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Chromatographic Analysis for Targeted Metabolomics of Antioxidant and Flavor-Related Metabolites in Tomato

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[Abstract] Targeted metabolomics is a useful approach to evaluate crop breeding studies. Antioxidant and flavor-related traits are of increasing interest and are considered quality traits in tomato breeding. The present study presents chromatographic methods to study antioxidants (carotenoids, vitamin C, vitamin E, phenolic compounds, and glutathione) and flavor-related characters (sugars and organic acids) in tomato. Two different extraction methods (for polar and apolar entities) were applied to isolate the targeted compounds. The extraction methods developed in this work were time and cost-effective since no further purification was needed. Carotenoids, vitamin C, glutathione, and phenolic acids were analyzed by HPLC-PDA using a RP C18 column at an appropriate wavelength for each compound. Vitamin E and sugars were analyzed by HPLC with RP C18 and NH2 columns and detected by FLD and RI detectors, respectively. In addition, organic acids were analyzed with GC-FID using a Rtx 5DA column after derivatization with MSTFA. As a result, sensitive analytical methods to quantify important plant metabolites were developed and are described herein. These methods are not only applicable in tomato but are also useful to characterize other species for flavor-related and antioxidant compounds. Thus, these protocols can be used to guide selection in crop breeding.

Keywords: HPLC, GC, Carotenoids, Vitamins, Phenolic compounds, Glutathione, Sugars, Organic acids

[Background] Metabolomics is an applied biochemical approach which has gained attention for its potential to aid crop breeding studies. In tomato breeding, both antioxidant and flavor-related metabolites are of increasing interest because of consumer preferences for improved crop quality. Humans cannot synthesize antioxidant molecules themselves; therefore, these molecules must be provided by the external daily diet (Lobo *et al.*, 2010). Flavor is a complex trait that also affects the consumer marketability of tomato (Kader, 2008). Thus, the quantification of these traits is important. Because the metabolome is complex and consists of a wide variety of compounds including lipid-soluble metabolites, aqueous polar metabolites, stable and unstable metabolites, as well as acidic and basic metabolites, many methods for both extraction and detection of plant metabolites are available. These methods encompass a range of different chromatographic techniques with different extraction methods depending on the type of metabolite. In the literature, different techniques including ultrasonication (Tan *et al.*, 2021), supercritical CO_2 extraction (Pellicanò *et al.*, 2020), water-induced hydrocolloidal

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complexation (Nagarajan *et al.*, 2020), and solid phase extraction (Figueira *et al.*, 2017) have been used to extract antioxidant molecules or flavor-related metabolites. Most of these extraction methods include many steps, sometimes require special chemicals and equipment, or entail pre-purification procedures. As a result, many of these methods are expensive and time consuming. Similarly, different chromatographic techniques have been used to quantify metabolites. The most commonly used method for both targeted (Dumont *et al.*, 2020, Tohge *et al.*, 2020) and untargeted metabolic profiling (Capanoglu *et al.*, 2008, Treutler *et al.*, 2016) is mass spectrophotometry coupled with either liquid or gas chromatography. The nuclear magnetic resonance technique (NMR) can also be used for metabolomics (Ingallina *et al.*, 2020, Masetti *et al.*, 2020). These methods not only require very expensive equipment but also require expertise. An alternative is the use of spectrophotometric methods which can be applied to evaluate lycopene content (Migalatev, 2017), total antioxidant capacity (Martínez *et al.*, 2020), total phenolic acids and flavonoid content (Alenazi *et al.*, 2020). Although spectrophotometric methods are easy and cheap, they are not as sensitive as chromatographic methods. Moreover, quantification of individual molecules such as specific phenolic acids or sugars is not feasible.

Given the limitations of the available extraction and analysis protocols, the current work was designed to establish rapid, easy and relatively cheap extraction methods for two groups of compounds, polar and apolar metabolites. In addition, cheap, easy and sensitive chromatographic methods (HPLC and GC) were developed to detect and quantify the different types of antioxidant and flavor-related metabolites. Apolar extracts were used for analysis of carotenoids with a HPLC method modified from those described by Ishida *et al.* (2001) and Serino *et al.* (2009). Polar extracts were used for the analysis of vitamin C, vitamin E, phenolic acids, reduced and oxidized glutathione, and sugars using HPLC. The HPLC methods for these compounds were modified from those described in previous studies for vitamin C (Li and Chen, 2001a and 2001b), vitamin E (Turner and Burri, 2012; Bakre *et al.*, 2015), phenolic acids (Gómez-Alonso *et al.*, 2007), glutathione (Khan *et al.*, 2011), and sugars (Petkova *et al.*, 2013). Organic acids were also detected from polar extracts using a GC method modified from those described in previous studies (Roessner *et al.*, 2001; Namgung *et al.*, 2010). All protocols were developed using fully ripe fruits (at market stage) from a tomato inbred backcross line population as samples. This interspecific population was derived from *S. lycopersicum* by *S. pimpinellifolium* cross (Celik *et al.,* 2017) and provided sufficient variation for the metabolites of interest to test the new protocols.

Materials and Reagents

A. Plant Material

An interspecific IBL (inbred backcross line) population (BC_2F_6) derived from the cross *S. lycopersicum* cv. Tueza (recurrent parent) × *S. pimpinellifolium* (LA1589) (donor parent) was used as plant material. This population was developed in previous studies and is described more fully in the literature (Celik *et al.*, 2017; Gürbüz Çolak *et al.*, 2020a and 2020b). Tueza is a cultivated fresh market tomato line with large (150-160 g), red, slightly flattened round fruits. LA1589 is a wild tomato accession with small (0.8-1 g), red, round fruits. The IBL population and parents were grown in the

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greenhouse in Antalya, Turkey. Ten plants per genotype were grown in double rows with 140 and 30 cm between wide and narrow rows, respectively. Plants were spaced at 40 cm intervals within rows. For basal fertilization, 500 kg 15:15:15 (N:P:K) fertilizer and 50 t of composted manure were applied per ha. Drip irrigation was used with fertigation (1.4 dS m-1 EC value) at each irrigation using 1-2-1 fertilizer until first fruit set, 2-1-1 fertilizer until first fruit ripening and 1-1-2 fertilizer after first fruit ripening.

B. Major Ingredients

1. C18 column (GL Sciences, catalog number: 5020-03946)
2. NH2 column (GL Sciences, catalog number: 5020-05546)
3. RTx 5DA column (Restek, catalog number: 10523)
4. Syringe (BD, Emerald, catalog number: 307742)
5. Syringe filter (Millipore, catalog number: Z227412)
6. Polyamide membrane filter (45 µm, 47 mm) (Sartorius, catalog number: 25006-47-N)
7. Acetonitrile (VWR, catalog number: 97065)
8. Ammonium dihydrogen phosphate (Merck, catalog number: 1.01126.0500)
9. Butylated hydroxytoluene (BHT) (Sigma-Aldrich, catalog number: W218405)
10. Chloroform (VWR, catalog number: JT9175)
11. Dichloromethane (VWR, catalog number: BDH23373)
12. Ethyl acetate (VWR, catalog number: JT9282)
13. Hexane (VWR, catalog number: BDH24575)
14. Methanol (VWR, catalog number: BDH20864)
15. Methoxamine hydrochloride (Sigma-Aldrich, catalog number: M6524)
16. N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Sigma-Aldrich, catalog number: 69479)
17. Ortho-phosphoric acid (Merck, catalog number: 100573)
18. Potassium dihydrogen phosphate KH_2PO_4 (Riedel-de Haën, catalog number: 04243)
19. Pyridine (Sigma-Aldrich, catalog number: 270970)
20. Triethylamine (Merck, catalog number: 8083520500)
21. Trifluoroacetic acid (Sigma-Aldrich, catalog number: T6508)
22. 0.1 M KH_2PO_4, pH = 7.0 (see Recipes)
23. 0.05% Trifluoroacetic $acid_{(aq)}$ (see Recipes)

C. Chemicals used as control molecules

1. β-carotene (Carl Roth, catalog number: 5669.1)
2. Apigenin (Applichem, catalog number: A3641)
3. Caffeic acid (Sigma-Aldrich, catalog number: C0625)
4. Catechin (Applichem, catalog number: A4325)
5. Chlorogenic acid (Sigma-Aldrich, catalog number: 00500590)

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6. Chrysin (Fluka, catalog number: 9582)
7. Cinnamic acid (Sigma-Aldrich, catalog number: W228826)
8. Citric acid (Carl Roth, catalog number: X863.1)
9. Coumaric acid (Carl Roth, catalog number: 9906.1)
10. Cyanidine (Carl Roth, catalog number: 4545.1)
11. Epicatechin (Applichem, catalog number: A3424)
12. Epigallocatechin (Applichem, catalog number: A2010)
13. Ferulic acid (Sigma-Aldrich, catalog number: 128708)
14. Fructose (Sigma-Aldrich, catalog number: F0127)
15. Fumaric acid (Sigma-Aldrich, catalog number: 47910)
16. Gallic acid (Sigma-Aldrich, catalog number: 91215)
17. Glucose (Sigma-Aldrich, catalog number: G8270)
18. Hydroxybenzoic acid (Sigma-Aldrich, catalog number: W398608)
19. Lactic acid (Sigma-Aldrich, catalog number: L1750)
20. Lutein (Applichem, catalog number: A1283)
21. Luteolin (Applichem, catalog number: A3424)
22. Lycopene (Carl Roth, catalog number: 5670.1)
23. Malic acid (Carl Roth, catalog number: 8684.1)
24. Malvidin (Applichem, catalog number: A8720)
25. Myricetin (Carl Roth, catalog number: 6461.1)
26. Oxidized glutathione (Sigma-Aldrich, catalog number: G4376)
27. Pelargonidin (Carl Roth, catalog number: 4540.1)
28. Peonidin (R&D, catalog number: 0942)
29. Pterostilben (Sigma-Aldrich, catalog number: P1499)
30. Quercetin (Applichem, catalog number: A3415)
31. Reduced glutathione (Sigma-Aldrich, catalog number: G4251)
32. Resveratrol (Sigma-Aldrich, catalog number: R5010)
33. Salicyclic acid (Sigma-Aldrich, catalog number: W398500)
34. Shikimic acid (Carl Roth, catalog number: 7305.2)
35. Sinapic acid (Carl Roth, catalog number: 5317.1)
36. Sucrose (Sigma-Aldrich, catalog number: S5016)
37. Syringic acid (Carl Roth, catalog number: 5361.1)
38. Tartaric acid (Carl Roth, catalog number: K302.1)
39. Vanillic acid (Carl Roth, catalog number: 3685.1)
40. Vitamin C (Sigma-Aldrich, catalog number: A5960)
41. Vitamin E (Sigma-Aldrich, catalog number: T3251)
42. Zeaxanthin (Carl Roth, catalog number: 5672.2)
43. Extraction solvent 1 (see Recipes)
44. Extraction solvent 2: Chloroform: methanol: water (1:3:1, v:v:v) (see Recipes)

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Equipment

1. -80 °C freezer
2. Analytical balance (Mettler Toledo, model: AB54-S)
3. Centrifuge (Beckman Coulter, model: Allegra X-15R)
4. Centrifuge (Thermo Scientific, model: SL 16)
5. GC/FID (Shimadzu, model: GC 2010 plus) with RTx 5DA column (0.25 mm × 0.25 mm × 30 m) (Restek; catalog number: 10523)
6. HPLC (Shimadzu, model: LC20-AT; refractive index (RI) detector model: RID 10A; photodiode array (PDA) detector model: SPD M20A; fluorescence detector model: RF 20A) with C18 column (5 µm-25 × 4.6 mm) (GL Sciences, catalog number: 5020-03946) and NH2 column (5 µm-25 × 4.6 mm) GL Sciences; catalog number: 5020-05546)
7. Knife grinder (Retsch, model: GM200)
8. Lyophilizer (Christ, model: Epsilon 1-4 LSC)
9. Orbital shaker (IKA, model: KS260)
10. Vacuum evaporator (Labconco, model: Centrivap)
11. Vacuum filtration (Do-Chrom, model: FB01)

Software

1. LC Solution (Shimadzu, https://www.shimadzu.com/an/lcms/opensolution/opensol4.html)
2. GC Solution (Shimadzu, https://www.shimadzu.com/an/gc/advflowtech/sw-dl.html)

Procedure

A. Extraction of Metabolites

1. Collect 10 ripe tomato fruits (average fresh weight was 65 g), bulk and dice. Take 100 g sample and lyophilize for two days (make sure they are completely dry). Grind the dried samples with a knife grinder and obtain a fine powder.
2. Weigh 1 g of dried sample and add 5 ml of extraction solvent 1 (see Recipes) to extract the apolar metabolites. Extract the metabolites by shaking on an orbital shaker at 400 rpm at 18 °C overnight.
3. Centrifuge the samples at 3,724 × *g* at 4 °C for 20 min. Save the supernatant and do a second overnight extraction with the pellet using the same solvent.
4. Centrifuge the samples again and combine the extracts. Aliquot the extracts (1 ml) and store at -80 °C until the analysis.
5. To extract the polar metabolites, use the pellet obtained in Step A4 and follow the same steps 1-4 using extraction solvent 2 (see Recipes). Aliquot the extracts (1 ml) and store at -80 °C until

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the analysis.

6. See Note 1.

B. HPLC Analysis of Metabolites

Carotenoids

Two different methods were used for the analysis of carotenoids: method 1 for the analysis of lycopene and β-carotene; method 2 for the analysis of lutein and zeaxanthin.

Method 1:

1. Prepare standard solutions of lycopene in methanol:acetone (1:1, v:v) at 1, 5, 10, 25, 50 and 100 ppm concentrations, and β-carotene in dichloromethane at 1, 5, 10, 25, 50, 100 ppm concentrations.
2. Add 0.05% trimethylamine to ethyl acetate and acetonitrile. Filter methanol, ethyl acetate and acetonitrile through membrane filter using vacuum filtration.
3. Use methanol:ethyl acetate:acetonitrile at 50:40:10 (v:v:v) ratio as the mobile phase for isocratic elution.
4. Use RP column and set the column temperature at 30 °C. Set the flow rate as 1.5 ml/min.
5. Filter the sample and the standard solutions through syringe filter. Inject 20 µl of the sample or the standard solution. Detect lycopene and β-carotene at 469 nm.
6. See Notes 2 and 3.

A chromatogram for 100 ppm standard molecules (Figure 1), standard curves (Figure 2) and the sample chromatogram for tomato (Figure 3) are shown.

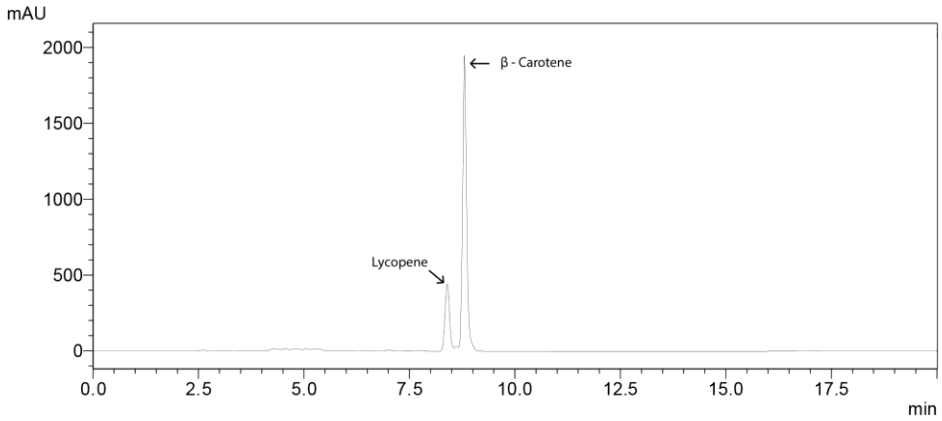

Figure 1. Chromatogram of lycopene and β-carotene standards

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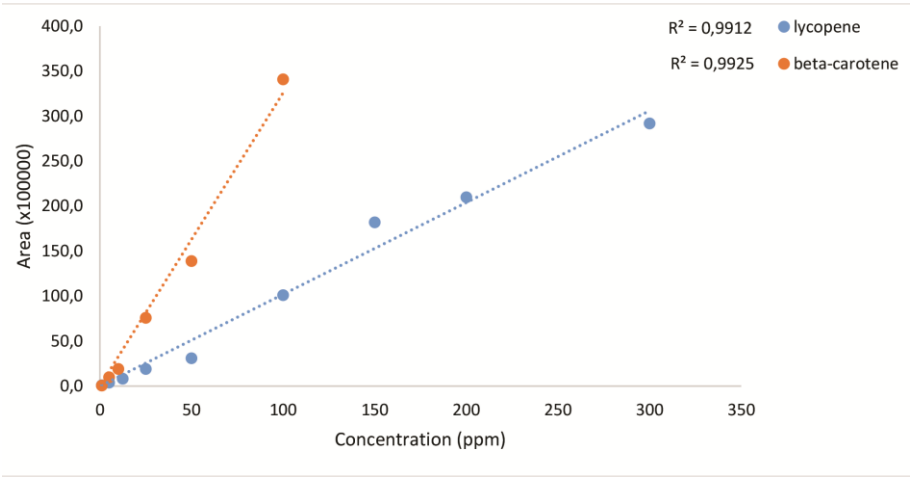

Figure 2. Standard curves of the carotenoids

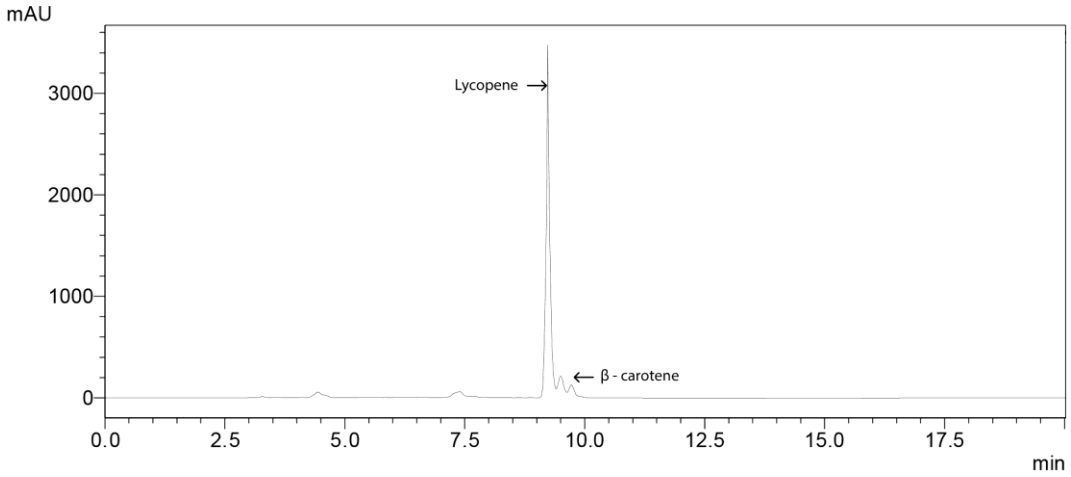

Figure 3. Sample chromatogram for tomato sample showing lycopene and β-carotene peaks

Method 2:

1. Prepare standard solutions of lutein and zeaxanthin in dichloromethane containing 0.01% BHT at 0.5, 1, 5, 12.5, 50, 100, 200 and 300 ppm concentrations
2. Add 0.05% trimethylamine to acetonitrile. Filter methanol and acetonitrile through membrane filter using vacuum filtration.
3. Use methanol:acetonitrile at 90:10 (v:v) ratio as the mobile phase for isocratic elution.
4. Use a RP column and set the column temperature at 30 °C. Set the flow rate as 1 ml/min.
5. Filter the sample and the standard solutions through syringe filter. Inject 20 µl of the sample or the standard solution. Detect lutein and zeaxanthin at 469 nm.
6. See Notes 2 and 3.

Chromatogram for 50 ppm standard molecules (Figure 4), standard curves (Figure 5) and the sample chromatogram for tomato (Figure 6) are shown.

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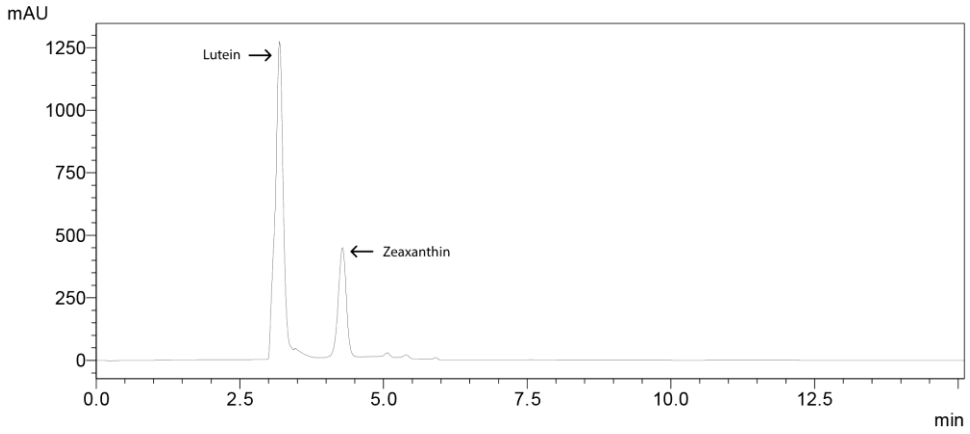

Figure 4. Chromatogram of lutein and zeaxanthin standards

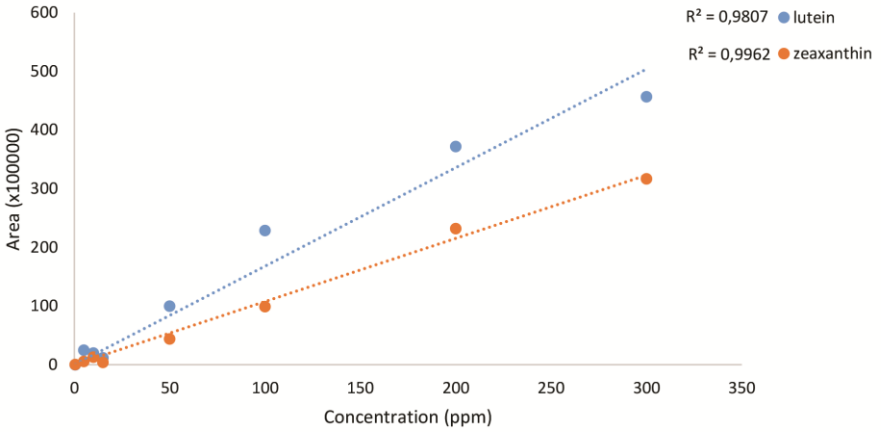

Figure 5. Standard curves of the carotenoids

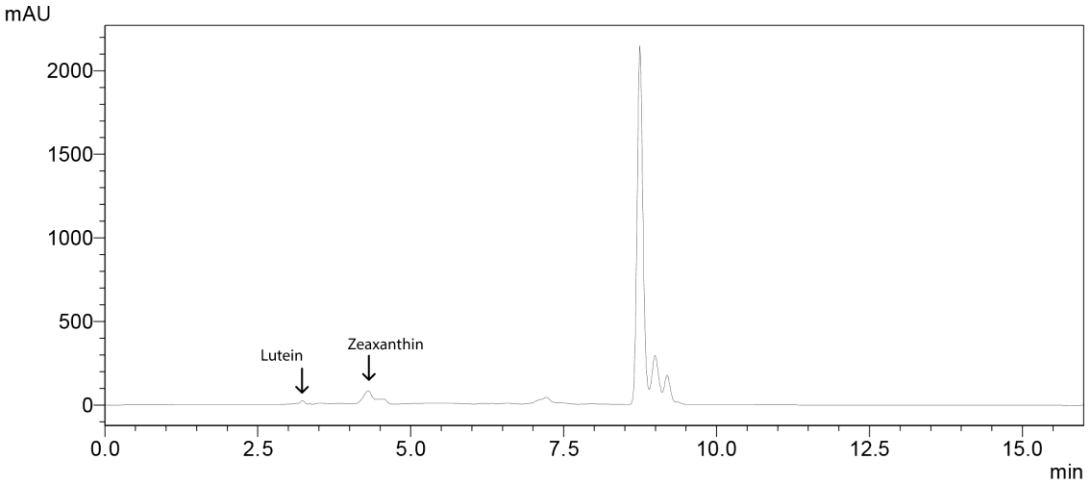

Figure 6. Sample chromatogram for tomato with lutein and zeaxanthin peaks

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Vitamin C

1. Prepare standard solutions of vitamin C in ultrapure water at 10, 50, 100, 150, 200 and 250 ppm concentrations.
2. Filter methanol and phosphate buffer through membrane filter using vacuum filtration.
3. Use methanol: KH_2PO_4 10:90 (v:v) ratio as the mobile phase for isocratic elution.
4. Use RP column and set the column temperature at 40 °C. Set the flow rate as 1 ml/min.
5. Filter the sample and the standard solutions through syringe filter. Inject 20 µl of the sample or the standard solution. Detect vitamin C at 265 nm.
6. See Note 2.

Chromatogram for 100 ppm standard molecule (Figure 7), standard curve (Figure 8) and the sample chromatogram for tomato (Figure 9) are shown.

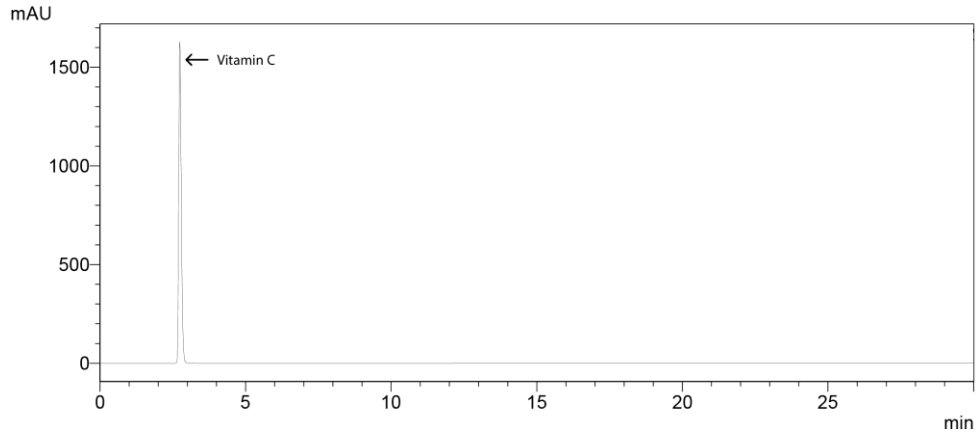

Figure 7. Chromatogram of vitamin C standard

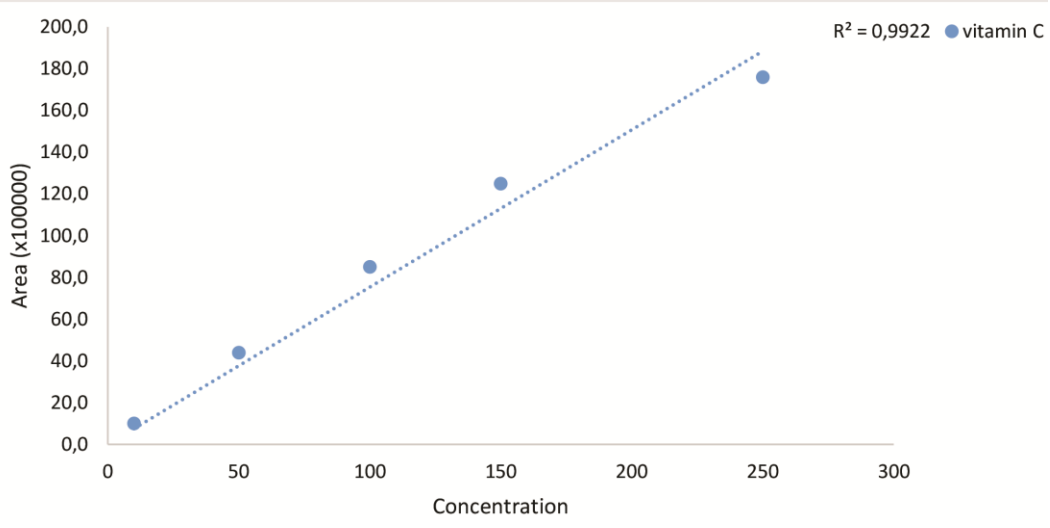

Figure 8. Standard curve for vitamin C

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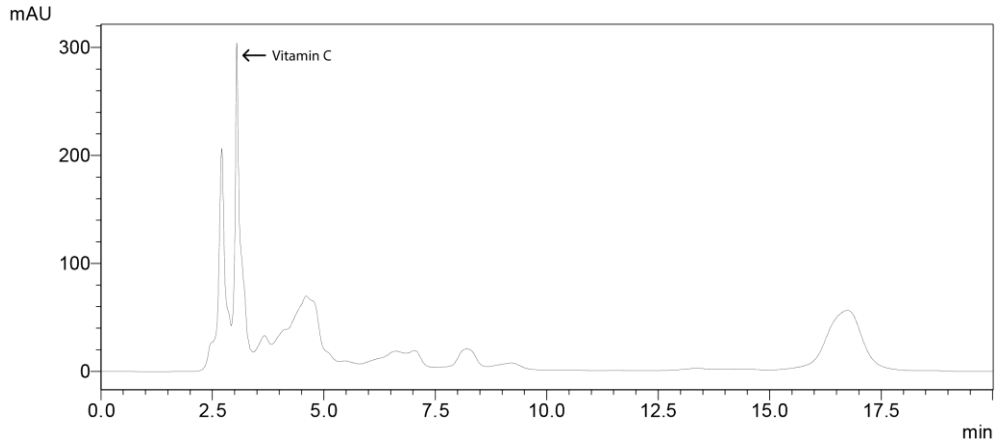

Figure 9. Sample chromatogram for tomato with vitamin C peak

Vitamin E

1. Prepare standard solutions of vitamin E in acetonitrile:methanol (80:20, v:v) at 10, 50, 100, 150, 200, 250, 300 and 500 ppm concentrations.
2. Filter methanol and acetonitrile through membrane filter using vacuum filtration.
3. Use methanol:acetonitrile 25:75 (v:v) ratio as the mobile phase for isocratic elution.
4. Use RP column and set the column temperature at 40 °C. Set the flow rate as 1.5 ml/min.
5. Filter the sample and the standard solutions through a syringe filter. Inject 20 µl of the sample or the standard solution. Detect vitamin E with the fluorescence detector at 300 nm excitation and 360 nm emission.
6. See Note 2.

Chromatogram for 500 ppm standard molecule (Figure 10), standard curve (Figure 11) and the sample chromatogram for tomato (Figure 12) are shown.

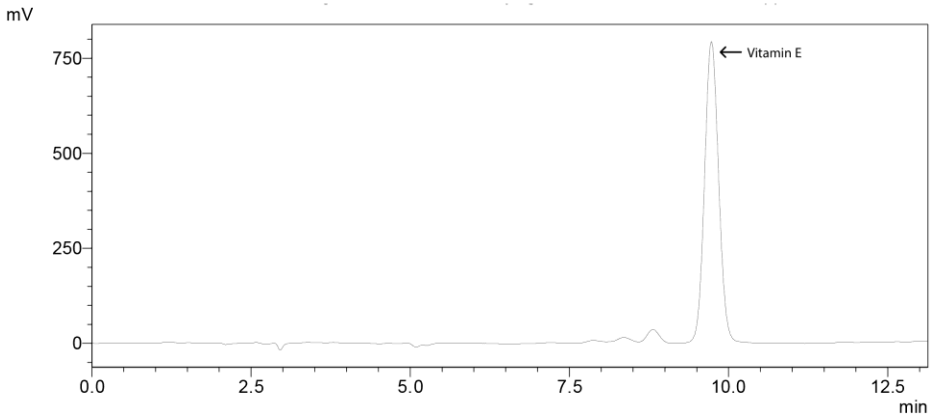

Figure 10. Chromatogram of vitamin E standard

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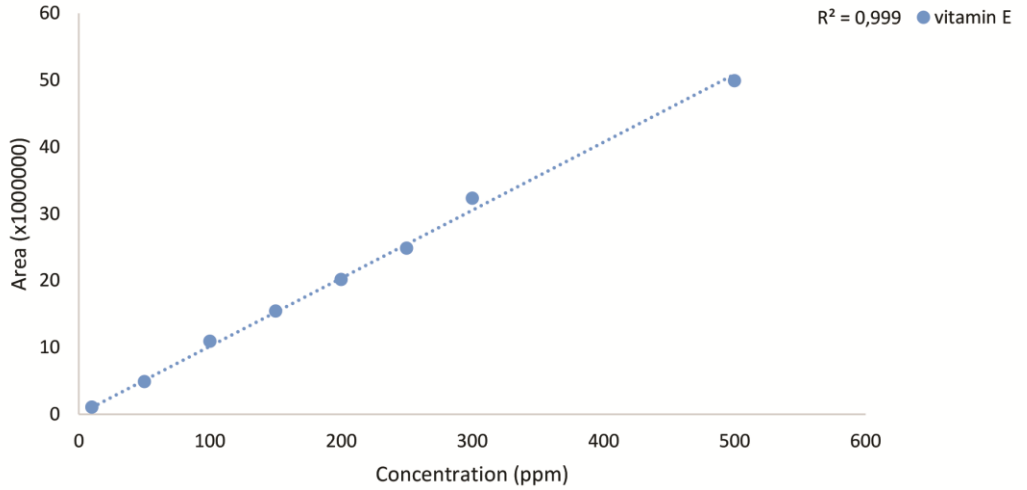

Figure 11. Standard curve of vitamin E

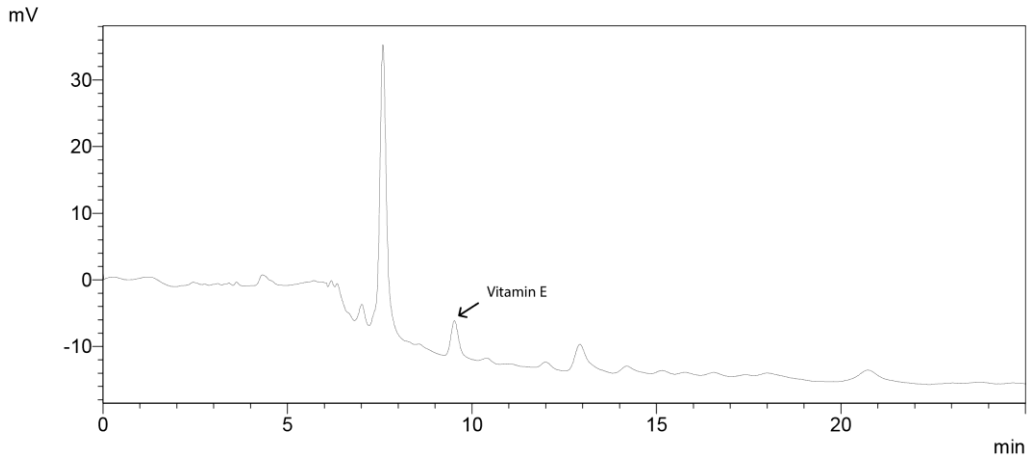

Figure 12. Sample chromatogram for tomato with vitamin E peak

Phenolic acids

1. Prepare standard solutions of phenolic acids in methanol at 1, 5, 10, 20, 30, 40, and 50 ppm concentrations.
2. Filter $NH_4H_2PO_4$, acetonitrile and H_3PO_4 through membrane filter using vacuum filtration.
3. Use $NH_4H_2PO_4$ (Mobile phase A), acetonitrile (Mobile phase B), and H_3PO_4 (Mobile phase C) as the mobile phases for gradient elution. See Table 1 for gradient elution parameters.

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Table 1. Gradient elution parameters

Time (min)	Flow rate (ml/dk)	Mobile Phase A %	Mobile Phase B %	Mobile Phase C %
0	1	100	0	0
2	1	100	0	0
5	1	92	8	0
17	1	0	14	86
22	1	0	18	82
29.5	1	0	21	70
55	1	0	33	67
70	1	0	50	50
75	1	0	50	50
78	1	20	80	0
81	1	20	80	0
86	1	100	0	0

4. Use RP column and set the column temperature at 35 °C. Set the flow rate as 1 ml/min.
5. Filter the sample and the standard solutions through a syringe filter. Inject 20 µl of the sample or the standard solution. Detect phenolic acids with PDA detector at different wavelengths. Epigallocatechin, epicatechin and chrysin are detected at 204 nm. Hydroxybenzoic acid, vanillic acid, myricetin and quercetin are detected at 254 nm. Gallic acid, catechin, syringic acid and cinnamic acid are detected at 280 nm. Chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, sinapic acid, resveratrol, apigenin and pterostilben are detected at 320 nm. Luteolin is detected at 360 nm, while cyaniding, pelargonidin and peonidin are detected at 520 nm.
6. See Note 2.

Chromatogram for 50 ppm standard molecules (Figure 13), standard curves (Figure 14) and the sample chromatogram for tomato (Figure 15) are shown.

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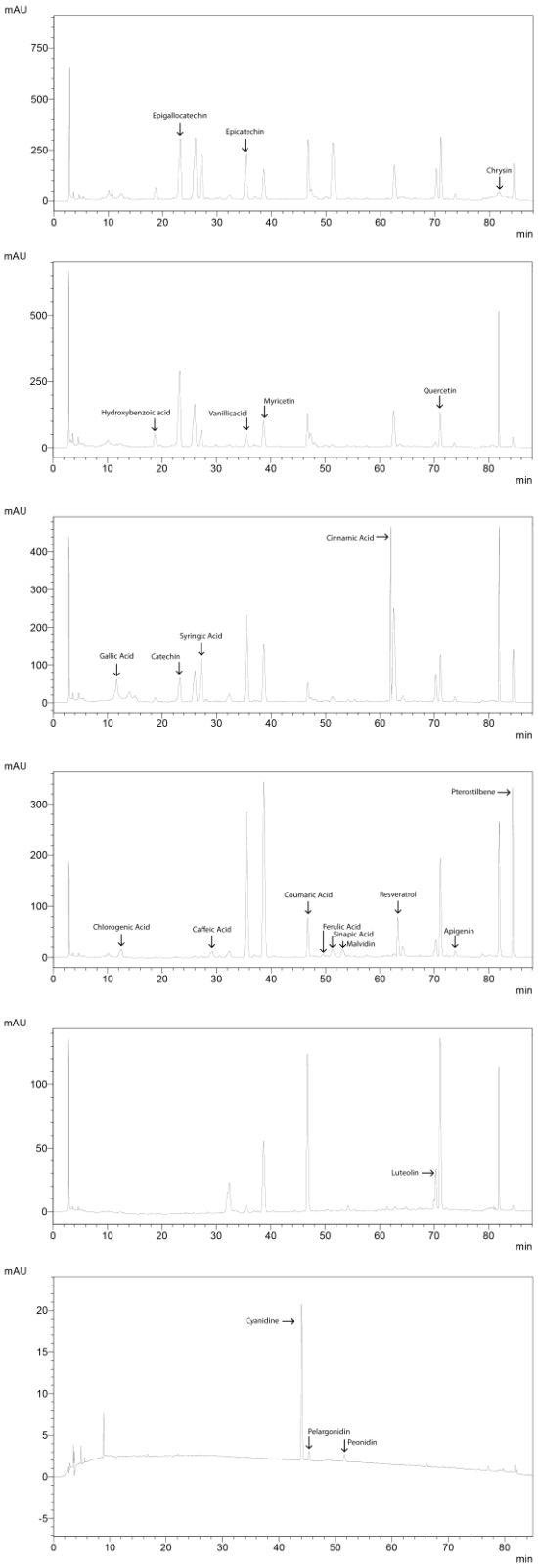

Figure 13. Chromatograms of standard molecules

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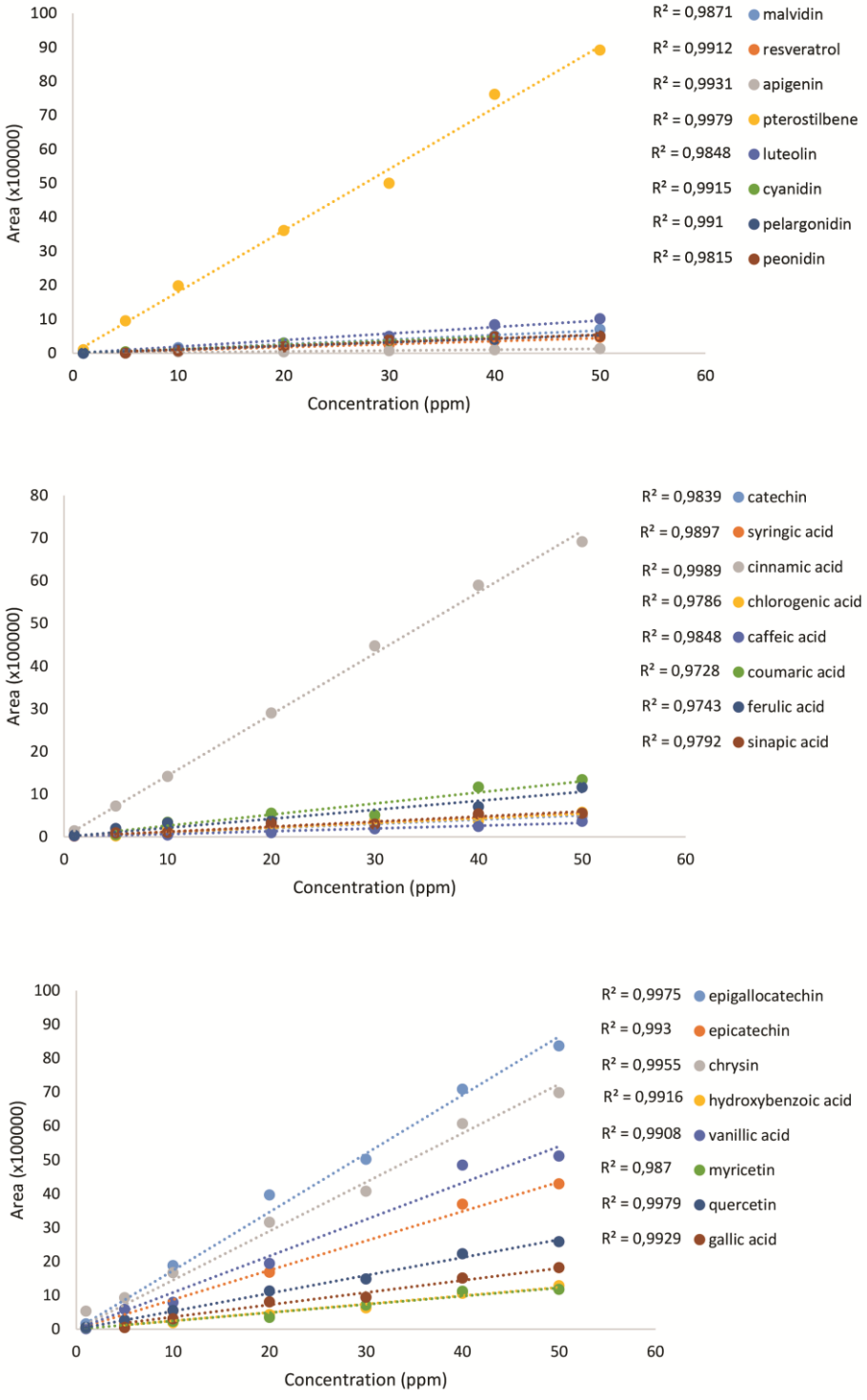

Figure 14. Standard curves of phenolic acids

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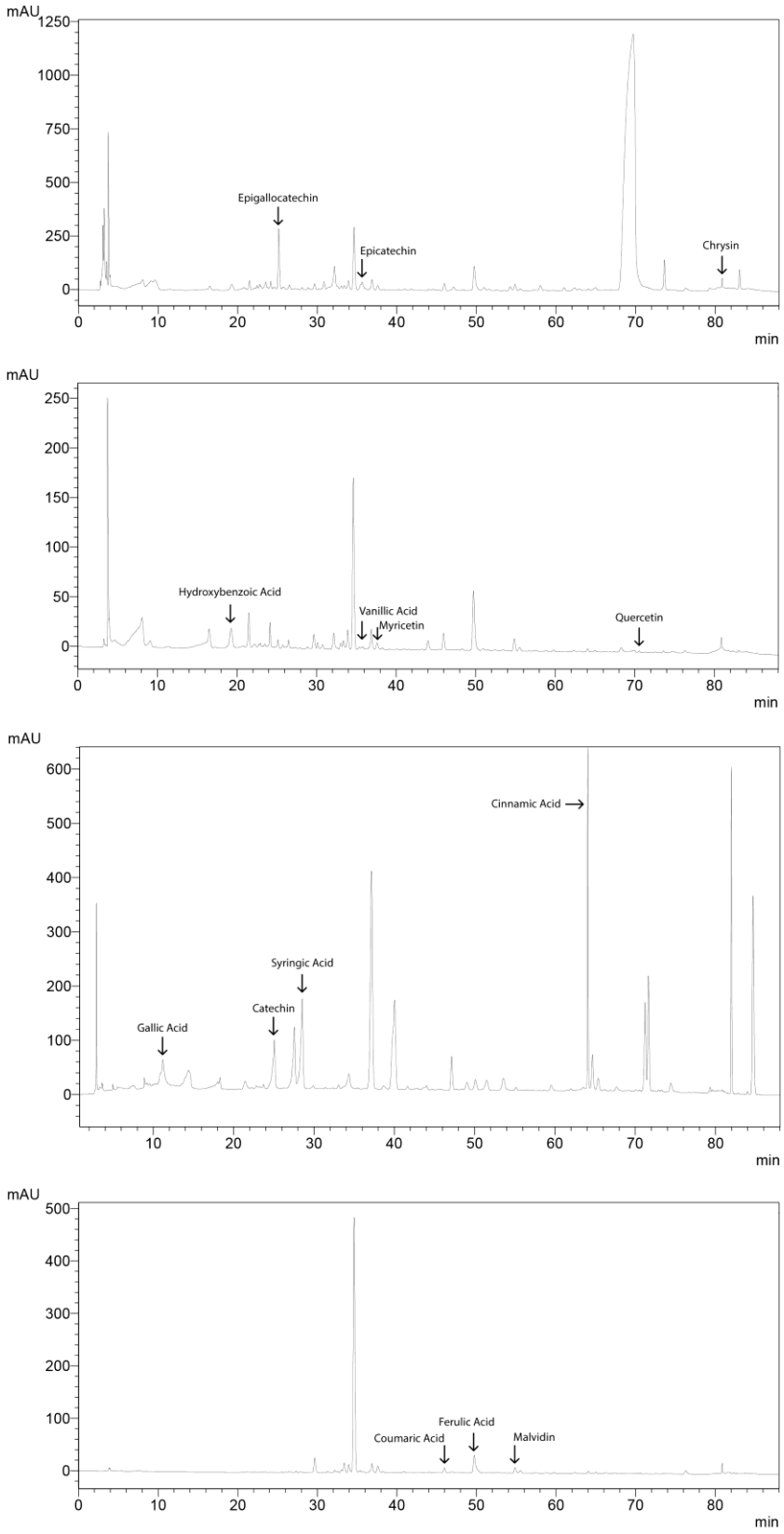

Figure 15. Sample chromatogram for tomato showing peaks for phenolic acids

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Glutathione

1. Prepare standard solutions of phenolic acids in methanol at 10, 50, 100, 150, 200, 250 ppm concentrations.
2. Filter trifluoroacetic acid and methanol through membrane filter using vacuum filtration.
3. Use trifluoroacetic acid:methanol (97:3, v:v) as the mobile phase for isocratic elution.
4. Use RP column and set the column temperature at 35 °C. Set the flow rate as 0.2 ml/min.
5. Filter the sample and the standard solutions through a syringe filter. Inject 20 µl of the sample or the standard solution. Detect oxidized and reduced form of glutathione with PDA detector at 208 nm.
6. See Note 2.

Chromatogram for 50 ppm standard molecules (Figure 16), standard curves (Figure 17) and the sample chromatogram for tomato (Figure 18) are given below.

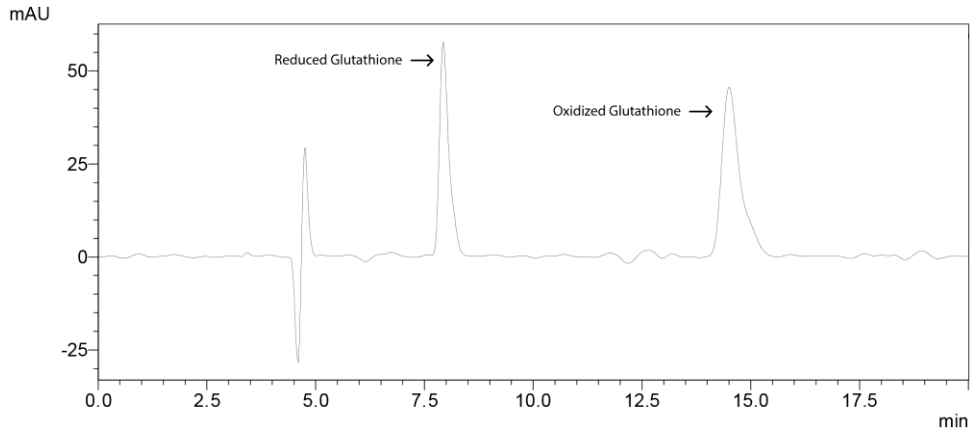

Figure 16. Chromatogram of reduced and oxidized glutathione standards

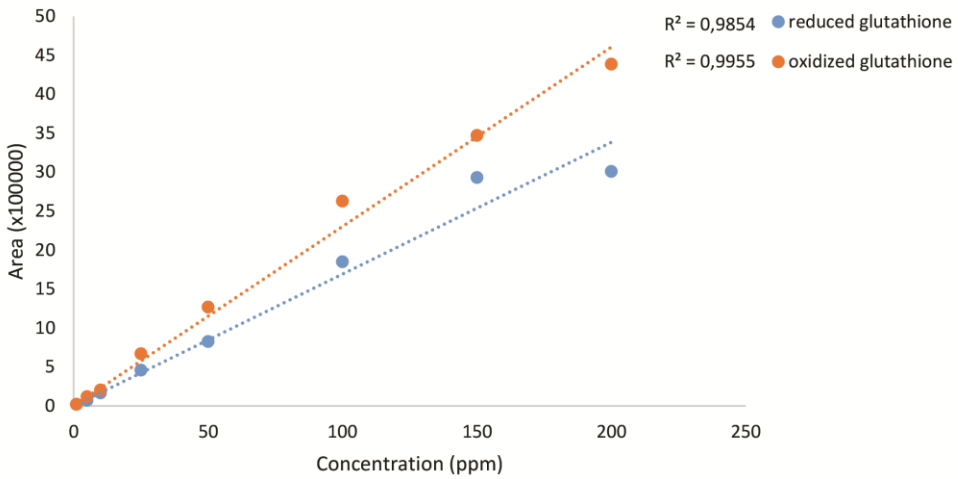

Figure 17. Standard curves of reduced and oxidized glutathione

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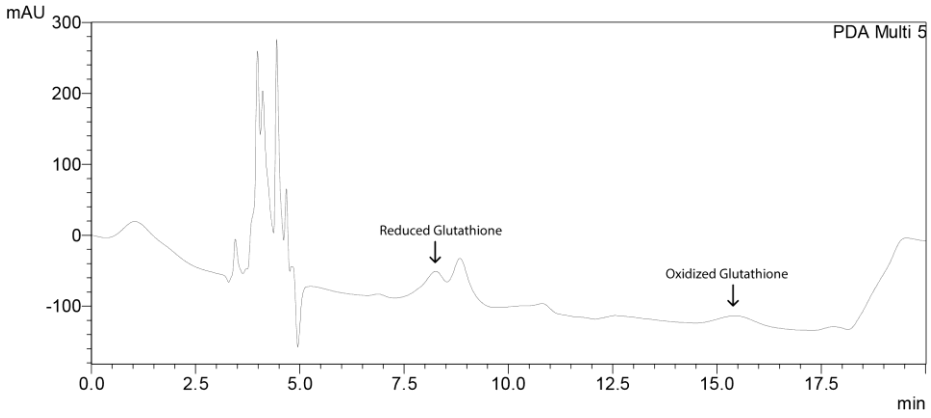

Figure 18. Sample chromatogram for tomato with glutathione results

Sugars

1. Prepare standard solutions of sugars (glucose, fructose and sucrose) in ultrapure water at 100, 150, 200, 250, 300, 500, 750, 1,000, 1,500, 2,000, 2,500 ppm concentrations.
2. Filter trifluoroacetic acid and methanol through membrane filter using vacuum filtration.
3. Use acetonitrile: water (90:10, v:v) as the mobile phase for isocratic elution.
4. Use RP column and set the column temperature at 40 °C. Set the flow rate as 1 ml/min.
5. Filter the sample and the standard solutions through a syringe filter. Inject 20 µl of the sample or the standard solution. Detect sugars with RI detector using positive mode.
6. See Notes 2 and 4.

Chromatogram for 50 ppm standard molecules (Figure 19), standard curves (Figure 20) and the sample chromatogram for tomato (Figure 21) are given below.

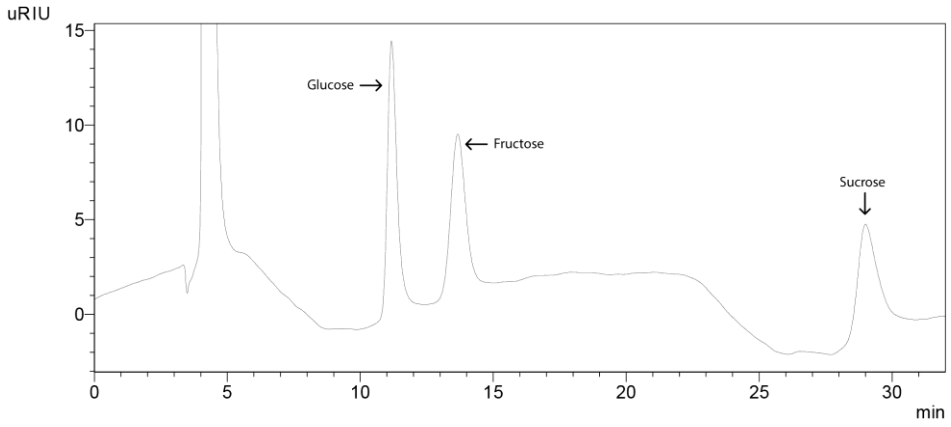

Figure 19. Chromatogram of glucose, fructose and sucrose standards

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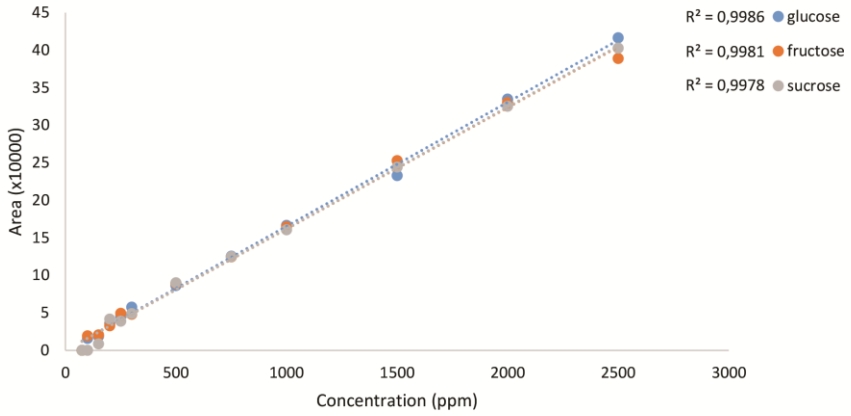

Figure 20. Standard curves of sugars

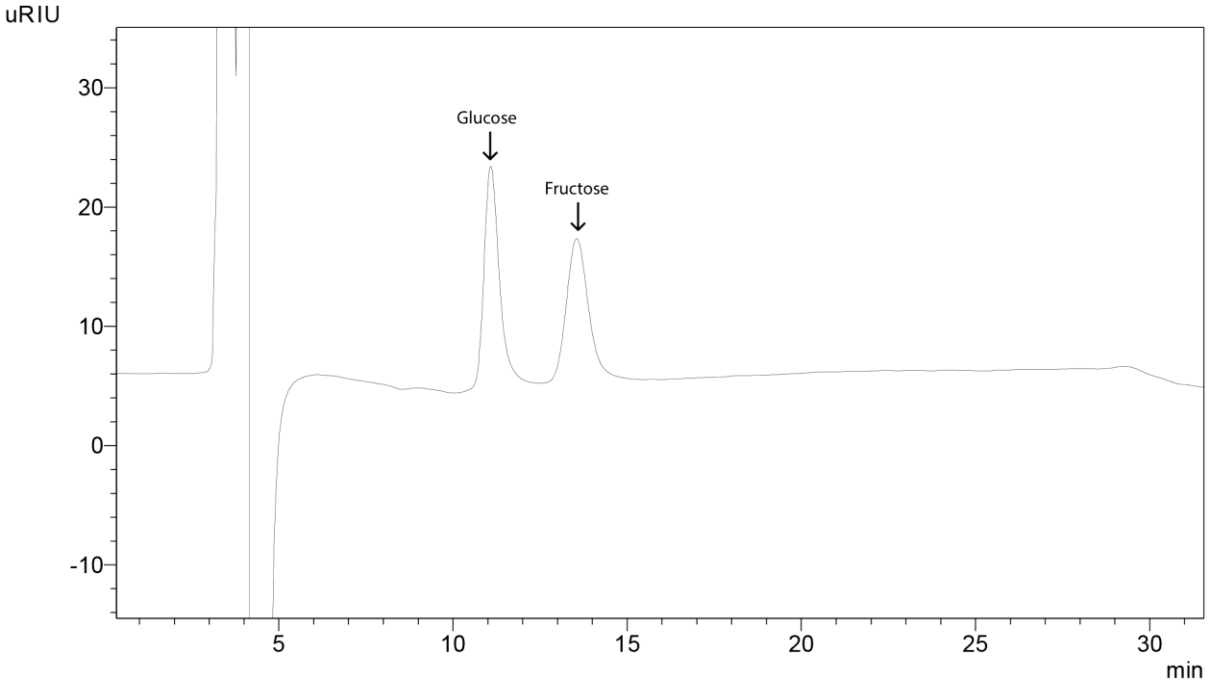

Figure 21. Sample chromatogram for tomato showing peaks for sugars. Sucrose was not detected in fully ripe fruit.

C. GC Analysis of Metabolites

Organic acids

1. Weigh standard molecules and dissolve them in methoxamine hydrochloride (40 µl, 20 mg/ml in pyridine) at 0.25, 0.5, 1, 2.5, 5, 7.5, 10 mg/ml concentrations.
2. Take 100 µl of the sample. Evaporate the organic solvent of the sample using a vacuum evaporator at 30 °C until completely dry.
3. Dissolve the tomato sample in methoxamine hydrochloride (40 µl, 20 mg/ml in pyridine) in an ultrasonic bath for 5 min.
4. Derivatize both the samples and standard molecules at 37 °C for 90 min.

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5. Perform the second derivatization with MSTFA. Add 60 µl MSTFA to the samples and the standard molecules, and incubate at 37 °C for 30 min.
6. Centrifuge the samples at 25,830 × *g*, 5 min.
7. Inject the supernatant into GC-FID.
8. Analyze the organic acids on an Rtx 5DA column with a thermogradient program. Set the column temperature from 100 °C (1 min hold) to 150 °C at a rate of 5 °C min^{-1}, from 150 °C (1 min hold) to 280 °C at a rate of 5 °C min^{-1}, 2 min hold at the final temperature.
9. Hold the injection port temperature at 250 °C, and detector temperature at 300 °C. Use nitrogen as the carrier gas, and adjust the split ratio 1/25.
10. See Notes 5 and 6.

Chromatogram for 1 mg/ml standard molecules (Figure 22), standard curves (Figure 23) and the sample chromatogram for tomato (Figure 24) are given below.

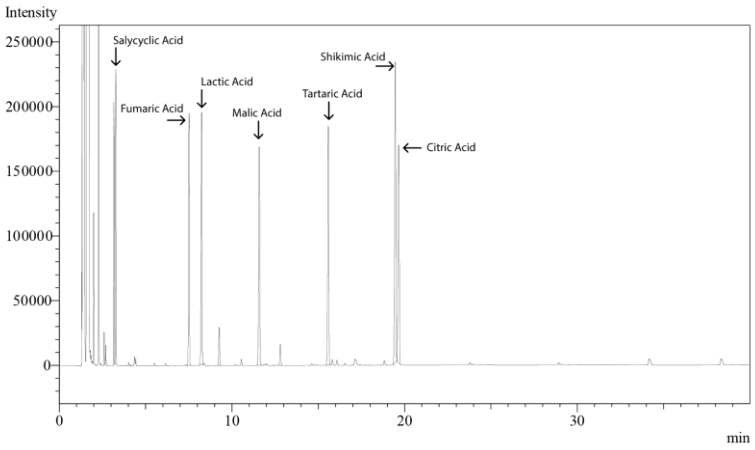

Figure 22. Chromatogram of organic acid standards

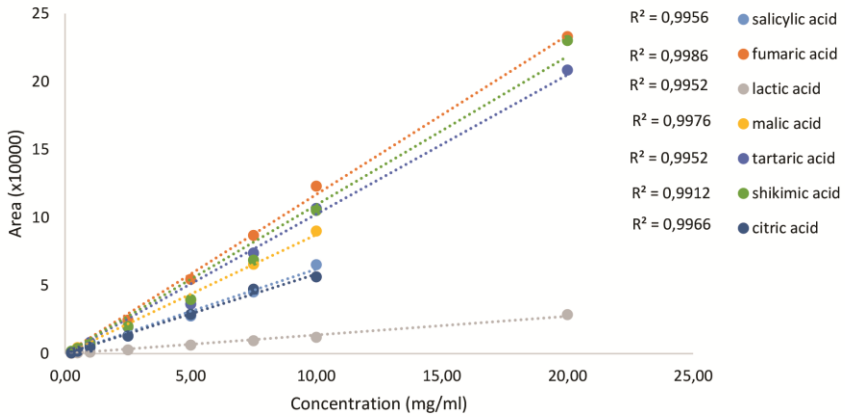

Figure 23. Standard curves of organic acids

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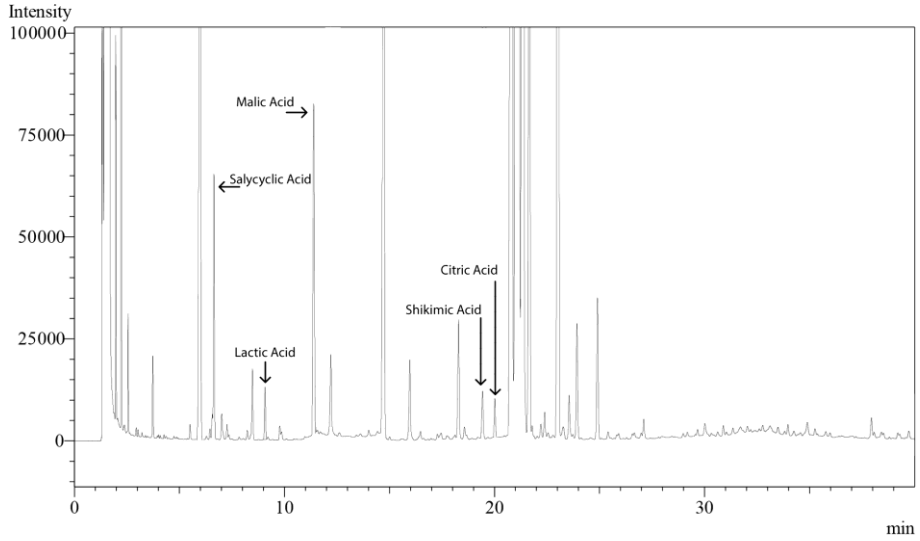

Figure 24. Sample chromatogram for tomato showing peaks for organic acids

Data analysis

Experiments were repeated and data recorded for three replicates for control molecules. Draw standard curves using LC solution for HPLC data and GC Solution for GC data. These software programs calculate the average value of the triplicate analysis and plot the curves based on the area and concentrations automatically. Also, the software programs calculate metabolite concentrations in the samples automatically using the standard curves. Average metabolite contents of the parental lines and population are shown in Table 2.

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Table 2. Mean metabolite content measured in the IBL population and parents

Metabolites	*S. lycopersicum*	*S. pimpinellifolium*	Population
Carotenoids			
Lycopene	16141.58	26733.95	16919.46
β-carotene	56.62	36.06	45.98
Lutein	3.25	5.06	3.83
Zeaxanthin	3.26	2.78	3.60
Vitamins			
Vitamin C	19.11	20.17	17.84
Vitamin E	3.61	20.28	20.40
Phenolic acids			
Epigallocatechin	1.92	2.19	2.61
Epicatechin	0.24	5.89	2.45
Chrysin	0.42	2.07	80.12
Hydroxybenzoic acid	6.60	1.37	46.80
Vanillic acid	12.8	2.05	61.12
Myricetin	10.00	40.00	3.06
Quercetin	1.88	1.67	1.025
Gallic acid	31.46	1.06	5.07
Catechin	0.37	0.59	26.53
Syringic acid	3.96	20.40	89.46
Cinnamic acid	0.06	0.11	0.98
Chlorogenic acid	19.20	0.83	0.53
Caffeic acid	9.54	3.32	3.99
Coumaric acid	0.24	2.13	1.87
Ferulic acid	2.08	31.00	3.29
Sinapic acid	1.47	1.29	1.60
Malvidin	0.76	0.75	3.48
Resveratrol	-	-	-
Apigenin	2.18	1.24	13.04
Pterostilbene	-	-	-
Luteolin	0.62	0.20	0.72
Cyanidin	-	-	-
Pelargonidin	-	-	-
Peonidin	-		
Glutathione			
Reduced glutathione	17.75	10.79	52.17
Oxidized glutathione	5.18	0.09	56.14
Sugars			
Glucose	8738.04	4153.29	6596.45

Fructose	8401.38	3967.70	5839.52
Sucrose	-	-	-
Organic acids			
Salicylic acid	0.01	0.02	0.03
Fumaric acid	-	-	-
Lactic acid	0.31	0.04	0.18
Malic acid	6.19	0.86	3.58
Tartaric acid	-	-	-
Shikimic acid	0.94	0.00	1.17
Citric acid	10.40	8.51	7.59

* Quantities are given as mg 100 g^{-1} DW.

** Resveratrol, pterostilbene, cyanidin, pelargonidin, peonidin, sucrose, fumaric acid and tartaric acid were not detected in the tomato samples.

Notes

1. It is better to do extraction in the dark and at low temperatures (+4 °C) since some metabolites are affected by light and high temperatures.
2. It is better to centrifuge the sample (25,830 × *g*, 1-2 min) once more before syringe filtration to extend column life.
3. A RP C30 column can provide better resolution but is more expensive than a RP C18 column.
4. It is better to wait for conditioning of the RI detector before analysis. Sometimes it is better to leave the detector overnight with mobile phase at low flow rate (*e.g.*, 0.1 ml/min-0.5 ml/min).
5. Helium can be used instead of nitrogen as the carrier gas.
6. Freshly prepare methoxamine hydrochloride in pyridine.
7. Use only ultrapure water for extraction and analysis.

Recipes

1. Extraction solvent 1
   a. Dichloromethane: hexane (1:1, v:v) containing 0.01% BHT to avoid oxidation reactions while extracting the metabolites
   b. Measure 50 ml of dichloromethane
   c. Add 0.01 g BHT into the dichloromethane and mix until BHT dissolves
   d. Add 50 ml of hexane and shake well
2. Extraction solvent 2: Chloroform: methanol: water (1:3:1, v:v:v)
   a. Measure 20 ml of chloroform
   b. Add 60 ml of methanol and mix well
   c. Place the solution on a shaker and add water slowly into the solution to avoid phase separation

3. 0.1 M KH_2PO_4, pH = 7.0
   a. Weigh 13.609 g KH_2PO_4 in a beaker
   b. Add 800 ml of water and mix until KH_2PO_4 dissolves
   c. Bring the pH to 7.0
   d. Make up volume to 1,000 ml with water
4. 0.05% Trifluoroacetic $acid_{(aq)}$
   a. Measure 950 ml of water into a volumetric flask
   b. Add 0.5 ml of trifluoroacetic acid and bring the volume to 1,000 ml with water

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Competing interests

The authors declare no financial and non-financial competing interests.

Ethics

No human and/or animal subjects were used in these protocols.

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