

# Determination of Phenolic Compounds in Apple Orchard Soil

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## Key Words

Accelerated Solvent Extraction, HPLC, Polar Compounds, Environmental Analysis, Soil Quality

## Goal

To develop an efficient and simple method for the determination of phenolic compounds (i.e., gallic acid, caffeic acid, 4-hydroxybenzoic acid, syringic acid, ferulic acid, salicylic acid, phloroglucinol, cinnamic acid, catechin, quercetin, vanillin, phloridzin, and phloretin) in apple orchard soil

## Introduction

Phenolic compounds generated from rhizosphere exudation and decomposition of plant material may contribute to replant disease in susceptible plants, including apple trees.<sup>1</sup> Most phenolic substances are water-soluble and aromatic (structures shown in Figure 1); therefore, reversed-phase high-performance liquid chromatography (HPLC) with UV detection is the analytical technique of choice.<sup>2</sup>

Conventional extraction methods for soil samples—such as sonication, hot reflux, soxhlet, and immersion—are time consuming and do not always deliver the desired reproducibility. Thermo Scientific™ Dionex™ ASE™ Accelerated Solvent Extractors have the advantages of short extraction time, low solvent consumption, high extraction efficiency, excellent reproducibility, and time-saving automation. Additionally, Dionex ASE systems have been widely applied to environmental, food, and pharmaceutical extractions and analyses.<sup>3</sup>

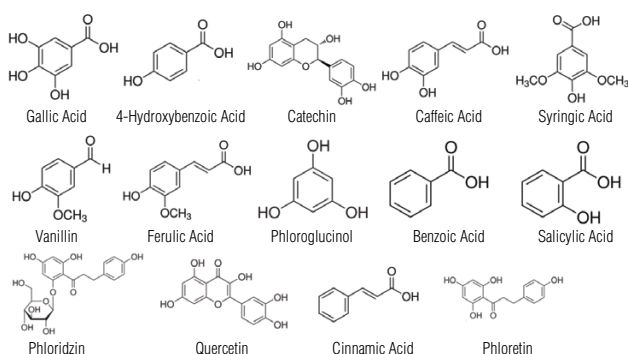


Figure 1. Structures of phenolic compounds.



## Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system, including:
  - LPG-3400RS Quaternary Rapid Separation Pump
  - SRD-3400 Integrated Solvent and Degasser Rack
  - WPS-3000TRS Rapid Separation Well Plate Autosampler, Thermostatted, with 25  $\mu$ L sample loop
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment
  - DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082-0010) with 2.5  $\mu$ L flow cell
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System Software, version 6.80 or higher
- Dionex ASE 350 Accelerated Solvent Extractor equipped with 66 mL Stainless Steel Extraction Cell Kit (P/N 068100)
- 250 mL Clear Collection Bottles for Dionex ASE Systems 350/300/150/100 (P/N 056284)

## Consumables

- Thermo Scientific™ Target2™ Nylon Syringe Filters, 0.45 µm, 30 mm (P/N F2500-1)
- Diatomaceous Earth (DE) Dispersant for Dionex ASE Systems (P/N 062819)

## Reagents and Standards

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH<sub>3</sub>CN), HPLC Grade (Fisher Scientific P/N AC610010040)
- Methanol (CH<sub>3</sub>OH), 99.9%, HPLC Grade (Fisher Scientific P/N AC610090040)
- Ethanol (CH<sub>3</sub>CH<sub>2</sub>OH), Ethyl Alcohol Denatured (Fisher Scientific P/N A407-1)
- Acetic Acid (CH<sub>3</sub>COOH), Glacial, HPLC Grade (Fisher Scientific P/N A35-500)
- Sodium Sulfate Anhydrous (Na<sub>2</sub>SO<sub>4</sub>), ≥99.0% (Fisher Scientific P/N S421-500)
- Gallic Acid Monohydrate (Fisher Scientific P/N A122-500)
- Catechin, 98% (Fisher Scientific P/N NC9236881)
- Vanillin (Fisher Scientific P/N V10-100)
- *trans*-Ferulic Acid (Fisher Scientific P/N 50-014-36252)
- 4-Hydroxybenzoic Acid, 99% (Fisher Scientific P/N AC120991000)
- Caffeic Acid, 98% (Fisher Scientific P/N 50-121-6645)
- Syringic Acid, 97% (Fisher Scientific P/N AC13289-0100)
- Phloroglucinol, 99% (Fisher Scientific P/N AC24176-0250)
- Benzoic Acid (Fisher Scientific P/N A68-30)
- Salicylic Acid (Fisher Scientific P/N A277-500)
- Phloridzin, 99% (Fisher Scientific P/N 50-750-9316)
- Quercetin, 99% (Fisher Scientific P/N ICN15200310)
- Cinnamic Acid, ≥98% (Fisher Scientific P/N AC15875-1000)
- Phloretin, 98% (Fisher Scientific P/N AC30765-5000)

## Conditions

### Dionex ASE System (Applicable to Figures 3–4)

System Pressure:	10 MPa (1500 psi)
Oven Temperature:	120 °C
Sample Size:	40 g
Heat Time:	5 min
Static Time:	5 min
Static Cycles:	2
Rinse Volume:	40 mL (60% of extraction cell volume)
Extraction Solvent:	Ethanol
Nitrogen Purge:	300 s
Extraction Time:	20 min
Cell Size:	66 mL

### Chromatographic (Applicable to Figures 2–4)

Column:	Thermo Scientific™ Acclaim™ 120, C18, 3 µm Analytical, 3.0 × 150 mm (P/N 063691)
Mobile Phase:	A. Acetonitrile B. Acetic acid solution (dilute 20 mL of glacial acetic acid to 1000 mL with DI water, pH 2.6)
Gradient:	0–5 min, 5% A; 25–30 min, 35% A; 35–40 min, 90% A; 40.1–45 min, 5% A
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temperature:	30 °C
Detection:	UV absorbance at 280 nm

## Preparation of Standard Solutions

### Stock Standard Mix 1

To prepare the Stock Standard Mix 1 of 14 analytes (i.e., gallic acid, 4-hydroxybenzoic acid, catechin, caffeic acid, syringic acid, vanillin, ferulic acid, phloroglucinol, benzoic acid, salicylic acid, phloridzin, quercetin, cinnamic acid, and phloretin), weigh 30 mg of each standard and dilute to 100 mL in a volumetric flask with methanol. The concentration of each analyte in Stock Standard Mix 1 will be 300 µg/mL.

### Stock Standard Mix 2

Dilute 10 mL of Stock Standard Mix 1 (300 µg/mL each) to 100 mL in a volumetric flask with DI water. The concentration of each analyte in Stock Standard Mix 2 will be 30 µg/mL.

### Mixed Working Standard Solutions for Calibration

To prepare five working standard solutions for calibration with 0.3, 1.5, 3.0, 6.0, and 9.0 µg/mL concentrations of each analyte, add the proper amount of Stock Standard Solution 2 and dilute with DI water.

## Sample Preparation

The soil samples used in this study were collected in an apple orchard located at Taishan Mount, Shandong Province, People's Republic of China.

Clean each extraction cell with soap and water prior to use and rinse thoroughly with DI water. Weigh 40 g of air-dried soil sample and mix with the appropriate amount of DE Dispersant for Dionex ASE Systems. Transfer the mixture to a 66 mL stainless steel cell equipped with an attached bottom cell cap and a cellulose fiber filter on the bottom. The mixture will nearly fill the cell. Perform the extraction using the specified conditions and collect the extract in the 250 mL clear collection bottle.

Add 1.5 g of  $\text{Na}_2\text{SO}_4$  (anhydrous) to the collected liquid extract. After shaking by hand for ~1 min, dry the extract using a rotary evaporator, then dissolve the remnants with 1 mL of methanol. Prior to HPLC analysis, filter the solution through a 0.45  $\mu\text{m}$  Target2 nylon syringe filter.

To prepare the spiked soil samples, add 400  $\mu\text{g}$  of phloridzin and 40  $\mu\text{g}$  of phloretin to 40 g of soil sample. Prepare the sample as described above. The spike concentrations are 10  $\mu\text{g}/\text{g}$  for phloridzin and 1  $\mu\text{g}/\text{g}$  for phloretin.

## Results and Discussion

### Separation of Phenolic Compounds

Because some phenolic compounds are structurally similar, their analysis requires high chromatographic selectivity and resolution. Experiments show that an acidic mobile phase benefits the separation of phenolic acidic compounds on the C18 reversed-phase stationary phase; the peak resolutions ( $R_s$ ) of the 14 analytes (i.e., gallic acid, 4-hydroxybenzoic acid, catechin, caffeic acid, syringic acid, vanillin, ferulic acid, phloroglucinol, benzoic acid, salicylic acid, phloridzin, quercetin, cinnamic acid, and phloretin) are all  $\geq 3.8$  when the pH = 2.6. Figure 2 shows that under the optimized chromatographic conditions, baseline separation of the 14 analytes was achieved using the Acclaim 120, C18 column.

### Optimization of the Dionex ASE System Method

Four solvents—methanol, ethanol, acetone, and methylene dichloride—were evaluated as extraction solvents used in the Dionex ASE system for extracting phloridzin and phloretin from an apple orchard soil sample. Phloridzin and phloretin, which are the characteristic phenolic compounds of *Malus* plants and present at significant concentrations in the bark and roots of an apple tree, may enter the soil through rhizosphere exudation and decomposition of plant material, resulting in reduced growth and a decline in production of the apple tree.<sup>4</sup> Figure 3 shows the extraction of vanillin (Peak 1), phloridzin (Peak 2), and phloretin (Peak 3) using methanol, ethanol, methylene chloride, and acetone. Although the extraction of vanillin using ethanol was not as good as that using acetone, ethanol was used as the extraction solvent because it provided the best extraction efficiency for phloridzin and phloretin.

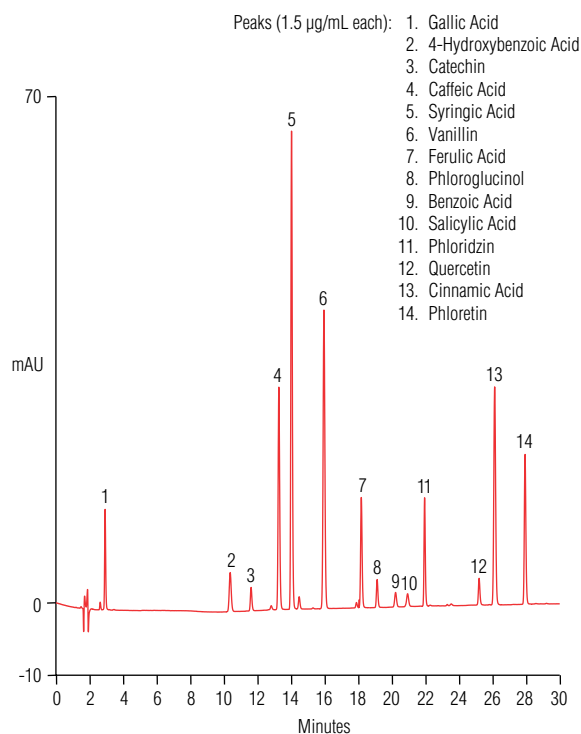


Figure 2. Phenolic compound standards.

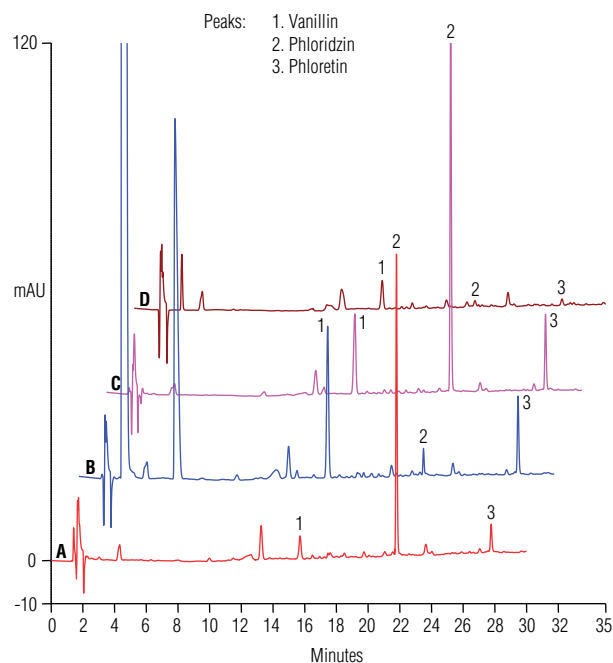


Figure 3. An apple orchard soil sample analyzed using different solvents: (A) methanol, (B) acetone, (C) ethanol, and (D) methylene dichloride as the Dionex ASE system solvent.

Dionex ASE system conditions were optimized experimentally in terms of oven temperature, static time, and cycle time. These optimized accelerated solvent extraction conditions are shown in the Conditions section for the Dionex ASE System. The extraction procedure required ~20 min and ~80 mL ethanol per sample.

### Reproducibility, Linearity, and Detection Limits

Method reproducibility was estimated by making seven consecutive injections of a calibration standard with a concentration of 1.5 µg/mL for each compound. The RSDs for retention time were all <0.10 and for peak area were all <1.21, demonstrating good reproducibility.

Calibration linearities of the 14 analytes were investigated by making three consecutive injections of a mixed standard prepared at five different concentrations. The external standard method was used to establish the calibration curve and quantify these compounds in apple orchard soil samples. Excellent linearities were observed from 0.3 to 9.0 µg/mL when plotting the concentration versus peak area, and the coefficients of determination were  $\geq 0.9978$  for all 14 compounds (Table 1). The method detection limits (MDLs) of each analyte were estimated using a signal-to-noise ratio (S/N) = 3. The calculated S/Ns and MDLs are summarized in Table 2.

### Sample Analysis

Figure 4 shows that three target analytes were found in the apple orchard soil sample: vanillin (Peak 1), phloridzin (Peak 2), and phloretin (Peak 3) were found with concentrations of 0.033, 0.154, and 0.018 µg/g, respectively. Recoveries for the detected phloridzins standards—phloridzin and phloretin—in the sample are 93% and 88%, respectively, demonstrating good accuracy of the Dionex ASE system HPLC method.

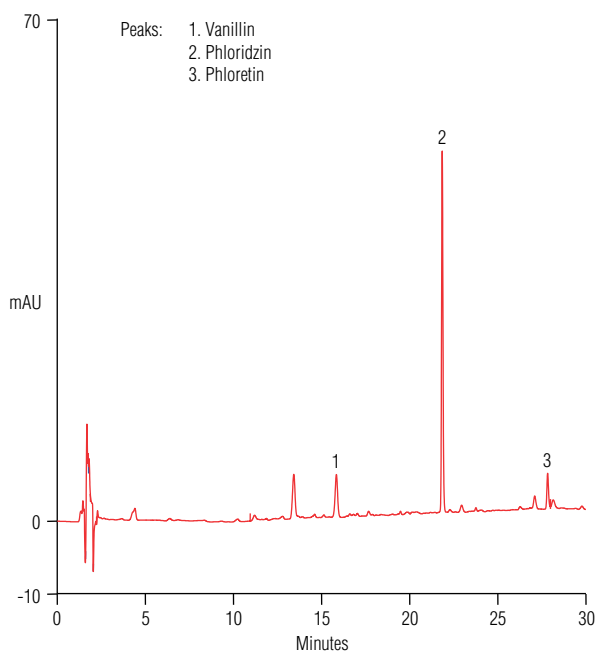


Figure 4. The apple orchard soil sample.

Table 1. Method calibration data.

Analyte	Regression Equation	$r^2$
Gallic Acid	$A = 0.6663c - 0.0164$	0.9998
4-Hydroxybenzoic Acid	$A = 0.5071c - 0.0036$	0.9991
Catechin	$A = 0.2324c + 0.0010$	0.9992
Caffeic Acid	$A = 2.2349c + 0.0062$	0.9998
Syringic Acid	$A = 4.4435c + 0.0326$	0.9997
Vanillin	$A = 3.3851c + 0.0280$	0.9997
Ferulic Acid	$A = 1.0592c + 0.0026$	0.9998
Phloroglucinol	$A = 0.2703c + 0.0030$	0.9981
Benzoic Acid	$A = 0.1752c + 0.0006$	0.9978
Salicylic Acid	$A = 0.1775c - 0.0017$	0.9990
Phloridzin	$A = 0.8844c + 0.0050$	0.9998
Quercetin	$A = 0.2683c - 0.0011$	0.9992
Cinnamic Acid	$A = 2.5296c + 0.0138$	0.9998
Phloretin	$A = 1.4841c + 0.0108$	0.9997

Table 2. S/Ns of phenolic compounds (0.3 µg/mL each) and calculated MDLs for each compound.

Analyte	Concn (µg/mL)	S/N	MDL (µg/L) <sup>a</sup>
Gallic Acid	0.3	63.5	0.01
4-Hydroxybenzoic Acid		24.9	0.04
Catechin		15	0.06
Caffeic Acid		141	0.006
Syringic Acid		305	0.003
Vanillin		190	0.004
Ferulic Acid		73	0.01
Phloroglucinol		17.5	0.05
Benzoic Acid		9.1	0.1
Salicylic Acid		8.2	0.1
Phloridzin		69	0.01
Quercetin		17	0.06
Cinnamic Acid		139	0.007
Phloretin		96	0.01

<sup>a</sup> The MDLs of each analyte were estimated using a S/N = 3.

## Conclusion

This work describes an efficient HPLC method with UV detection combined with a Dionex ASE system for the determination of phenolic compounds in soil samples. The entire analysis process, including sample preparation and separation for one soil sample, can be accomplished within 80 min.

## References

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