
7 chapter

# High-Performance Liquid Chromatography

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## 7.1 INTRODUCTION

### 7.1.1 Background

High-performance liquid chromatography (HPLC) has many applications in food chemistry. Food components that have been analyzed with HPLC include organic acids, vitamins, amino acids, sugars, nitrosamines, certain pesticides, metabolites, fatty acids, aflatoxins, pigments, and certain food additives. Unlike gas chromatography, it is not necessary for the compound being analyzed to be volatile. It is necessary, however, for the compounds to have some solubility in the mobile phase. It is important that the solubilized samples for injection be free from all particulate matter, so centrifugation and filtration are common procedures. Also, solid-phase extraction is used commonly in sample preparation to remove interfering compounds from the sample matrix prior to HPLC analysis.

Many food-related HPLC analyses utilize reversed-phase chromatography in which the mobile phase is relatively polar, such as water, dilute buffer, methanol, or acetonitrile. The stationary phase (column packing) is relatively nonpolar, usually silica particles coated with a $C_8$ or $C_{18}$ hydrocarbon. As compounds travel through the column, they partition between the hydrocarbon stationary phase and the mobile phase. The mobile phase may be constant during the chromatographic separation (i.e., isocratic) or changed stepwise or continuously (i.e., gradient). When the compounds elute separated from each other at the end of the column, they must be detected for identification and quantitation. Identification often is accomplished by comparing the volume of liquid required to elute a compound from a column (expressed as retention volume or retention time) to that of standards chromatographed under the same conditions. Quantitation generally involves comparing the peak height or area of the sample peak of interest with the peak height or area of a standard (at the same retention time). The results are usually expressed in milligrams per gram or milliliters of food sample.

### 7.1.2 Reading Assignment

Ismail, B.P. 2017. Basic principles of chromatography. Ch. 12, in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

Reuhs, B.L. 2017. High performance liquid chromatography. Ch. 13, in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

## 7.2 DETERMINATION OF CAFFEINE IN BEVERAGES BY HPLC

### 7.2.1 Introduction

The caffeine content of beverages can be determined readily by simple filtration of the beverage prior to separation from other beverage components using reversed-phase HPLC. An isocratic mobile phase generally provides for sufficient separation of the caffeine from other beverage components. However, separation and quantitation are much easier for soft drinks than for a beverage such as coffee, which has many more components. Commercially available caffeine can be used as an external standard to quantitate the caffeine in the beverages by peak height or area.

### 7.2.2 Objective

To determine the caffeine content of soft drinks by reversed-phase HPLC with ultraviolet (UV) detection, using peak height and area to determine concentrations

### 7.2.3 Chemicals

| | *CAS no.* | *Hazards* |
|---|---|---|
| Acetic acid ($CH_3COOH$) | 64-19-7 | Corrosive |
| Caffeine | 58-08-2 | Harmful |
| Methanol, HPLC grade ($CH_3OH$) | 67-56-1 | Extremely flammable, toxic |

### 7.2.4 Hazards, Precautions, and Waste Disposal

Adhere to normal laboratory safety procedures. Wear safety glasses at all times. Methanol waste must be handled as hazardous waste. Other waste likely may be put down the drain using a water rinse, but follow good laboratory practices outlined by environmental health and safety protocols at your institution.

### 7.2.5 Reagents

(** It is recommended that these solutions be prepared by the laboratory assistant before class.)

- Mobile phase**
  Deionized distilled (dd) water: HPLC-grade methanol: acetic acid, 65:35:1 (v/v/v), filtered through a Millipore filtration assembly with 0.45-μm nylon membranes and degassed
- Caffeine solutions of varying concentration for standard curve**
  Prepare a stock solution of 20 mg caffeine/100 mL dd water (0.20 mg/mL). Make standard solutions containing 0.05, 0.10, 0.15, and 0.20 mg/mL, by combining 2.5, 5.0, 7.5, and 10 mL of stock solution with 7.5, 5.0, 2.5, and 0 mL dd water, respectively.

### 7.2.6 Supplies

(Used by students)

- Disposable plastic syringe, 3 mL (for filtering sample)

- Hamilton glass HPLC syringe, 25 µL (for injecting sample if using manual sample loading)
- Pasteur pipettes and bulb
- Sample vials for autosampler (if using autosampler)
- Soft drinks, with caffeine
- Syringe filter assembly, (Syringe and 0.45 µm filters)
- Test tubes, e.g., 13 × 100-mm disposable culture tubes (for filtering sample)

### 7.2.7 Equipment

- Analytical balance
- HPLC system, with UV–vis detector
- Membrane filtering and degassing system

### 7.2.8 HPLC Conditions

| | |
|---|---|
| Column | Waters µBondapak $C_{18}$ (Waters, Milford, MA) or equivalent reversed-phase column |
| Guard column | Waters Guard-Pak Precolumn Module with $C_{18}$ Guard-Pak inserts or equivalent |
| Mobile phase | dd $H_2O$: HPLC-grade methanol: acetic acid, 65:35:1 (v/v/v) (combine and then filter and degas) |
| Flow rate | 1 mL/min |
| Sample loop size | 10 µL |
| Detector | Absorbance at 254 nm or 280 nm |
| Sensitivity | Full-scale absorbance = 0.2 |
| Chart speed | 1 cm/min |

### 7.2.9 Procedure

(Instructions are given for manual injection and for analysis in triplicate)

1. Filter beverage sample.
   (a) Remove plunger from a plastic 3-mL syringe, and connect syringe filter assembly (with a membrane in place) to the syringe barrel.
   (b) Use a Pasteur pipette to transfer a portion of beverage sample to the syringe barrel. Insert and depress syringe plunger to force sample through the membrane filter and into a small test tube.
2. Flush Hamilton HPLC syringe with filtered sample, and then take up 15–20 µL of filtered sample (try to avoid taking up air bubbles).
3. With HPLC injector valve in *LOAD* position, insert syringe needle into the needle port all the way.
4. Gently depress syringe plunger to completely fill the 10-µL injector loop with sample.
5. Leaving the syringe in position, *simultaneously* turn valve to *INJECT* position (mobile phase now pushes sample onto the column) and depress chart marker button on the detector (to mark start of run on chart recorder paper).
6. Remove syringe. (Leave valve in the *INJECT* position so that the loop will be continuously flushed with mobile phase, thereby preventing cross contamination.)
7. After caffeine peak has eluted, return valve to *LOAD* position in preparation for next injection.
8. Repeat Steps 3–7, injecting each caffeine standard solution in duplicate or triplicate. (Note: The laboratory assistant can inject all standard solutions prior to the laboratory session).

### 7.2.10 Data and Calculations

1. Chromatograms and peak area reports will be printed out for you by the laboratory assistant. Use peak areas of the standards to construct your standard curve. Use the equation of the line for your standard curve to calculate the concentration of caffeine in your sample. Use the appropriate dilution factor when calculation the caffeine concentration in your sample. Complete the tables below with all data.

Standard curve:

| *Caffeine conc. (mg/ml)* | *Rep* | *Peak area ($cm^2$)* |
|---|---|---|
| 0.05 | 1 | |
| | 2 | |
| | 3 | |
| 0.10 | 1 | |
| | 2 | |
| | 3 | |
| 0.15 | 1 | |
| | 2 | |
| | 3 | |
| 0.20 | 1 | |
| | 2 | |
| | 3 | |

Sample: (Complete this table for each type of sample)

| *Rep* | *Retention time (min)* | *Peak area ($cm^2$)* | *Conc. based on area from std. curve (mg/ml)* | *Conc. based on area after considering dilution factor (mg/mL)* | *Conc. based on area (mg/100 mL)* |
|---|---|---|---|---|---|
| 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| | $\bar{X}$ | | | | |
| | SD = | | | | |

2. Using the mean values, calculate the concentration of caffeine in your sample expressed in terms of milligrams caffeine in a 12-oz. can (1 mL = 0.0338 oz).

### 7.2.11 Questions

1. Based on the triplicate values and the linearity of your standard curves, which of the two methods used to calculate concentration seemed to work best in this case? Is this what you would have expected, based on the potential sources of error for each method?
2. Why was it important to filter and degas the mobile phase and the samples?
3. How is the "reversed-phase" HPLC used here different from "normal phase" with regard to stationary and mobile phases and order of elution?
4. Mobile phase composition:
   (a) If the mobile phase composition was changed from 65:35:1 (v/v/v) to 75:25:1 (v/v/v) water to methanol to acetic acid, how would the time of elution (expressed as retention time) for caffeine be changed, and why would it be changed?
   (b) What if it was changed from 65:35:1 (water: methanol: acetic acid) to 55:45:1? How would that change the retention time and why?

## 7.3 SOLID-PHASE EXTRACTION AND HPLC ANALYSIS OF ANTHOCYANIDINS FROM FRUITS AND VEGETABLES

### 7.3.1 Introduction

Anthocyanins are naturally occurring plant pigments known for their diverse colors depending on solution pH. Analysis for anthocyanins is often difficult due to their similar molecular structure and polarity and their diversity of sugar and/or organic acid substituents. Color intensity is a common means of analyzing for anthocyanins since monomeric anthocyanins are colored bright red at low pH values from 1 to 3 (oxonium or flavylium forms) and are nearly colorless at higher pH values from 4 to 6 (carbinol or pseudobase forms). A pure anthocyanin in solution generally follows Beer's law; therefore concentration can be estimated from an extinction coefficient when an authentic standard is not available. However, many standards are commercially available with cyanidin-3-glucoside used most often for quantification purposes.

Red-fleshed fruits and vegetables contain many different anthocyanin forms due to their diverse array of esterified sugar substituents and/or acyl-linked organic acids. However, most foods contain up to six anthocyanin aglycones (without sugar or organic acid substituents, referred to as anthocyanidins) that include delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Fig. 7.1). Sample preparation for anthocyanin analysis generally involves solid-phase extraction of these compounds from the food matrix followed by acid hydrolysis to remove sugar and/or organic acid linkages. Anthocyanidins are then easily separated by reversed-phase HPLC.

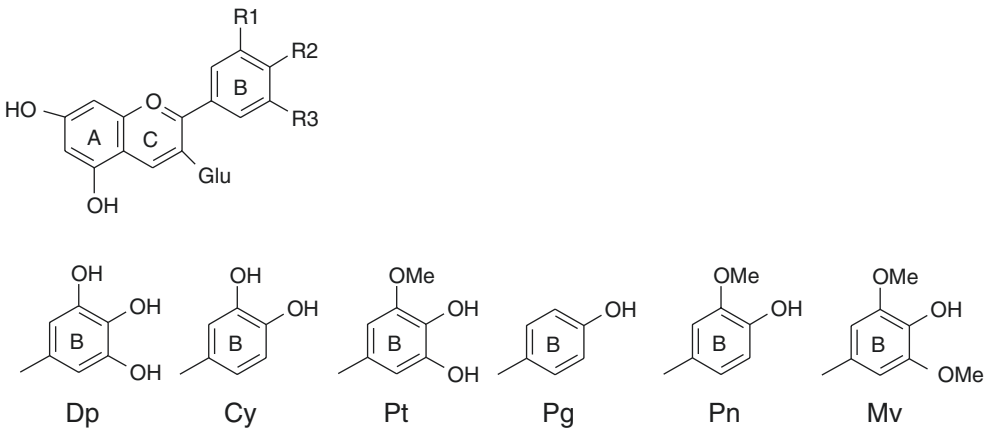
The use of solid-phase extraction (SPE) is a common chromatographic sample preparation technique used to remove interfering compounds from a biological matrix prior to HPLC analysis. This physical extraction technique is similar to an actual separation on a reversed-phase HPLC column. Although many SPE stationary phases exist, the use of reversed-phase $C_{18}$ is commonly employed for food analysis. On a relative basis, anthocyanins are less polar than other chemical constituents in fruits and vegetables and will readily bind to a reversed-phase $C_{18}$ SPE cartridge. Other compounds such as sugars, organic acids, water-soluble vitamins, or metal ions have little or no affinity to the cartridge. After the removal of these interferences, anthocyanins can then be efficiently eluted with alcohol, thus obtaining a semipurified extract for HPLC analysis.

Separation of compounds by HPLC involves the use of a solid support (column) over which a liquid mobile phase flows on a continuous basis. Chemical interactions with an injected sample and the stationary and mobile phases will influence rates of compound elution from a column. For compounds with similar polarities, the use of mixtures of mobile phases (gradient elution) is often employed. Reversed-phase stationary phases are most common for anthocyanin separations, and are based on column hydrophobicity of a silica-based column with varying chain lengths of *n*-alkanes such as $C_8$ or $C_{18}$. By setting initial chromatographic conditions to elute with a polar (water) mobile phase followed by an organic (alcohol) mobile phase, anthocyanins will generally elute in order of their polarity.

You will be analyzing anthocyanins isolated from fruits or vegetables for anthocyanidins (aglycones) following SPE and acid hydrolysis to remove sugar glycosides. After sugar hydrolysis, samples will be injected into an HPLC for compound separation. Depending on plant source, you will obtain between one and six chromatographic peaks representing common anthocyanidins found in edible plants.

### 7.3.2 Objective

Isolate and quantify anthocyanidin concentration from common fruits and vegetables by reversed-phase




7.1 figure

Anthocyanin structures. Common substitutions on the B-ring include delphinidin (*Dp*), cyanidin (*Cy*), petunidin (*Pt*), pelargonidin (*Pg*), peonidin (*Pn*), and malvidin (*Mv*)

HPLC with Vis detection, using spectrophotometric absorbance readings and extinction coefficients of anthocyanidins to determine standard concentrations.

### 7.3.3 Chemicals

| | *CAS no.* | *Hazards* |
|---|---|---|
| Hydrochloric acid (HCl) | 7647-01-0 | Corrosive |
| Methanol ($CH_3OH$) | 67-56-1 | Extremely flammable, toxic |
| *o*-Phosphoric acid ($H_3PO_4$) | 7664-38-2 | Corrosive |

### 7.3.4 Hazards, Precautions, and Waste Disposal

Adhere to normal laboratory safety procedures. Wear safety glasses at all times. Use hydrochloric acid under a fume hood. Methanol waste must be handled as hazardous waste. Other waste likely may be put down the drain using a water rinse, but follow good laboratory practices outlined by your environmental health and safety protocols.

### 7.3.5 Reagents

(** It is recommended that these solutions be prepared by a laboratory assistant before class.)

- 4 *N* HCl in water (for anthocyanin hydrolysis)**
- 0.01 % HCl in water (for sample extraction)**
- 0.01 % HCl in methanol (for elution from $C_{18}$ cartridge)**
- Mobile Phase A: 100 % water (pH 2.4 with *o*-phosphoric acid)**
- Mobile Phase B: 60 % methanol and 40 % water (pH 2.4 with *o*-phosphoric acid)**

[Each mobile phase should be filtered through a 0.45-µm nylon membrane (Millipore) and degassed while stirring using either a nitrogen sparge, under vacuum (ca. 20 min/l of solvent), or by sonication.]

### 7.3.6 Supplies

- Beaker, Pyrex, 500 mL (for boiling water for hydrolysis)
- Blender, kitchen scale, for sample homogenization
- Disposable plastic syringe, 3–5 mL (for filtering sample)
- Filter paper (Whatman #4) and funnels
- Fruit or vegetable that contains anthocyanins (blueberries, grapes, strawberries, red cabbage, blackberries, cherries, or commercial juices that contain anthocyanins)
- Hamilton glass HPLC syringe, 25 µl (for injecting sample)
- Reversed-phase $C_{18}$ cartridge (for SPE, e.g., Waters $C_{18}$ Sep-Pak, WAT051910)
- Syringe filter (0.45-µm PTFE, polytetrafluoroethylene)
- Test tubes, screw cap, with lids (for anthocyanin hydrolysis)

### 7.3.7 Equipment

- Analytical balance
- Hot plate
- HPLC system, gradient, with Vis detector (520 nm)
- Membrane filtering and degassing system
- Spectrophotometer and cuvettes (1-cm path length)

### 7.3.8 HPLC Conditions

| | |
|---|---|
| Column | Waters Nova-Pak $C_{18}$ (WAT044375) or equivalent reversed-phase column |
| Guard column | Waters Guard-Pak Precolumn Module with $C_{18}$ Guard-Pak inserts |

| | |
|---|---|
| Mobile phase | Phase A, 100% water; Phase B, 60% methanol and 40% water (both adjusted to pH 2.4 with *o*-phosphoric acid) |
| Flow rate | 1 mL/min |
| Sample loop size | Variable: 10–100 µl |
| Detector | Visible at 520 nm |
| Gradient conditions | Linear ramp. Hold time at 100% Phase B after 15 min may vary with column length and/or column packing material |

| *Time (min)* | *% Phase A* | *% Phase B* |
|---|---|---|
| 0 | 100 | 0 |
| 5 | 50 | 50 |
| 10 | 50 | 50 |
| 15 | 0 | 100 |
| 35 | 0 | 100 (end) |
| 37 | 100 | 0 (equilibration) |

### 7.3.9 Procedure

#### 7.3.9.1 *Sample Extraction*

(Note to the instructor: Several different commodities can be evaluated or the experiment replicated as needed.)

1. Weigh ca. 10 g of fruit or vegetable containing anthocyanins (record exact weight) and place in blender. Add ca. 50 mL of water containing 0.01% HCl and blend thoroughly (acidified acetone, methanol, or ethanol are also suitable substitutions for water). Fruit juices that contain anthocyanins can be used without further preparation.
2. Filter homogenate through filter paper and collect aqueous filtrate.
3. Keep refrigerated until needed.

#### 7.3.9.2 *Solid-Phase Extraction*

1. Condition a reversed-phase SPE cartridge by first washing with 4 mL of 100% methanol followed by 4 mL of water acidified with 0.01% HCl.
2. *Slowly* pass 1–2 mL of juice or filtrate (record exact volume) through the SPE cartridge being careful not to lose visible pigment. Anthocyanins will adhere to the SPE support and less polar compounds such as sugars, organic acids, and ascorbic acid will be removed.
3. Slowly pass an additional 4 mL of water (acidified with 0.01% HCl) through the cartridge to remove residual water-soluble components. Remove residual moisture from the cartridge by pushing air through the cartridge with an empty syringe or by flushing the cartridge with nitrogen gas until dry.
4. Elute anthocyanins with 4 mL of 0.01% HCl in methanol and collect for subsequent hydrolysis.

#### 7.3.9.3 *Acid Hydrolysis*

[Note to the instructor: It is recommended that a previously extracted sample be acid hydrolyzed before class to save time].

1. Pipette 2 mL of anthocyanins, dissolved in methanol, into a screw-cap test tube and add an equal volume of aqueous 4*N* HCl (final acid concentration = 2*N*) for a twofold dilution factor (see calculations below).
2. Under a fume hood, tightly cap the screw-cap vial and place in boiling water for ca. 90 min.
3. Remove test tubes and cool to room temperature before opening the vial. Filter an aliquot through a 0.45-µm PTFE syringe filter for analysis by HPLC.
4. Inject the filtered extract into the HPLC column and record peak areas for quantification of each compound (see Fig. 7.2).

### 7.3.10 Data and Calculations

Authentic standards for select anthocyanins can be obtained from several sources and should be used according to manufacturer's suggestions. If using anthocyanin glycosides, then the acid hydrolysis procedure should be conducted prior to HPLC analysis. Some anthocyanin suppliers include: Polyphenols Laboratories, Sandnes, Norway; INDOFINE Chemical Company, Somerville, NJ.

Cyanidin is a common anthocyanin present in large concentrations in many fruits and vegetables and will be used for sample calculations. A standard solution of cyanidin should be prepared in Mobile Phase A (water at pH 2.4) to establish a standard curve. Unless the actual concentration is known from the manufacturer, the standard should be quantified by determining its absorbance on a spectrophotometer at 520 nm against a blank of the same solvent. Using the molar extinction coefficient for cyanidin (obtained from manufacturer or expressed as cyanidin-3-glucoside equivalents, $\varepsilon = 29{,}600$ for a 1-*M* solution and 1-cm light path), the concentration is calculated using Beer's law, $A = \varepsilon bc$, using the following calculation:

$$\text{mg / L Cyanidin} = \frac{(\text{Absorbance at } \lambda_{max})(1000)(\text{MW})}{\varepsilon}$$

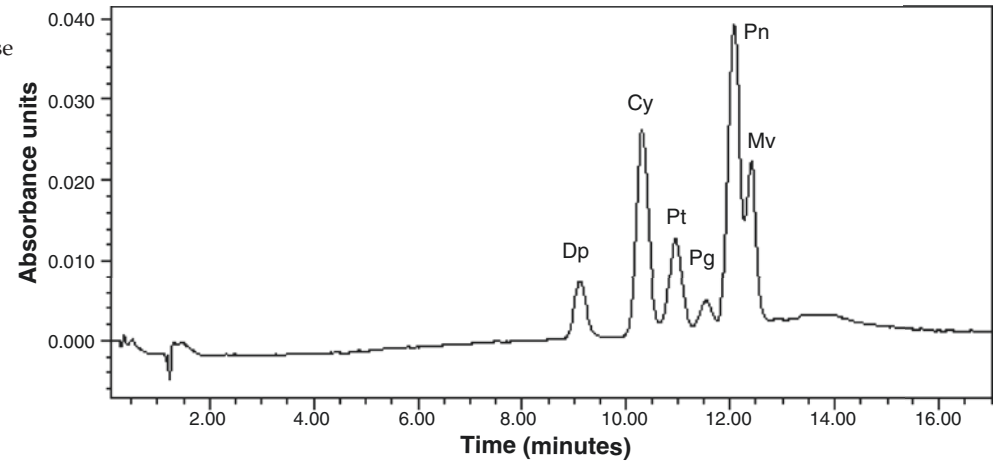
where:

MW ~ 457 g/mol
ε ~ 29,600

1. Inject a series of standard concentrations into the HPLC to generate a standard curve. (*Note*: These procedures can be performed by a laboratory assistant prior to the laboratory session.)
2. Chromatograms and peak area reports will be printed out for you by the laboratory assistant. Use peak areas of the standards to construct

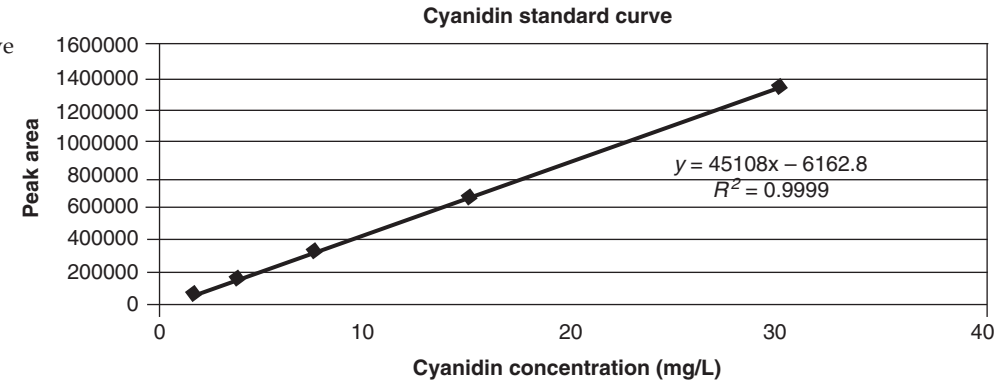
7.2 figure

Typical reversed-phase HPLC chromatograph of anthocyanidins (grape)




7.3 figure

Typical standard curve for cyanidin




your standard curve. Use the equation of the line for your standard curve to calculate the anthocyanin concentration of your acid hydrolyzed samples (Fig 7.3). Use appropriate dilution factors.

3. Express relative concentrations of each identifiable compound as cyanidin equivalents (mg/L) based on their peak area (unless commercial standards are available for each peak in the chromatograph).

| *Peak* | *Peak area* | *Relative concentration (mg/L)* |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |

$$\text{mg/L Cyanidin (in unknown)} = \frac{\text{Peak area} \times 2}{\text{Slope of standard curve}} \times \text{Sample dilution factors}$$

Peak area multiplied by 2 will compensate for the two-fold dilution incurred during acid hydrolysis. Sample dilution factors are calculated based on weight of fruit/vegetable per volume of extraction solvent (sample weight + solvent volume/sample weight). Single-strength fruit juices would have a sample dilution factor of 1.

### 7.3.11 Questions

1. Based on chemical structure, why do anthocyanidins elute in their respective order?
2. Predict how each compound would elute from a normal-phase column.
3. If the retention time of a compound that had absolutely *no* affinity to the column was 1.5 min and the flow rate was 1 mL/min, what is the total volume of mobile phase contained in the column, tubing, and pumps? Are you surprised at this number? Why or why not?
4. What would the chromatograph look like if you injected 40 µl of a sample as compared to 20 µl?
5. What would the chromatograph look like if Mobile Phases A and B were reversed (i.e., beginning with 100% Phase B and increasing Phase A over time)?

## RESOURCE MATERIALS

AOAC International (2016) Official methods of analysis, 20th edn., (On-line) Method 979.08. Benzoate, caffeine, and saccharin in soda beverages. AOAC International, Rockville, MD

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