Development and Validation of an HPLC-UV Method for Determination of Eight Phenolic Compounds in Date Palms

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Development and Validation of an HPLC-UV Method for Determination of Eight Phenolic Compounds in Date Palms

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A simple, precise, accurate, and selective method was developed and validated for determination of eight phenolic compounds (gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) in date palms. Separation was achieved on an RP C18 column using the mobile phase methanol–water with 2% acetic acid (18 + 82, v/v). This method was validated according to the requirements for new methods, which include accuracy, precision, selectivity, robustness, LOD, LOQ, linearity, and range. The method demonstrated good linearity over the range 1–1000 ppm of gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffeic acid, and syringic acid with r^2 greater than 0.99, and in the range of 3–1000 ppm for p–coumaric acid, ferulic acid, and sinapic acid with r^2 greater than 0.99. The recovery of the eight phenolic compounds ranged from 97.1 to 102.2%. The method is selective because adjacent peaks of phenolic compounds were well separated with good resolution. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators proved that the method is robust and rugged.

Interest in phytochemical content and antioxidant activity of date palms has increased greatly in recent years. The majority of antioxidant activity in date palm fruits originates from phenolic compounds (1). Phenolic compounds are secondary metabolites of the plant that play important roles in disease resistance and protection against pests (2, 3). Phenolic compounds are a complex and important group of naturally occurring products (4). Many phenolic compounds are present in date palm fruits, including, among others, gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid (5–9). Figure 1 shows the structures of these phenolic compounds. The main characteristic of phenolic compounds is their ability to trap free radicals. Phenolic compounds scavenge free radicals such as peroxide, hydroperoxide, or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (10). In this respect, determination of the amount of phenolic compounds in date palms is important. Therefore, an accurate, precise, and selective method is required to determine the concentrations of these phenolic compounds in date palms.

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Additionally, the method should be sensitive with low LOD and LOQ so that low concentrations of these phenolic compounds can be determined, as their concentrations in date palms depend greatly on the maturity and harvesting season. The objectives of this work, therefore, were to develop and validate a sensitive, selective, precise, accurate, robust, rugged, and a linear (with wide dynamic range) method for simultaneous determination of eight phenolic compounds that are present in date palms. These phenolic compounds were selected as they are present in many types of date palms (5–9). RP-HPLC with a UV detector and isocratic elution was used for analysis of the phenolic compounds. The method is simple because this type of HPLC is available in most analytical laboratories. Validation of the method was conducted in accordance with requirements of new methods, which include linearity and range, accuracy, precision, selectivity, ruggedness, robustness, LOD, and LOQ. In the scientific literature, many methods have been used for determination of one or more of these compounds using HPLC (11–18). However, a method dealing with simultaneous analysis of the eight phenolic compounds described in this work was not reported so far.

Experimental

Instrumentation and Reagents

An HPLC system (Merck Hitachi LaChrome Elite; Sigma-Aldrich, Tokyo, Japan) with an L–2130 pump, L-2200 autosampler, L-2300 column oven, L-2490 UV detector, and Ezochrom Elite software was used. The C18 column (250 × 4.6 mm id, 5 μm) was from Waters Corp. (Milford, MA). Methanol HPLC grade was from J.T. Baker (Phillipsburg, NJ). Acetic acid and the phenolic compounds (gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid, all HPLC grade) were purchased from Merck (Darmstadt, Germany).

HPLC

UV detection was used at 280 nm, and isocratic elution was used at a flow rate of 1.0 mL/min. The injection volume was 20 μL.

Preparation of the Mobile Phase and Standard Solutions

The HPLC mobile phase was prepared by mixing 180 mL methanol with 820 mL water for HPLC (Sigma-Aldrich) and adding 2 mL acetic acid. Stock standard solutions of the phenolic compounds with a concentration of 1000 ppm were

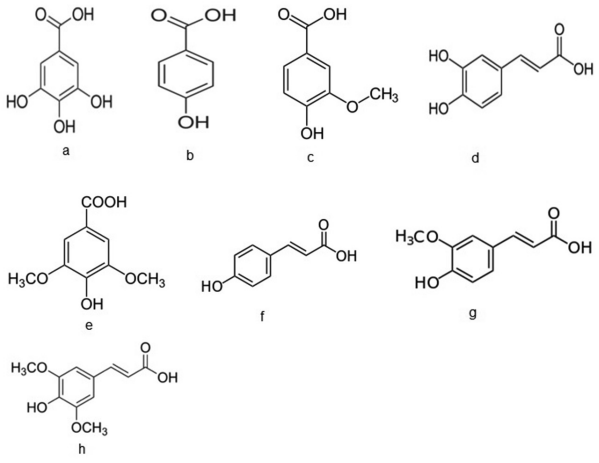

Figure 1. Structures of the phenolic acids analyzed in this study: (a) gallic acid, (b) *p*-hydroxybenzoic acid, (c) vanillic acid, (d) caffeic acid, (e) syringic acid, (f) *p*-coumaric acid, (g) ferulic acid, and (h) sinapic acid.

prepared by dissolving 100 mg of each phenolic compound in 100 mL methanol. Six solutions of the phenolic compounds with concentrations of 1, 3, 5, 100, 300, 500, and 800 ppm were prepared from the stock standard solution by dilution using mobile phase as the diluent. These solutions were used for linearity and range studies of the method. For the recovery study, three solutions of these phenolic compounds spiked in blank (distilled water) at three concentrations (5.0, 100.0, and 1000.0 ppm) were prepared. These solutions used for the recovery study were also used for the precision study.

To determine LOD and LOQ of the phenolic compounds using this method, solutions with low concentrations that are expected to produce a response of 3–20 times baseline noise were prepared. LOD of a phenolic compound was selected as the concentration that gives an S/N ratio of 3–10, while LOQ was selected as the concentration that gives an S/N ratio of 10–20.

Palm Date Material

Fruits of two date palm cultivars were investigated in this study: Rotab and Ahmar Balade. Date samples (1.0 kg each) were collected in September 2011 from Jericho/Palestine and stored in a freezer at −15°C until analysis.

Preparation of Date Palm Samples for HPLC Analysis

About 100 g of the edible part of date palm fruits was crushed and blended for 3 min. The date palm was then extracted with 300 mL methanol–water (4 + 1, v/v) at room temperature for 4 h. The extracts were then filtered using suction filtration, and the supernatant was concentrated under reduced pressure at 40°C for 2 h using a rotary evaporator to obtain the date palm crude extract. The crude extract was dissolved in the mobile phase and analyzed by HPLC using the proposed method.

Results and Discussion

Method Development

Preliminary studies involved trying octylsilyl (C8) and C18 RP columns and testing several mobile phase compositions for the separation of the eight phenolic compounds with good chromatographic parameters (e.g., minimal peak tailing, good symmetry, and good resolution between adjacent peaks). A C18 column (5 µm particle size, 250 × 4.6 mm id) as the stationary phase with the mobile phase methanol–water with 2% acetic acid (18 + 82, v/v) at a flow rate of 1.0 mL/min afforded the best separation of the eight phenolic compounds with good resolution. Acetic acid in the mobile phase gave sharper peaks for the phenolic compounds, while mobile phase without acetic acid gave very broad peaks (low theoretical plate numbers) with very poor resolution. Figure 2 shows chromatograms of the eight phenolic compounds separated using the method developed in this study, while Figures 3 and 4 show chromatograms for the phenolic compounds detected in two types of date palm extracts.

Method Validation

After method development, validation of the method for determination of the eight phenolic compounds was performed in accordance with requirements for new methods, which include accuracy, precision, selectivity, robustness, ruggedness, linearity and range, LOD, and LOQ.

Linearity and range.—Linearity is the ability of a method to elicit test results that are directly proportional to analyte concentration within a given range. Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and

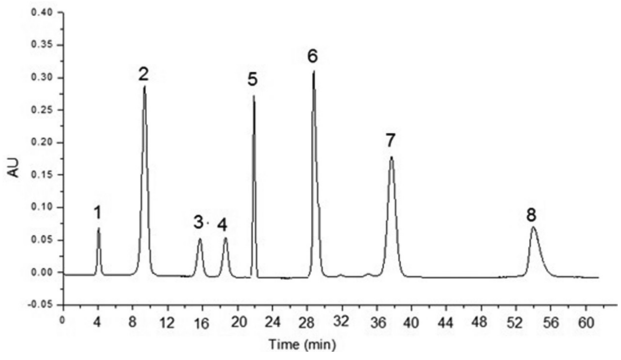

Figure 2. Chromatogram of the eight phenolic acids separated using the developed method. Mobile phase: methanol–water with 2% of acetic acid (18 + 82, v/v), flow rate 1.0 mL/min, and injection volume 20 µL. Column: C18, 5 µm, 25 cm length × 4.6 mm id. UV detection: 280 nm. Analytes separated: (1) gallic acid, (2) *p*-hydroxybenzoic acid, (3) vanillic acid, (4) caffeic acid, (5) syringic acid, (6) *p*-coumaric acid, (7) ferulic acid, and (8) sinapic acid.

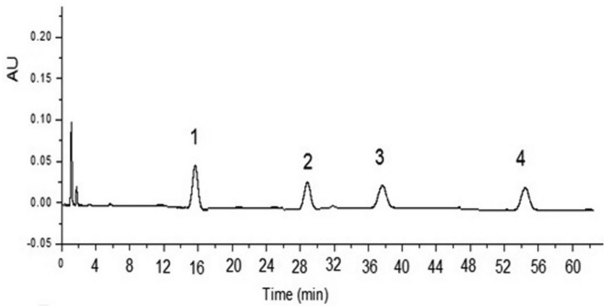

Figure 3. Chromatogram of phenolic compounds detected in Rotab date palm sample obtained from the West Bank; conditions are the same as in Figure 2: (1) vanillic acid, (2) *p*-coumaric acid, (3) ferulic acid, and (4) sinapic acid.

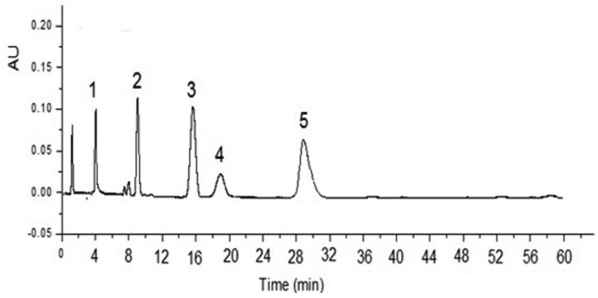

Figure 4. Chromatogram of phenolic compounds detected in Ahmar Balade date palm sample obtained from the West Bank; conditions are the same as in Figure 2: (1) gallic acid, (2) *p*-hydroxybenzoic acid, (3) vanillic acid, (4) caffeic acid, and (5) *p*-coumaric acid.

linearity using the method as written. A minimum of five concentration levels, along with certain minimum specified ranges, are required. Acceptance criteria for linearity are that the correlation coefficient (r^2) is not less than 0.99 for the least squares regression (19).

To evaluate linearity of the method, different calibration standards of the phenolic compounds were analyzed by HPLC-UV, and the responses were recorded. A plot of the peak areas of the each compound versus concentration (in ppm) was found to be linear in the range of 1–1000 ppm of gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffeic acid, and syringic acid with r^2 greater than 0.99, and in the range of 3–1000 ppm for *p*-coumaric acid, ferulic acid, and sinapic acid with r^2 greater than 0.99. These results demonstrated linearity of this method for determination of the eight phenolic compounds over a wide dynamic range.

Accuracy (recovery).—The accuracy of an analytical method measures the agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and value found (i.e., accuracy is a measure of exactness of an analytical method). Accuracy is measured as the percentage of analyte recovered after spiking into a blank sample. To document accuracy, a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations, three replicates for each) are analyzed. Accuracy evaluation is performed at three concentrations covering the range of the method. At each level studied, replicate samples are analyzed. The RSD of the replicates provides the analysis variation and gives an indication of the precision of the test method. Moreover, the mean of the replicates, expressed as percentage of spike level, indicates the accuracy of the test method. The mean recovery of the assay should be within 100 ± 5.0% at each concentration over the studied range (20–22).

For determination of the percentage recovery of the phenolic compounds, these compounds were spiked in mobile phase at three concentrations (5.0, 100.0, and 1000.0 ppm) followed by HPLC-UV analysis. The average recovery for each level was calculated as the proportion of the area of the peak of each compound resulting from the spiked solution to the area of the peak resulting from a standard solution. The average recovery and the RSD for each level was calculated. Results showed that the current method has good recovery (from 98.4–101.1, 99.0–102.2, 99.6–101.5, 98.8–102.0, 98.1–101.5, 97.1–100.2, 97.1–101.5, and 97.1–101.5% for gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid, respectively) at the three concentration levels studied (5.0, 100.0, and 1000.0 ppm) with RSD lower than 1.0% (Table 1).

Precision.—Precision is the measure of the degree of repeatability of an analytical method under normal operation and is usually expressed as the RSD for a statistically significant number of samples. There are two types of precision: repeatability and intermediate precision (ruggedness).

Repeatability is the closeness of agreement between mutually independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time. It is determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each). RSD for replicate injections should not be greater than 1.5% (23).

Repeatability of the current method for determination of the eight phenolic compounds was evaluated by calculating the RSD of the peak areas of six replicate injections for three standard solutions with three concentrations (5.0, 100.0, and 1000.0 ppm), which was found to be less than 1.0% (data not shown). These results showed that the current method for determination of the phenolic compounds is repeatable.

Intermediate precision (also called ruggedness) of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions. The method of analysis should not be prone to day-to-day or place-to-place variations. The test conditions consist of different laboratories, analysts, instruments, days, etc. Ruggedness is a measure of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. The RSD of results obtained under various normal test conditions, e.g., different laboratories or different analysts, should not be more than 2.0% (23). Intermediate precision of the current method was evaluated by calculating the recovery of the phenolic compounds at three concentration levels (5.0, 100.0, and 1000.0 ppm) by another analyst on a different day. Results of this study showed that the recovery obtained by the second analyst was comparable to that obtained by the first analyst and ranged from 98.3 to 101.8% with an RSD of less than 1.0% (data not shown), indicating that this method is rugged.

Selectivity.—Selectivity is the ability to assess the analyte unequivocally in the presence of other analytes and other components that may be expected to be present in the sample matrix (24). It is a measure of the degree of interferences from such components to ensure that a response is due to a single component only. Selectivity of the current method was demonstrated by good separation of the adjacent peaks of phenolic compounds with good resolution. Resolution between gallic acid and *p*-hydroxybenzoic acid was 3.2, between *p*-hydroxybenzoic acid and vanilic acid 6.8, between vanilic acid and caffeic acid 1.8, between caffeic acid and syringic acid 2.8, between syringic acid and *p*-coumaric acid 3.6, between *p*-coumaric acid and ferulic acid 4.4, and between ferulic acid and sinapic acid 5.1. (Figures 2 and 3).

Robustness.—Robustness measures how a method stands up to slight variations in the operating parameters of the method like flow rate, wavelength, and percentage of mobile phase composition. Robustness of the current method was

Table 1. Recovery of the eight phenolic compounds at three concentration levels

	Recovery, %		
	Concentration, ppm		
Phenolic compound	5.0	100.0	1000.0
Gallic acid	99.6, 98.7, 99.0	98.4, 99.9, 100.1	101.1, 100.1, 99.6
Mean	99.1	99.5	100.3
SD	0.46	0.93	0.76
RSD, %	0.46	0.93	0.76
p-Hydroxybenzoic acid	99.0, 99.9, 100.7	100.9, 100.7, 99.8	101.9, 102.2, 100.8
Mean	99.9	100.5	101.6
SD	0.85	0.59	0.73
RSD, %	0.85	0.58	0.73
Vanilic acid	100.8, 99.9, 100.3	99.7, 99.8, 99.6	101.5, 101.0, 100.8
Mean	100.3	99.7	101.1
SD	0.45	0.10	0.36
RSD, %	0.10	0.10	0.36
Caffeic acid	101.5, 100.8, 99.9	99.7, 98.8, 99.8	102.0, 101.2, 101.8
Mean	100.7	99.4	101.7
SD	0.80	0.55	0.42
RSD, %	0.80	0.55	0.41
Syringic acid	101.5, 100.8, 99.6	98.4, 98.7, 99.9	99.5, 98.1, 99.2
Mean	100.6	99.0	98.9
SD	0.96	0.79	0.74
RSD, %	0.95	0.80	0.75
p-Coumaric acid	97.1, 97.8, 98.1	98.1, 97.7, 98.7	100.0, 100.2, 99.6
Mean	97.7	98.2	99.9
SD	0.51	0.50	0.31
RSD, %	0.52	0.51	0.31
Ferulic acid	99.5, 99.7, 99.4	97.7, 97.2, 97.1	101.5, 101.0, 100.8
Mean	99.5	97.3	101.1
SD	0.15	0.32	0.36
RSD, %	0.15	0.33	0.36
Sinapic acid	99.9, 98.8, 99.0	97.7, 97.6, 98.1	101.4, 101.1, 100.1
Mean	99.2	97.8	100.9
SD	0.58	0.26	0.68
RSD, %	0.59	0.27	0.67

investigated by measuring the recovery percentage of the phenolic compounds at three concentration levels using the same developed method in this study but deliberately changing one chromatographic condition each time. The chromatographic conditions that were changed are flow rate (0.8 and 1.2 mL/min versus the original 1.0 mL/min), volume fraction of methanol (17 and 19% versus the original 18%), and wavelength (278 and 282 nm versus the original 280 nm). Results showed that separation is not affected by slightly changing the chromatographic conditions; resolution between adjacent peaks remained good. Additionally, the recovery of the phenolic compounds at the three concentration levels was not affected significantly by changing the chromatographic conditions (flow rate, percentage of acetonitrile, and wavelength; data not shown).

Table 2. LOD and LOQ of the eight phenolic compounds using the developed method

Phenolic compound	LOD, ppm	LOQ, ppm
Gallic acid	0.2	0.5
p-Hydroxybenzoic acid	0.3	0.6
Vanilic acid	0.3	0.6
Caffeic acid	0.4	0.7
Syringic acid	0.4	0.7
p-Coumaric acid	0.4	0.8
Ferulic acid	0.4	0.8
Sinapic acid	0.5	1.0

LOD and LOQ.—LOD is the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. It can be determined by preparing a solution that is expected to produce a response and is about 3 to 10 times baseline noise. The solution is injected three times, and the S/N for each injection is recorded. The concentration of the solution is considered as the LOD when the S/N is between 3 and 10. LOQ can be determined in the same manner but with an S/N of 10–20.

LOD and LOQ of the phenolic compounds using this method was determined by preparing dilute solutions of the phenolic compounds (0.1, 0.3, 0.4, 0.5, and 1.0 ppm), injecting these solutions into the liquid chromatograph, and recording the S/N for the phenolic compounds peaks at each concentration. Results showed that LOD and LOQ of the eight phenolic compounds are low (Table 2), which permits the determination of these phenolic compounds at low concentrations.

Analysis of the Phenolic Acids in Date Palm Extracts

The applicability of the developed method was tested by the identification and quantitation of the phenolic compounds in two date palm extracts (Rotab and Ahmar Balade types). Identification of the phenolic compounds was based on retention times in comparison with standards. The quantitation was carried out using the external standard method. The concentration of each of the phenolic compounds was calculated using peak area and the calibration curves obtained from the phenolic compound standard solutions. The amount of phenolic compound was expressed as mg/100 g of date palm dry weight (DW). Figures 3 and 4 show the chromatograms of the phenolic compounds detected in these two date palm types, while Table 3 shows the concentrations (as mg/100 g DW) of these compounds detected in the two date palm types.

Results showed that there is a clear difference in the profile of the phenolic compounds of the two varieties of date palm investigated in this study (Ahmar Balade and Rotab). Gallic acid, *p*-hydroxybenzoic acid, and caffeic acid were detected in Ahmar Balade but not in Rotab date palm cultivar, while ferulic acid and sinapic acid were detected in Rotab but not in Ahmar Balade. Vanillic acid and *p*-coumaric acid were detected in both cultivars, while syringic acid was not detected in the two cultivars. The concentration range for these phenolic compounds in the two date palm cultivars was 0.29 to 0.49 mg/100 g of

Table 3. Amounts of phenolic acids determined in the extracts of Rotab and Ahmar Balade date palm types

	Date palm type	
Phenolic compound	Rotab, mg/100 g DW	Ahmar Balade, mg/100 g DW
Gallic acid,	ND[a]	0.43
p-Hydroxybenzoic acid	ND	0.46
Vanillic acid	0.36	0.49
Caffeic acid	ND	0.32
Syringic acid	ND	ND
p-Coumaric acid	0.32	0.40
Ferulic acid	0.30	ND
Sinapic acid	0.29	ND

[a] ND = Not detected.

date palm (DW basis; Table 3). These results are in agreement with other studies previously published; a study by Al Harthi et al. (7) has shown the presence of phenolic acids like gallic acid, vanillic acid, caffeic acid, *p*-coumaric acid, and syringic acid in four different date palm varieties grown in Oman.

Conclusions

A simple, accurate, precise, and selective HPLC method was developed and validated for the determination of eight phenolic compounds (gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) that are present in date palms. The method is linear for determination of the phenolic compounds with a wide dynamic range (1–1000 ppm). This method is also accurate with recovery of these phenolic compounds within 97.1 to 102.2%. Precision of the method is confirmed by low RSD of replicate injections of solutions of the eight phenolic compounds at three concentration levels. The method is selective as adjacent peaks of the phenolic compounds are well separated. Low LOD and LOQ of the eight phenolic compounds enable the detection and quantitation of these compounds at low concentrations.

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