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Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography

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Abstract

An HPLC method is reported for the separation and quantification of five major polyphenolic groups found in fruits and related products: single ring phenolic acids (hydroxybenzoic acid and hydroxycinnamic acid derivatives), flavan-3-ols, flavonols, anthocyanins, and dihydrochalcones. A binary mobile phase consisting of 6% acetic acid in 2 mM sodium acetate aqueous solution (v/v, final pH 2.55) (solvent A) and acetonitrile (solvent B) was used. The use of sodium acetate was new and key to the near baseline separation of 25 phenolics commonly found in fruits. A photodiode array detector was used and data were collected at four wavelengths (280, 320, 360, and 520 nm). This method was sensitive and gave good separation of polyphenolics in apple, cherry, strawberry, blackberry, grape, apple juice, and a processing by-product. The improved separation has led to better understanding of the polyphenolic profiles of these fruits. Individual as well as total phenolic content was obtained, and the latter was close to and correlated well with that obtained by the Folin–Ciocalteu method (FC). The HPLC data can be used as a total phenolic index (TPI) for quantification of fruit phenolics, which is advantageous over the FC because it has more information on individual compounds.

Keywords: Optimization; Mobile phase composition; Total phenolic index; Fruit analysis; Polyphenols

1. Introduction

The potential benefits of antioxidant phytochemicals in health maintenance have been increasingly recognized in recent years. Sufficient evidence has shown that harmful free radicals play an important role in most major health problems such as cancer, cardiovascular disease, and degenerative diseases associated with aging. Flavonoids and other plant polyphenolics are especially important antioxidants because of their high redox potentials, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [1]. In addition, they have a metal chelating potential [2]. The antioxidant activity of the dietary polyphenolics is considered to be much greater than that of the essential vitamins, therefore

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contributing significantly to the health benefits of fruits [3].

More than 5000 polyphenolics, including over 2000 flavonoids have been identified, and the number is still growing [4]. Fruits are particularly rich in polyphenolics. Phenolics of single-ring structure such as hydroxybenzoic acids and hydroxycinnamic acids are found in nearly all fruits (Fig. 1). Flavonoids can be further classified into anthocyanidins, flavan-3-ols, flavones, flavanones, and flavonols. Some of the flavonoids such as flavan-3-ols can be found as dimers, trimers, and polymers (Fig. 1). Many of the polyphenolics are often associated with sugar moieties, which further diversifies the polyphenolic profiles of fruits [5]. The diversity of the polyphenolics in fruits, therefore, is still a challenge to the analytical chemist. The total phenolic content (TPC), measured by the Folin–Ciocalteu (FC) method only gives an estimation of the phenolic content. It does not separate nor does it give quantitative measurement of individual compounds. Despite a great number of investigations, the separation and quantification of different polyphenolics remain difficult, especially the simultaneous determination of polyphenolics of different groups [6–8].

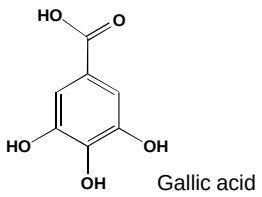
Among the different methods, HPLC has been a method of choice for the separation and quantification of polyphenolics in fruits. The chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase C18 column; UV-Vis diode array detector, and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). The separation normally requires 1 h at a flow rate of 1.0–1.5 ml/min. Solvent A usually includes aqueous acids or additives such as phosphate. Solvent B is normally pure or acidified methanol or acetonitrile. Among the many separation systems, only a few procedures were developed to specifically measure polyphenolic concentrations in several commonly consumed foods [5]. Most of these methods have been developed to measure different groups of polyphenolics in a single plant, or a single or a few groups in multiple plant sources, which most often are non-food plants. Van Sumere et al. [9] indeed developed a good method that separated nearly 50 phenolic compounds from the rose flower pedals. However, fruit polyphenols such as procyanidins, chlorogenic acid, and phloretin-glycosides were not included in their method. A method by Paganga et al. [10] and two other recent HPLC methods by Schieber et al. [8] and Shui and Leong [11] were developed for the separation and measurement of prominent food flavonoids that are members of the subgroups of flavonoids mentioned earlier; anthocyanins and procyanidins, however, were not included in their methods. Some methods such as those developed by Escarpa and Gonzalez [6,7], separated multiple groups of the most prominent phenolics with a relatively short analysis time, an obvious advantage for those who are interested in analyzing the major phenolic components. In shortening the analysis time, however, some minor or unknown compounds may have been missed due to co-elution. The co-elution may also affect the quantification of known compounds. Obtaining good resolution is considered the main difficulty for a method that is targeted for separation of multiple polyphenolic groups [11].

In this paper, we report an HPLC method developed for the simultaneous determination of the five major groups of polyphenolics: single-ring phenolic acids, procyanidins, anthocyanidins, flavonols, and dihydrochalcones in several commonly consumed fruits and related products. The chromatographic data will also be used as an index for rapid and specific estimation of phenolics in comparison with the non-specific TPC by the FC method.

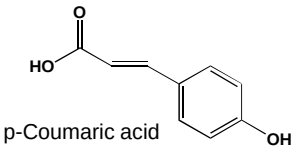
2. Experimental

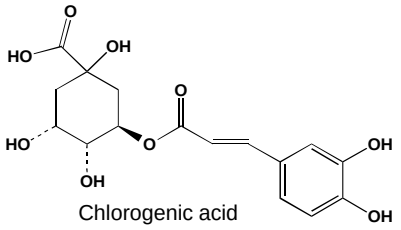
2.1. Chemicals and solvents

Gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, ferulic acid, caffeic acid, *p*-coumaric acid, *o*-coumaric acid, *trans*-cinnamic acid, ellagic acid, catechin, epicatechin, phloridzin, quercetin, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin-3-xyloside, and quercetin-3-rutinoside (rutin) were purchased from Sigma–Aldrich (Oakville, ON). Procyanidin B1 and B2, quercetin-4-glucoside, quercetin-3-arabinoglucoside, kaempferol-3-galactoside, cyanidin chloride, cyanidin-3-galactoside, cyanidin-3- glucoside, cyanidin-3-rutinoside, malvidin-3-galactoside, pelargonidin-3-glucoside, and peonidin-3-glucoside were obtained from Indofine Chemical Company (Somerville, NJ). Water used for HPLC analysis was purified in-house from distilled water using a Barnstead NanoPure® system (Dubuque,



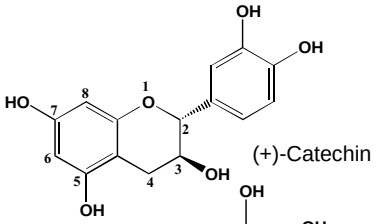
Hydroxybenzoic Acids

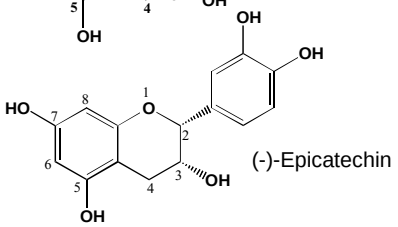


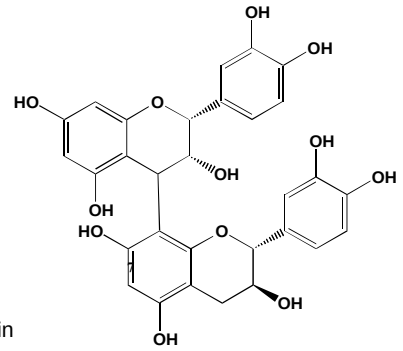


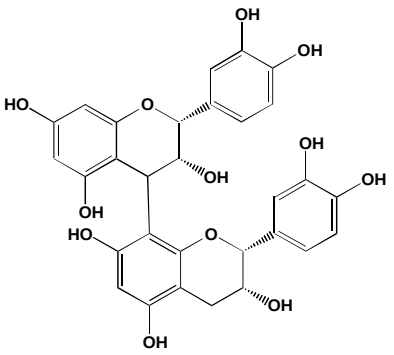
Hydroxycinnamic Acids

Single-Ring Phenolic Acids

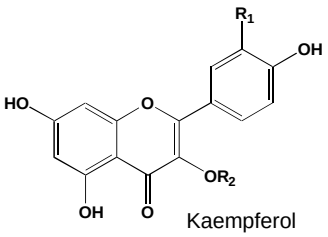








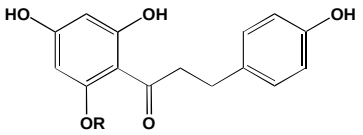
Flavan-3-ols and Procyanidins



	R1
Kaempferol	H
Quercetin	OH

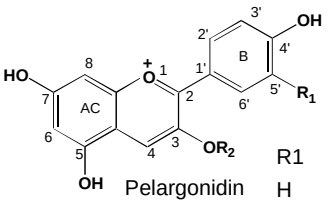
R2: Sugar unit in glycosides

Flavonols



Phlotetin: R=H
Phloredzin: R=glucose

Dihydrochalcones



	R1
Pelargonidin	H
Cyanidin	OH

R2: Sugar unit in glycosides

Anthocyanidins

Fig. 1. Major groups of polyphenolics found in apple and other fruits.

IA). HPLC grade solvents were purchased from Caledon Laboratories Ltd. (Georgetown, ON). Reagent grade sodium acetate was from Sigma–Aldrich (Oakville, ON).

2.2. Sample preparation

For apple samples, 10 commercially harvested red delicious apples from the McCallum Farm (Woodstock, ON) were peeled with a hand peeler (1–2 mm thickness). The peel and flesh were processed separately. Approximately 10 g of the peel or flesh from each of the 10 apples were pooled and ground in liquid nitrogen in a mortar, and then transferred to a centrifuge tube with 70% aqueous methanol added in a 1:1 (w/v) ratio. The mixture was homogenized using a Polytron® blender (Brinkmann Instruments, NY) and filtered first through a Whatman no. 1 filter paper under vacuum and then through a 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI). The final filtrate was stored at −20 °C prior to analysis. Fresh apple pomace and juice mix were supplied by a local juice company. Extraction of pomace followed the same procedures as stated above for the peel and flesh of apple. The juice was filtered through a 0.45 μm syringe filter before HPLC analysis.

Cherries (Vista) and strawberries (Kent) were harvested at commercial ripeness from Vineland Research Station (University of Guelph, Vineland, ON). Blueberries, blackberries, and red globe table grapes were purchased from a local supermarket. Cherries and grapes were de-pitted. Edible parts of 10 fruits from each species were randomly sampled. Ten fruits of each were cut into quarters, one quarter of each was pooled, and then blended with 50% methanol (1:10, w/v) in an Eberbach® mini-blender (Eberbach Co., Ann Arbor, MI). The extract was vacuum filtered through a Whatman no. 1 filter paper. Ten milliliter of each extract was concentrated in vacuo under 40 °C to dryness. The residue was reconstituted in 1 ml of methanol and filtered through a 0.45 μm syringe filter prior to analysis.

2.3. HPLC conditions and peak identification

An Agilent Technologies 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler, and a diode array detector (DAD), was used for quantification and identification of various polyphenolics in the samples. Separation of polyphenolics was carried out using a Phenomenex® Luna C18(2) column (250 mm × 4.6 mm i.d.; particle size, 5 μm) with a C18 guard column. The binary mobile phase consisted of 6% acetic acid in 2 mM sodium acetate (final pH 2.55, v/v, solvent A) and acetonitrile (solvent B). Solvent A was prepared first by making 2 mM sodium acetate water solution, which was then mixed with acetic acid at a ratio of 94:6 by volume. All solvents were filtered through a 0.45 μm membrane filter prior to analysis. The flow rate was kept constant at 1.0 ml/min for a total run time of 70 min. The system was run with a gradient program: 0–15% B in 45 min, 15–30% B in 15 min, 30–50% B in 5 min, and 50–100% B in 5 min. There was a 10 min post run at initial conditions for equilibration of the column. The injection volume for apple and related products was 10 μl, and for the others it was 20 μl. All standards except for the anthocyanins were dissolved in methanol. The latter were dissolved in methanol containing 1% HCl. The detector was set at 280, 320, 360, and 520 nm for simultaneous monitoring of different groups of polyphenolics. Identification of compounds was achieved by comparing their retention times and UV-Vis spectra with those of the standards in the library that was built by using the inline DAD with a 3D feature.

Polyphenolics were grouped into five categories and quantified based on the maximum UV-Vis absorption of each group. These five groups are: single ring phenolic acids (hydroxybenzoic acid and hydroxycinnamic acid derivatives), procyanidins (flavan-3-ols), flavonols, anthocyanidins, and dihydrochalcones (Fig. 1). These compounds were analyzed by HPLC at four different wavelengths with the diode array detector (Fig. 2). The hydroxybenzoic acid derivatives, flavan-3-ols (including their dimers), and dihydrochalcones were quantified at 280 nm (Fig. 2A, C, and D); hydroxycinnamic acid derivatives at 320 nm (Fig. 2B); flavonols at 360 nm (Fig. 2E); and anthocyanins at 520 nm (Fig. 2F). Accordingly, the concentrations of the hydroxybenzoic acids, hydroxycinnamic acids, dihydrochalcones, anthocyanins, all flavan-3-ols including their dimers or oligomers, and flavonols were expressed as gallic acid, chlorogenic acid, phloridzin, cyanidin-3-galactoside, epicatechin, and quercetin-3-galactoside equivalent, respectively.

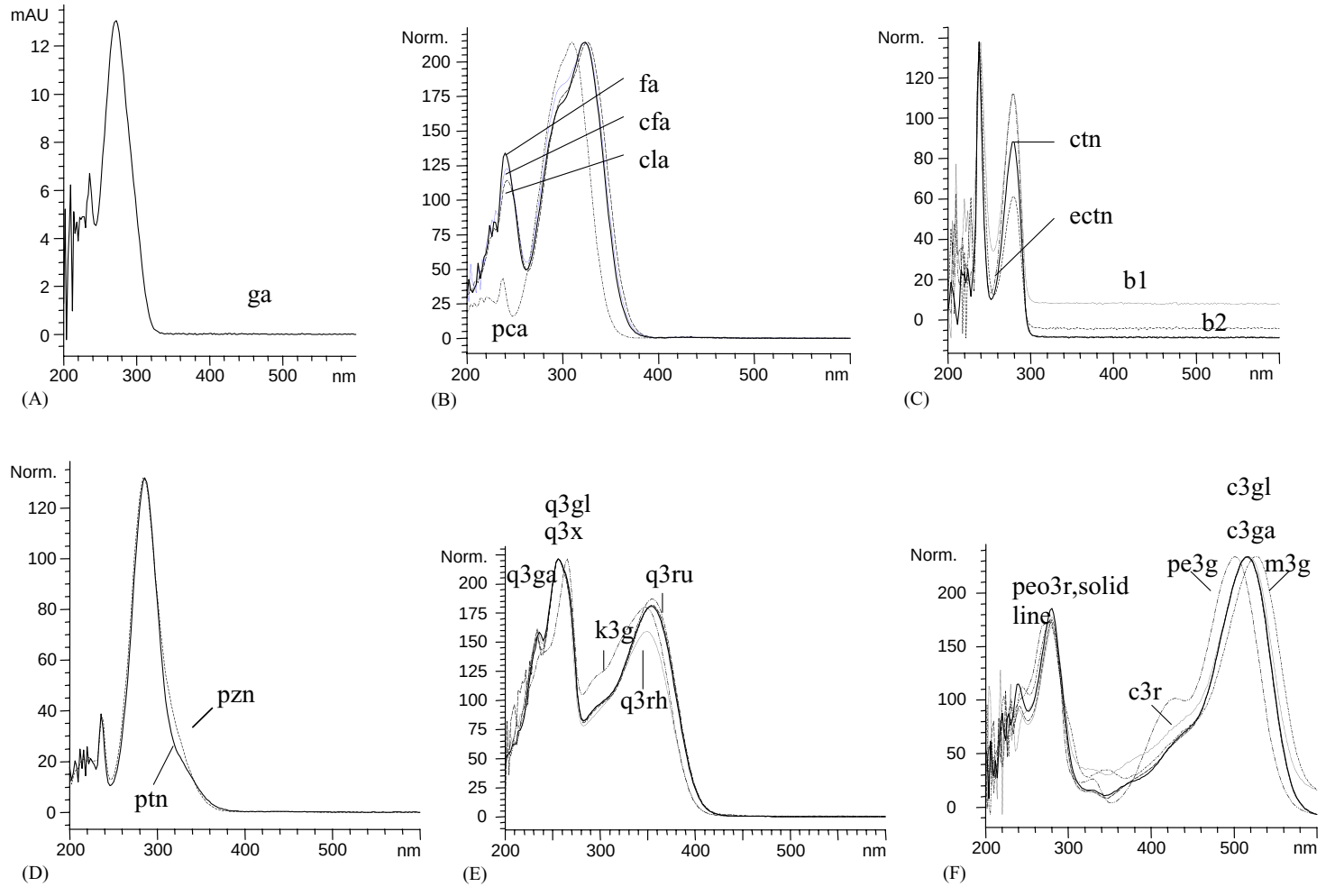


Fig. 2. UV-Vis absorbance patterns of five groups of polyphenolics. (A) Gallic acid; (B) hydroxycinnamic acids; (C) flavan-3-ols and dimers; (D) dihydrochalcones; (E) flavonols; and (F) anthocyanidins. ga, gallic acid; cla, chlorogenic acid; cfa, caffeic acid; fa, ferulic acid; ectn, epicatechin; ctn, catechin; b1, procyanidin B1; b2, procyanidin B2; pzn, phloridzin; ptn, phloretin; q3gl, quercetin-3-glucoside; q3x, quercetin-3-xyloside; q3ga, quercetin-3-galactoside; q3ru, quercetin-3-rutinoside; q3rh, quercetin-3-rhamnoside; k3g, kaempferol-3-glucoside; peo3r, peonidin-3-rutinoside; pe3g, pelargonidin-3-glucoside; c3r, cyanidin-3-rutinoside; c3gl, cyanidin-3-glucoside; c3ga, cyanidin-3-galactoside; m3g, malvidin-3-glucoside.

Table 1
Standard curves, detection limits, and method validation data

Name of phenolics	Wavelength (nm)	Linear range (μg/ml)	Formulae	R^2	LOD (μg/ml)	R.S.D.% (Rt, $n = 3$)	R.S.D.% (Area, $n = 3$)	Recovery (%)
Gallic acid	280	0.1–200	$Y = 22.22X + 48.06$	0.9996	0.05	1.4	1.0	108
Chlorogenic acid	320	0.2–100	$Y = 26.74X - 10.71$	0.9999	0.1	0.7	1.8	106
Quercetin-3-galactoside	360	0.5–100	$Y = 14.84X - 7.738$	0.9988	0.1	0.2	2.0	101
Epicatechin	280	1.0–200	$Y = 6.270X + 8.322$	0.9994	0.5	0.1	1.3	102
Phloridzin	280	0.1–200	$Y = 18.08X - 5.920$	0.9999	0.05	0.5	0.9	106
Cyanidin-3-galactoside	520	1.0–100	$Y = 20.04X + 6.561$	0.9997	0.5	1.4	5.8	110

The total concentration of the group was the sum of the individual concentrations of each group. The linear range, calibration formula, and the detection limit of the standards for each group are listed in Table 1. The detection limit was defined as the concentration at which the signal to noise ratio (*S*/*N*) was equal to or greater than three. In the calibration formula, *X* stands for the concentration of the analyte, and *Y* is the peak area. All samples were prepared and analyzed in duplicate.

2.4. Total phenolic content based on the colorimetric method

The Folin–Ciocalteu (FC) method [12] was modified for the analysis of TPC in all samples. Briefly, 0.2 ml solvent or sample, 1.0 ml FC reagent, and 0.8 ml Na_2CO_3 (7.5%) were mixed and allowed to stand for 30 min at room temperature. Absorption was measured at 765 nm in a Varian® Cary 3C spectrophotometer (Varian Analytical Instruments, CA). Results were expressed as microgram of gallic acid equivalent (GAE) per milliliter solution. Concentrations beyond the highest point (500 μg/ml) of the linear range of the standard curve were diluted before finally analyzed.

3. Results and discussion

3.1. Optimization of chromatographic conditions

HPLC equipped with a reversed phase C18 column is probably the most widely used chromatographic technique for the separation and analysis of polyphenolics. Many C18 columns offering relatively good separation efficiency are now available. Optimization of the mobile phase, however, is still an important step in the method development. Early in our study, the use of phosphate and other additives in solvent A was eliminated due to their effects on peak broadening and co-elution of some of the 25 standards. Initial trials using acetic acid, however, gave good resolution for the standard mixture; therefore, effort was made to optimize this system. Our standard mixture was designed to include the major groups of polyphenolics of fruits, especially those found in apple (Fig. 1).

Although acetic acid gave better resolution of the standards than other solvent A additives, it was found that the system still could not separate several key polyphenolics in the standard mixture. Several concentrations of acetic acid between 2 and 10%, and sodium acetate between 0.5 and 4 mM were evaluated, and the best separation was found with 6% acetic acid in 2 mM sodium acetate (v/v, final pH 2.55). In the linear gradient program (see HPLC conditions), virtually all the standard polyphenolics in the mixture were separated at the baseline with this mobile phase system (Fig. 3). Sodium acetate does have a disadvantage as an additive when the HPLC method is adopted for HPLC–ESI–MS because it is not volatile. A volatile salt, ammonium acetate, was therefore studied as an alternative, however, the separation was not good at the same (2 mM) or higher concentration (20 mM), particularly for the early-eluting compounds (peaks 3–11). Peaks 5 and 6 were co-eluted using low concentration ammonium acetate (2 mM), and peaks 7 and 8 were co-eluted at 20 mM. By setting the detector at multiple wavelengths, certain groups of compounds can be selectively detected, for example, anthocyanins were the only compounds detected at 520 nm (Fig. 3). Fronting of the anthocyanin peaks in Fig. 3 is caused by the impurities

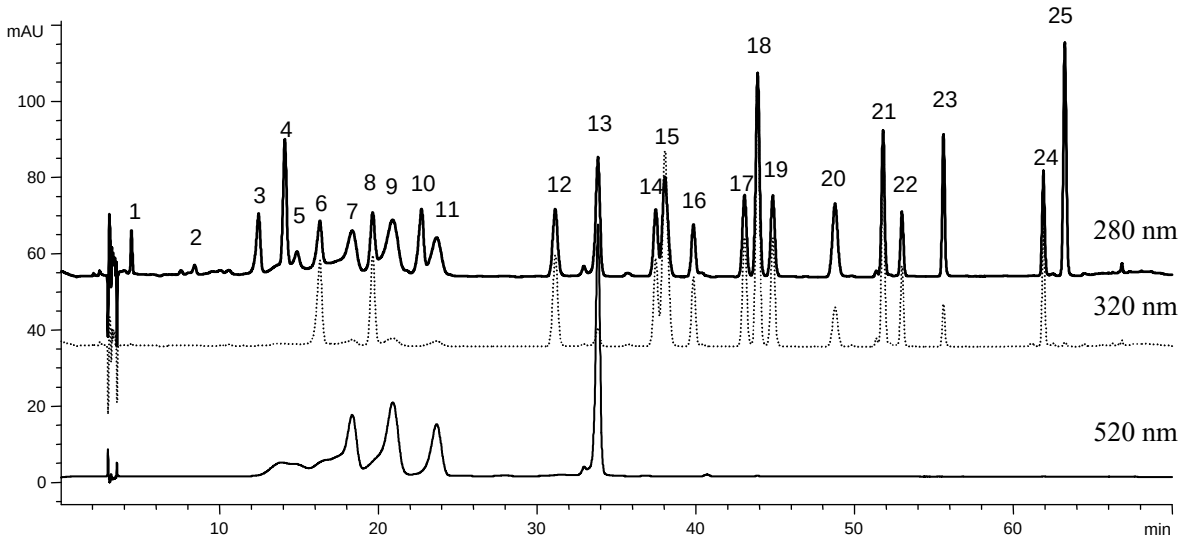


Fig. 3. Chromatograms of the standards run with 6% acetic acid in 2 mM sodium acetate additive at wavelengths of 280, 320, and 520 nm. (1) Gallic acid; (2) procyanidin B1; (3) catechin; (4) 4-hydroxybenzoic acid; (5) procyanidin B2; (6) chlorogenic acid; (7) cyanidin-3-galactoside; (8) caffeic acid; (9) cyanidin-3-glucoside; (10) epicatechin; (11) cyanidin-3-rutinoside; (12) *p*-coumaric acid; (13) cyanidin chloride; (14) quercetin-3-xyloside; (15) ferulic acid; (16) quercetin-3-arabinoglucoside; (17) quercetin-3-galactoside; (18) quercetin-3-glucoside; (19) quercetin-3-rutinoside; (20) *o*-coumaric acid; (21) quercetin-3-rhamnoside; (22) quercetin-4-glucoside; (23) phloridzin; (24) quercetin; (25) cinnamic acid.

(percentage not available from the supplier) in the standards.

During method development the detector was set at 280 nm. This wavelength was also used by Schieber et al. [8] in detecting 26 polyphenolics. In the Schieber et al. method, acetic acid was added to solvent A for a near-baseline separation of all 26 standards. However, anthocyanins were not included in the mixture of the method, therefore, it is difficult to know whether the method will separate the anthocyanins from nearby polyphenolics such as procyanidin B2, chlorogenic acid, and caffeic acid [8]. The current method, on the other hand, overcame such difficulty and separated the anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside) from each other at 520 nm, and from other nearby polyphenolics at 280 nm (Fig. 3). This gives the method advantages for simultaneous detection of major polyphenolics in fruits, particularly for those researchers that do not have a multiple wavelength or photodiode array detector. At 280 nm, although sensitivity of certain groups of polyphenolics may not be at the highest, major polyphenolics in common fruits can be detected. Acetate additives have not been used in improving the separation of the multiple groups of polyphenolics of fruits. Only one study used an acetate additive [13], however, the application of this method was to separate flavan-3-ols in tea infusions.

3.2. HPLC method validation

The between-day precisions of retention time were within 0.1–1.4% relative standard deviation (R.S.D.) for the six representative standards (Table 1). That of peak area were within 0.9 and 2.0% R.S.D. except cyanidin-3-galactocide, which was slightly higher (5.8%) (Table 1). The accuracy of the method was validated by analyzing a spiked sample. Known amounts of standards were mixed together at 100 μl of 1000 μg/ml in methanol per compound, and added to a juice sample (1 ml) with known contents of these target compounds. The recoveries of these standards were between 101 and 110% (Table 1). The limits of detection (LOD, $S/N = 3$) are listed in Table 1.

3.3. Quantification and identification

To achieve high sensitivity and simplicity for the method, quantification of the polyphenolics was based

on the maximum UV-Vis absorptions. As shown in Fig. 2, the maximum absorptions of the five major groups of polyphenolics included in this study fell into one of the four wavelengths. The λ_{max} for the single-ring phenolic acids other than the hydroxybenzoic acids was near 320 nm (Fig. 2B); for the hydroxybenzoic acids, flavan-3-ols (including the dimers), and dihydrochalcones λ_{max} was near 280 nm (Fig. 2A, C, and D, respectively); for the flavonols λ_{max} was 360 nm (Fig. 2E); and for the anthocyanins λ_{max} was near 520 nm (Fig. 2F). Standard curves of gallic acid, chlorogenic acid, phloridzin, cyanidin-3-galactoside, epicatechin, and quercetin-3-galactoside were generated, and concentrations of the polyphenolics were calculated accordingly, and the total concentrations of each group were summed in Table 2 as equivalents of the six respective standards. Using this method, in combination with the diode array detector, a simplified and fast estimation of concentrations of similar compounds as a group can be obtained. This is an improvement from the FC total phenolic content method. The total concentration of each group of polyphenolics can be obtained without complete identification of the individual compounds. The TPCs measured by HPLC were about 4.5–24% lower than those obtained from the FC method, however, the two sets of data correlated well ($R^2 = 0.9978$) (Table 2). The difference between the two methods might be due to undetected minor compounds in the HPLC method, or interference from proteins in the FC method. Protein was found to cause varied degrees of overestimation of the TPC in the FC method [14].

The high correlation between the HPLC and FC methods could be important and useful in the estimation of phenolics. The HPLC data can be used as total chromatographic index (TCI) for the quantification of fruit phenolics, making it a good tool towards total phenolic index (TPI). Escarpa and Gonzalez [14] published a paper, which indicated that such approach is viable. TPI has the advantage over TPC (total phenolic content determined by the FC method) in that it gives more specific information of individual compounds or groups. Variation between the two methods was significantly lower in our study than in that reported by the above authors for all fruit samples.

Table 2
Concentrations of phenolic compounds in fruits and fruit products (μg/g, wet weight) determined by HPLC and FC methods[a]

	Total benzoic acids	Total hydroxycinnamic acids	Total flavan-3-ols	Total anthocyanidins	Total flavonols	Total dihydrochalcones	TPI[b]	TPC[c]	TPC/TPI
Apple peel	ND[d]	50	1655	149	244	252	1756	2012	1.1
Apple flesh	ND	137	342	ND	4	28	411	447	1.1
Apple juice	ND	18	6	ND	3	23	50	62	1.2
Fresh apple pomace	ND	ND	22	29	423	238	712	830	1.2
Strawberry	3	5	ND	44	8	4	64	67	1.0
Cherry	299	386	ND	292	ND	ND	958	1046	1.1
Blueberry	212	312	ND	1213	ND	ND	1737	2078	1.2
Blackberry	ND	9	22	1923	49	ND	2003	2416	1.2
Red table grape	ND	16	ND	81	23	13	133	175	1.3

[a] The total concentrations were cumulative concentrations of each individual compounds of the same group quantified as equivalents of representative standards. Data for each compound were average of two replicates. Total benzoic acids include gallic acid, ellagic acid, hydroxybenzoic acids, and their derivatives; total hydroxycinnamic acids include chlorogenic acid, *p*-coumaric acid, ferulic acid, and their derivatives; total flavan-3-ols include catechin, epicatechin, and their dimmers; total anthocyanidins include cyandin-3-galactoside, cyanidin-3-glucoside, cyanidin-rutinoside, malvidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-rutinoside; total flavonols include kaempferol-3-glucoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rutinoside, quercetin-3-xyloside, quercetin-3-rhamnoside, and other quercetin derivatives; and total dihydrochalcones include phloridzin and other phloretin derivatives. Temporarily identified derivatives in each group were quantified using the standard compounds listed in Table 1 as equivalents.

[b] Total phenolic index measured by HPLC.

[c] Total phenolic content measured by the FC method (see text for details).

[d] Not detectable.

The TPCI/TPI ratios were between 1.0 and 1.3 (Table 2).

The identification of the polyphenolics was based on the retention time and their UV-Vis spectrum. Each standard was injected individually and the UV-Vis spectrum was obtained by using the inline diode array detector. These spectra were then saved into a spectral library that was built in-house for the polyphenolics. A compound can be identified with high confidence by comparing its UV spectrum with those stored in the library. A match in both UV spectrum and retention time gives positive identification of the compound. A match only in UV spectrum but not in retention time only gives semi-identification of the compound, i.e. identified as a derivative of that particular polyphenolic group. This provides a quick overview of the polyphenolic profile of a fruit before a standard is available. In fact, many standards are not commercially available. Such initial characterization can also provide helpful information for the final identification of the compounds of interest by other instrumentation such as mass spectrometry and NMR. Further information about a specific compound can be obtained by comparing the absorption pattern of the UV spectrum. As Fig. 2 shows, many compounds have more or less absorption at 280 nm. Therefore, it is difficult to distinguish compounds in the group A from those in groups C, D, and F. However, if a compound had even stronger absorption at near 520 nm in addition to the strong absorption at 280 nm, that compound is very likely an anthocyanin. If an additional absorption appeared at 240 nm, and there was no absorption at 360 nm, it would be considered to belong to the group C. Compounds of groups A and D do not have stronger absorption at 240 nm or 360 nm. The λ_{max} shifts and tails slightly toward longer wavelength for group D compounds than for group A compounds due to the additional chromaphore in the dihydrochalcones. The hydroxycinnamic acids had a strong λ_{max} at ca. 320 nm; however, the λ_{max} shifts toward longer wavelength for those with two hydroxy or methoxy groups (ferulic, caffeic, and chlorogenic acids). Therefore, these compounds can be distinguished from the *p*-coumaric acids (Fig. 2B). The usefulness of this technique can be exemplified by the identification of flavan-3-ol, quercetin, and phloretin derivatives (Figs. 4 and 5).

3.4. Application of the method

This method was developed primarily for the simultaneous analysis of polyphenolics in apples and related products; therefore, the standard mixture contained many polyphenolics that have been reported in apple. Nonetheless, many polyphenolics such as quercetin and its glycosides, anthocyanins, procyanidins, and hydroxycinnamic acids are common polyphenolics of many fruits, and we found that this method was equally good for the simultaneous analysis of polyphenolics in several commonly consumed fruits including strawberry, sweet cherry, high-bush blueberry, blackberry, and red table grape (Fig. 5). Fig. 4 shows the HPLC profiles of major polyphenolics in the peel and flesh of a red delicious apple. Catechin, epicatechin, and phloridzin were the major components in the peel, along with procyanidin B1, B2, chlorogenic acid, cyanidin-3-galactoside, caffeic acid, quercetin-3-glucoside, and quercetin-3-rutinoside. The polyphenolic profile of the flesh was quite different from that of the peel. Chlorogenic acid was the predominant polyphenolic compound in the flesh, whereas phloridzin predominated in the peel (Fig. 4). The peel, in general, had significantly higher TPCs and most of the individual compounds (Table 2), except that of chlorogenic acid (Fig. 4). It should be mentioned, however, that such a change of secondary metabolite concentrations may happen during fruit maturation. Other polyphenolics including procyanidins, phloretin, quercetin, and chlorogenic acid derivatives were also tentatively identified from the apple peel, flesh, apple juice, and pomace. Polyphenolics in apple pomace was of particular interest of us, because such a processing by-product may represent a great potential as a source of antioxidants.

The polyphenolic profiles differed in the fruits selected in this study. Fig. 5 shows the typical HPLC chromatograms of strawberry, sweet cherry, and red table grape extracts. Pelargonidin-3-glucoside and ellagic acid were typical of the strawberry (Fig. 5A), and cyanidin-3-rutinoside, *p*-coumaric acid, ferulic acid, and cinnamic acid were major polyphenolics in sweet cherry (Fig. 5B). The red table grape analyzed in this study contained mainly cyanidin-3-glucoside, peonidin-3-glucoside, and quercetin-3-xyloside (Fig. 5C). Although the chromatograms are not shown

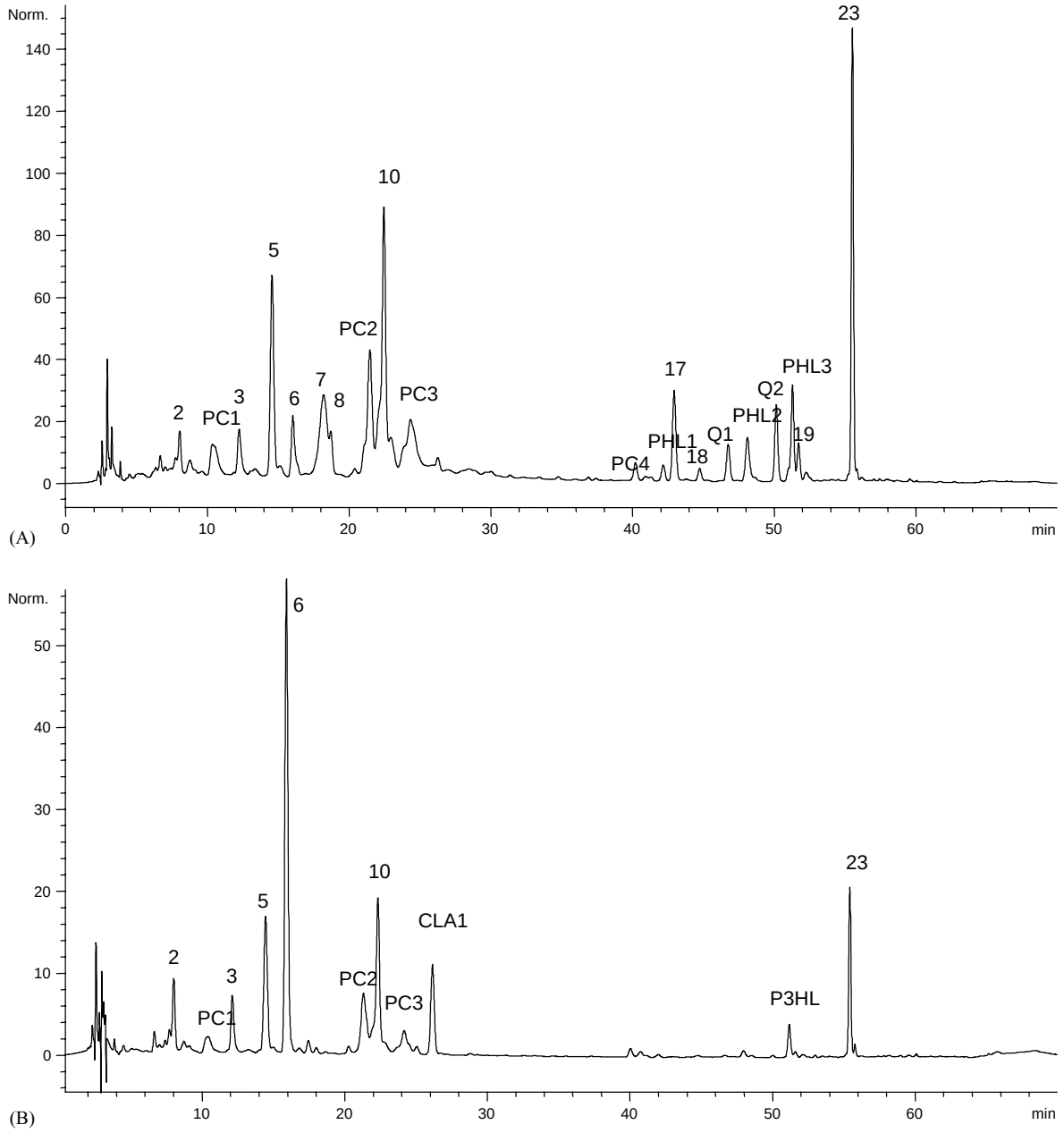


Fig. 4. HPLC profiles of polyphenolics in red delicious apple peel (A) and flesh (B) at wavelength of 280 nm. Peaks with numeric labels are the same compounds as designated in Fig. 3. PC1-PC4: procyanidin derivatives; PHL1-3: phloretin derivatives; Q1 and Q2: quercetin derivatives; CLA1: hydroxycinnamic acid derivative.

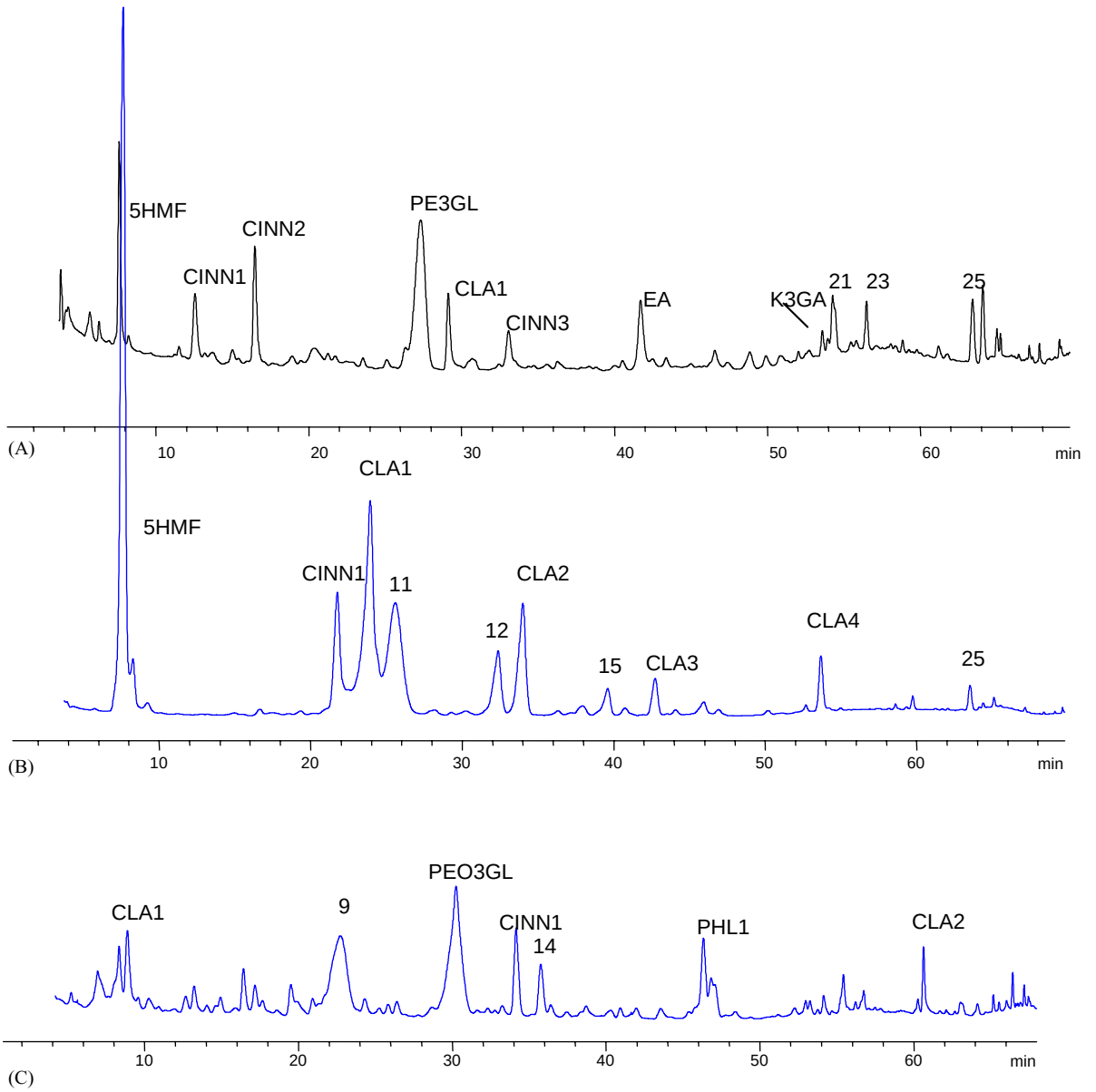


Fig. 5. HPLC profiles of polyphenolics in other commonly consumed fruits (280 nm). (A) Strawberry; (B) sweet cherry; (C) red table grape. Peaks with numeric labels are the same compounds as designated in Fig. 3. 5HMF: 5-hydroxymethylfurfural; PE3GL: pelargonidin-3-glucoside; EA: ellagic acid; K3GA: kaempferol-3-galactoside; PEO3GL: peonidin-3-glucoside; CINN1-CINN3: cinnamic acid derivatives; PHL1: phloretin derivative; CLA1-CLA4: hydroxycinnamic acid derivatives.

in this paper, the high-bush blueberry and blackberry contained various polyphenolics, particularly anthocyanins.

Although in general, good separations were achieved for the selected fruit samples, this method can still be slightly modified to meet the individual polyphenolic profiles of each fruit. The gradient program can also be changed to accommodate the elution of certain groups of polyphenolics, but sodium acetate seems to be critical for the separation.

4. Conclusion

Many good HPLC methods exist for the separation and quantification of different polyphenolic groups found in different fruits or other plants. Our method showed that good separation could be achieved by using sodium acetate in combination with acetic acid as a solvent A additive of the mobile phase. The method was sensitive and selective by using multiple wavelengths corresponding to the different UV-Vis maximum absorptions of the different polyphenolic groups in fruits. Certain polyphenolics can be used collectively as representative standards of a fruit in quantification as done in this study, however, this method still allows quantification of individual compounds. Such HPLC data can be used as TPI for the quantification of fruit phenolics. TPI will give more information on individual compounds or groups of compounds than the TPC by FC.

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