenols, alkaloids, and terpenoids. Although these compounds are not involved in normal growth, development, and reproduction, they still play a crucial role in the organism. For example, polyphenols act as pigments and can protect against disease. Secondary metabolites are also thought to be responsible for the purported health benefits associated with the consumption of botanicals, supplements, some foods, and beverages.[1–2] Interestingly, polyphenols are associated with the quality and sensory characteristics of tea, wine, and beer.[3–4]

A gradient high-performance liquid chromatography (HPLC) spectro-electro array platform combines the universality of diode array detection with the selectivity and sensitivity of coulometric electrode array electrochemical (EC) detection. This technique can be used to simultaneously measure hundreds of known and unknown secondary metabolites in a sample. Such metabolite profiles contain a wealth of useful information. When evaluated using chemometric modeling software, changes in the pattern of metabolites can be used to study product adulteration, contamination, composition, and stability, and—in the case of wine and juice—the effects of growing region and differences between the varietals used in production.

Goal

To investigate the use of a spectro-electro array platform to generate metabolic patterns that can be interrogated using chemometric modeling software. This metabolomic approach is then used to differentiate wines and teas, and to study adulteration and the effects of geography on varietals using fruit juice as an example.

Equipment

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

Consumables

- Centrifugal Filters, 0.22 µm, nylon
- 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
Ethanol	Fisher Scientific	P/N A995-4
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
Ethylenediaminetetraacetic Acid (EDTA)	Fisher Scientific	P/N S311-100
Dimethylformamide (DMF)	Fisher Scientific	P/N AC116220010

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
Temperature:	35° C
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, relative to Pd, in 60 mV increments

Standards Preparation

Depending on solubility, prepare stock standards in ethanol, methanol, or methanol/water solutions at 1 or 0.1 mg/mL. Prepare substock standards A-G by mixing aliquots of different individual standards into 10 mL volumetric glass. Add 0.5 mL preservative solution containing 2% ascorbic acid and 0.02% EDTA. Dilute to 10 mL with a solution of 25% methanol at pH 3.2 adjusted with phosporic acid. Then mix the substock standards and dilute in water to prepare working standards at 0.2, 0.5, and 1.0 mg/L. See Table 1 for standards preparation details.

Samples and Sample Preparation

- Five red wines (four Cabernet Sauvignon samples and one Burgundy sample)
- Green, white, and black teas; and the bergamot-flavored black tea, Earl Grey
- Orange fruit juice

Dilute wine samples 1:50 v/v with the preservative solution.

Prepare tea by steeping 0.5 g of tea with 75 mL of boiling water for 15 min. Then dilute that solution 10x with the preservative solution.

Centrifuge orange juice samples and then filter through a 0.22 µm filter at 4 °C prior to analysis.

Data Analysis and Processing

Analyze data using Chromeleon CDS and CoulArray software. Transfer EC array data to Pirouette® software for chemometric analysis using the CoulArray software version 2.0 software utility, Pattern-Recognition Setup Wizard. Tabularize UV data prior to transfer to Pirouette software.

Table 1. Details for standards preparation.

Compound Name	Stock Std Concn (mg/mL)	Solvent	Aliquot (mL) to 10 mL	Substock Concn (mg/L)
Mix A				
Gallic Acid	1	50% Methanol	0.10	10
3,4-Dihydroxybenzioc Acid	1	50% Methanol	0.10	10
Catechin	1	Methanol	0.20	20
Syringic Acid	1	50% Methanol	0.10	10
Caffeic Acid	1	50% Methanol	0.10	10
Umbelliferone	1	Methanol	0.10	10
Salicylic Acid	1	50% Methanol	0.20	20
Naringin	1	Ethanol	0.20	20
Fisetin	0.1	Ethanol	1.00	10
Luteolin	0.1	Ethanol	1.00	10
Isorhamnetin	0.1	Ethanol	1.00	10
Carvacrol	1	Methanol	0.10	10
Carnosic Acid	0.1	Methanol	1.00	10
Mix B				
4-Hydroxybenzyl Alcohol	1	50% Methanol	0.10	10
Chlorogenic Acid	1	Methanol	0.20	20
4-Hydroxyphenylacetic Acid	1	50% Methanol	0.10	10
Vanillic Acid	1	Methanol	0.10	10
Vanillin	1	Methanol	0.10	10
Sinapic Acid	1	Methanol	0.10	10
Ferulic Acid	1	Ethanol	0.10	10
4-Hydroxycoumarin	1	Methanol	0.20	20
Hesperidin	1	DMF or Formamide	0.20	20
Myricetin	0.1	Ethanol	1.00	10
Kaempferol	0.1	Ethanol	1.00	10
Thymol	1	Methanol	0.10	10
Mix C				
p-Aminobenzoic Acid	1	50% Methanol	0.10	10
Gentisic Acid	1	50% Methanol	0.10	10
2-Hydroxybenzyl Alcohol	1	50% Methanol	0.10	10
p-Hydroxybenzoic Acid	1	50% Methanol	0.10	10
4-Hydroxybenzaldehyde	1	50% Methanol	0.20	20
Syringaldehyde	1	Methanol	0.10	10
p-Coumaric Acid	1	Ethanol	0.20	20
Ethyl Vanillin Bourbanol	1	Methanol	0.10	10
Rosemarinic Acid	0.1	Ethanol	1.00	10
Quercetin Dihydrate	1	Ethanol	0.20	20
Eugenol	1	50% Methanol	0.20	20
Carnosol	0.1	50% Methanol	1.00	10

Compound Name	Stock Std Concn (mg/mL)	Solvent	Aliquot (mL) to 10 mL	Substock Concn (mg/L)
Mix D: UV Compounds				
3,4-Dimethoxybenzoic Acid	1	Methanol	0.10	10
Coumarin	1	Methanol	0.10	10
Methoxybenzaldehyde	1	Methanol	0.10	10
Cinnamic acid	1	50% Methanol	0.10	10
Apigenin	0.1	Ethanol	1.00	10
Chrysin	1	Ethanol	0.10	10
Mix E				
Rutin	0.1	Ethanol	1.00	10
Ellagic Acid Dihydrate	0.1	Ethanol	1.00	10
trans-Resveratrol	0.1	Ethanol	1.00	10
cis-Resveratrol	0.1	Ethanol	1.00	10
Mix F				
Isoxanthohumol	0.1	Ethanol	1.00	10
Xanthohumol	0.1	Ethanol	1.00	10
Mix G				
Gallocatechin	0.1	Methanol	1.00	10
Epigallocatechin	0.1	Methanol	1.00	10
Catechin	1	Methanol	0.10	10
Epicatechin	1	Methanol	0.10	10
Epigallocatechin Gallate	1	Methanol	0.10	10
Gallocatechin Gallate	1	Methanol	0.10	10
Epicatechin Gallate	1	Methanol	0.10	10
Catechin Gallate	0.1	Methanol	1.00	10

Results and Discussion

The spectro-electro array makes use of both spectrophotometric and EC data. While UV data provides identification and quantitation of the major components in a sample, EC array detection provides additional information:

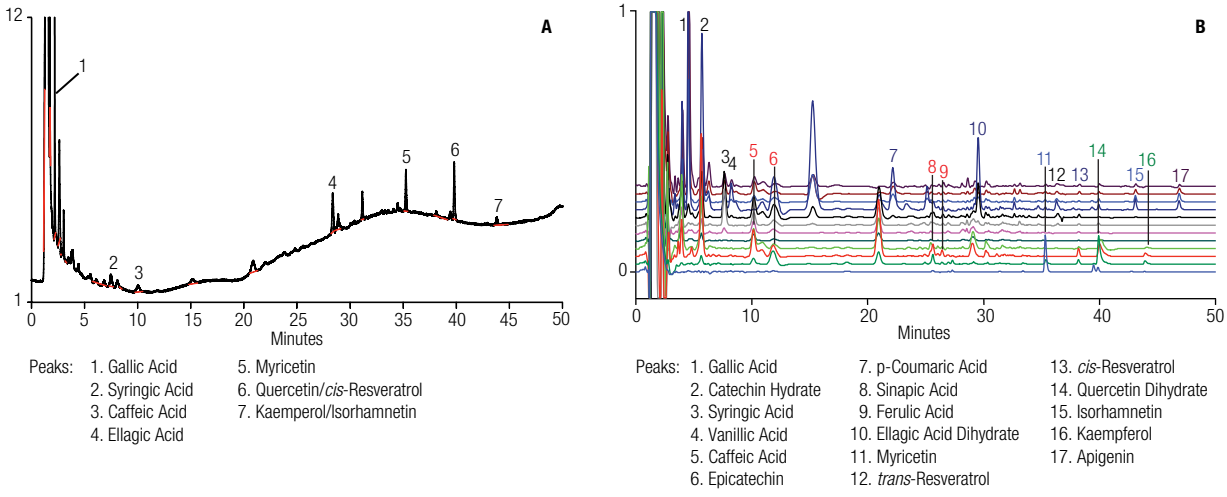
- The EC array is incredibly sensitive with low-pg limits of detection.
- It voltammetrically resolves compounds that coelute chromatographically.
- The EC array is fully gradient compatible, thereby extending the number of analytes that can be measured in a sample.
- The redox behavior of a compound reacting across the array provides qualitative information and can be used for analyte identification/authentication.

Table 2. Some of the more abundant analytes measured in different wine samples, and in good agreement with researched literature.[5–6]

Compound	Wine #1 Cabernet Sauvignon, Argentina (mg/L)	Wine #2 Cabernet Sauvignon, So. Africa (mg/L)	Wine #3 Cabernet Sauvignon, U.S. (mg/L)	Wine #4 Cabernet Sauvignon, Chile (mg/L)	Wine #5 Hearty Burgundy, U.S. (mg/L)
Apigenin	16	17.5	9.5	13	41
Caffeic Acid	8	13	5	17	3
Catechin Hydrate	37	26	26.5	24	22
Ellagic Acid Dihydrate	52	133	84	94	100
Epicatechin	19	15	16.5	11	4
Ferulic Acid	1	1	2	3	2
Gallic Acid	57	33.5	37	35	29.5
Isorhamnetin	6	5.5	2.5	6.5	2
Kaempferol	0.5	0.5	0.5	1	1
Myricetin	11	11	5	8	1.5
p-Coumaric Acid	8.5	16	2.5	14.5	3.5
Quercetin Dihydrate	13.5	15.5	3	14	4
cis-Resveratrol	1	1.5	0.5	2	0.5
trans-Resveratrol	2.5	2	1	2.5	1.5
Sinapic Acid	2	2	2	2	2
Syringic Acid	19	9.5	9	12	7
Vanillic Acid	6.5	4.5	2.5	8	4

Wine Analysis

A simple experiment examining the metabolite profiles of a selection of red wines was used to evaluate the application of the spectro-electro array platform to metabolomic studies. The general polyphenol method was used to analyze the five red wine samples. Several hundred analytes, including both known (Table 2) and unknown compounds, were measured in each sample (Figure 1).



Peaks: 1. Gallic Acid
2. Syringic Acid
3. Caffeic Acid
4. Ellagic Acid
5. Myricetin
6. Quercetin/*cis*-Resveratrol
7. Kaemperol/Isorhamnetin

Peaks: 1. Gallic Acid
2. Catechin Hydrate
3. Syringic Acid
4. Vanillic Acid
5. Caffeic Acid
6. Epicatechin
7. p-Coumaric Acid
8. Sinapic Acid
9. Ferulic Acid
10. Ellagic Acid Dihydrate
11. Myricetin
12. *trans*-Resveratrol
13. *cis*-Resveratrol
14. Quercetin Dihydrate
15. Isorhamnetin
16. Kaempferol
17. Apigenin

Figure 1. A Cabernet Sauvignon wine sample from Argentina analyzed by (A) UV detection at 254 nm and (B) EC array detection at low sensitivity. Note that compounds that coelute by UV detection are fully resolved using EC array detection (e.g., quercetin/*cis*-resveratrol and kaempferol/isorhamnetin).

Principal component analysis (PCA) was used to differentiate samples (Figure 2). Although this study is preliminary, it does show the capability of the system to differentiate samples by grape varietal/blend and by growing region. This approach is important when trying to authenticate a sample or identify product adulteration.

Tea Analysis

A similar approach was also applied to tea analysis to see whether the spectro-electro array platform can differentiate samples of green, white, and black tea, as well as the bergamot-flavored black tea, Earl Grey. Several hundred analytes were simultaneously measured in each sample, including both known (Table 3) and unknown analytes. A typical EC array chromatogram is presented in Figure 3.

To test the stability of the prepared sample extracts, tea samples were extracted and analyzed several days apart. PCA was then used to differentiate samples (Figure 4). As shown, the approach clearly distinguished between the metabolite profiles of green, white, and black teas. Furthermore, this approach also detected the subtle changes between the new extract and old extract within each tea type.

Although black tea and the Earl Grey bergamot-flavored black tea showed some similarity, they did show distinct clustering of samples by PCA. It is unclear whether such a difference in their metabolite patterns was a reflection of the addition of flavoring (bergamot orange extract) or differences between the flavored and unflavored black tea base. Regardless, subtle changes in metabolite profiles were easily identified using this approach.

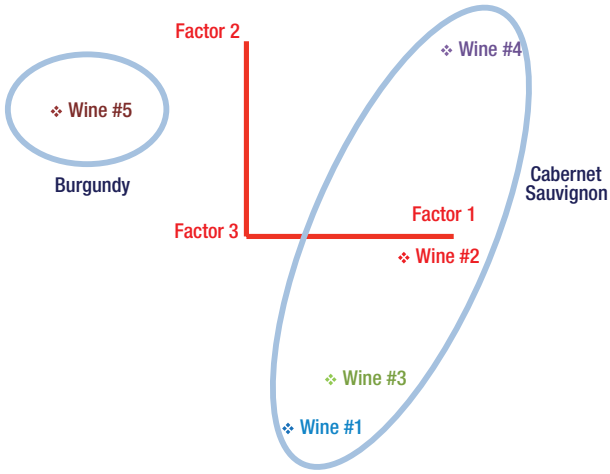


Figure 2. Initial study showing the PCA of wines.

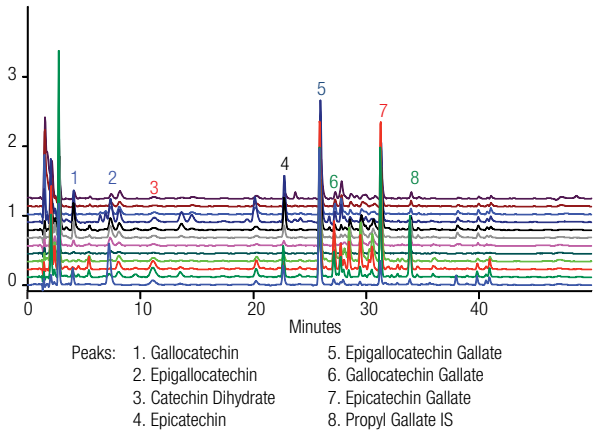


Figure 3. Green tea EC array chromatogram presented at low sensitivity showing the highly abundant catechins.

Table 3. Abundance of catechins in different teas. Data are in good agreement with the literature.[7–10]

Compound	Green Tea (mg/g)	Black Tea (mg/g)	White Tea (mg/g)
Catechin Hydrate	3.7	3.0	8.1
Epicatechin	50.8	9.3	39.8
Epicatechin Gallate	65.3	40.6	95.9
Epigallocatechin	49.2	2.5	32.3
Epigallocatechin Gallate	180	31.3	211
Gallocatechin	18.8	3.2	22.0
Gallocatechin Gallate	5.9	7.0	3.0

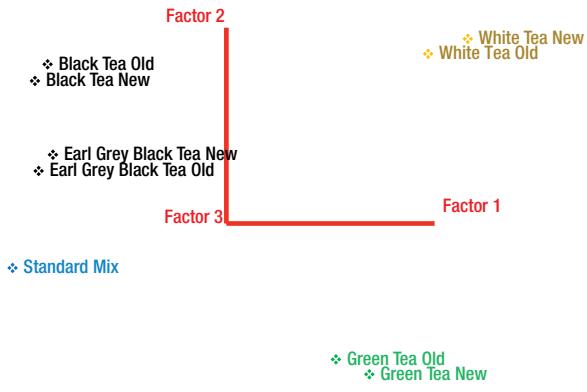


Figure 4. Initial study showing the PCA of teas.

Juice Analysis

A gradient HPLC spectro-electro array analytical method similar to the polyphenol method described above was used to study orange fruit juice adulteration.[11] The intent of this study was to combine this approach with PCA to identify the lowest level of adulteration—achieved either by blending with other juices or through the addition of orange peel or pulp wash—that can be detected in orange juice samples. Figure 5, Graph A shows distinct clustering of apple, grapefruit, and orange juice samples. Blending of as little as 10% grapefruit juice into orange juice was easily measured. Similarly, blending as little as 10% orange peel or 10% pulp wash into orange juice also was detected (Figure 5, Graph B).

Juice Varieties	
GF	Grapefruit Juice
OJ	Orange Juice
OJ10%GF	Orange Juice Blended with 10% Grapefruit Juice
POOL	Equal Blend of Several Orange Juice Samples

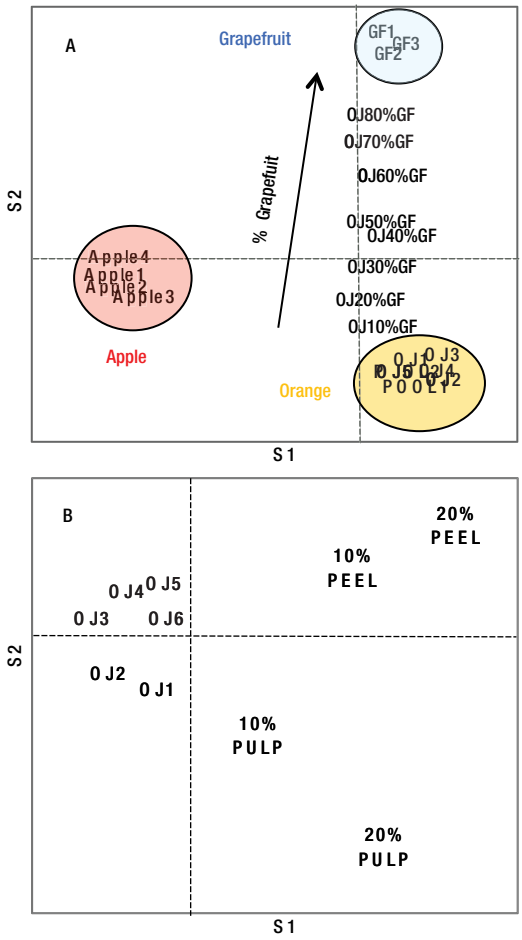


Figure 5. Measurement using the spectro-electro array platform combined with the PCA of orange juice adulteration by (A) blending with other juices and (B) adding orange peel or pulp wash. Note: This figure reproduced here with permission from Steven Nagy, Editor.[11]

Finally, data from the spectro-electro array platform was analyzed and displayed as a nearest-neighbor dendrogram showing the relationship between orange varietals and the geographic location of where the oranges were grown (Figure 6).

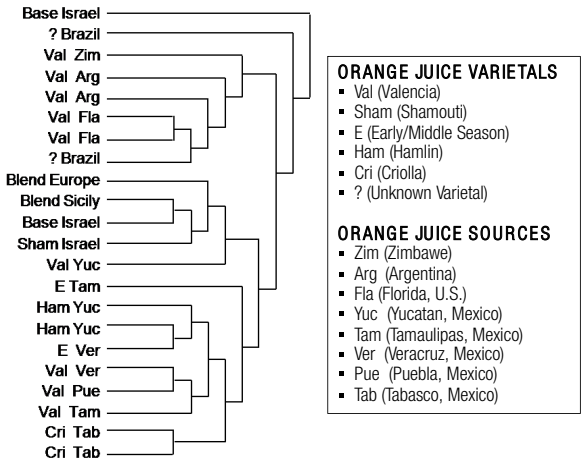


Figure 6. Nearest-neighbor dendrogram in which orange juice samples are classified by varietal and geography. Note: This figure reproduced here with permission from Steven Nagy, Editor.[11]

Conclusion

- Gradient HPLC with spectro-electro array detection is a simple approach that can be used to generate both targeted and information-rich metabolomic data. Metabolite profiles are generated with sensitive three-dimensional EC array data.
- Metabolomic data can be imported into pattern-recognition software and combined with PCA to readily identify product adulteration and authenticity.
- PCA easily differentiates a variety of wines and teas. Fruit juice adulteration using dilution with another juice or the inclusion of orange peel or pulp wash can be readily detected. It is possible to classify orange juice samples by varietal and geographical region.
- Although this work highlights the application of the method to beverages, this method is also applicable to other fields, including botanical/supplement testing, fuel/oil testing, drug testing, and counterfeit product identification.

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