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Simultaneous determination of phenolic compounds in Cynthiana grape (*Vitis aestivalis*) by high performance liquid chromatography–electrospray ionisation–mass spectrometry

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Abstract

Phenolic acids, flavanols, flavonols and stilbenes (PAFFS) were isolated from whole grapes, juice, or pomace and purified using enzymatic hydrolysis. Only anthocyanin mono-glucosides and a few of the oligomers from Cynthiana grape (*Vitis aestivalis*) were analysed. Flavonoid-anthocyanin mono-glucosides (FA) were isolated using methanol/0.1% hydrochloric acid extraction. In addition, crude extractions of phenolic compounds from Cynthiana grape using 50% methanol, 70% methanol, 50% acetone, 0.01% pectinase, or petroleum ether were also evaluated. Reverse phase high performance liquid chromatography (RP-HPLC) with photodiode array (PDA) detector was used to identify phenolic compounds. A method was developed for simultaneous separation, identification and quantification of both PAFFS and FA. Quantification was performed by the internal standard method using a five points regression graph of the UV–visible absorption data collected at the wavelength of maximum absorbance for each analyte. From whole grape samples nine phenolic compounds were tentatively identified and quantified. The individual phenolic compounds content varied from 3 to 875 mg kg^{-1} dry weight. For juice, twelve phenolic compounds were identified and quantified. The content varied from 0.07 to 910 mg kg^{-1} dry weight. For pomace, a total of fifteen phenolic compounds were tentatively identified and quantified. The content varied from 2 mg kg^{-1} to 198 mg kg^{-1} dry matter. Results from HPLC analysis of the samples showed that gallic acid and (+)-catechin hydrate were the major phenolic compounds in both whole grapes and pomace. Cyanidin and petunidin 3-*O*-glucoside were the major anthocyanin glucosides in the juice.

Keywords: Cynthiana grape; Extraction; Phenolic compounds; HPLC–ESI–MS

1 Introduction

Cynthiana (*Vitis aestivalis*) also known as Norton is a variety of grape that is native to North America and is renowned for its ability to produce an intense-coloured red wine. The local regions where Cynthiana is produced are Arkansas, Illinois, Indiana, Kansas, Louisiana, Maryland, Missouri, Oklahoma, New Jersey, Tennessee, Texas, Virginia and West Virginia (Roberts, 1999).

Phenolic compounds are a subdivision of the phytochemical group and the study have attracted passionate interest of both researchers and consumers due to their antioxidants and antimicrobial activities (Puupponen-Pimiä et al., 2001).

The antioxidant activities of phenolic compounds are attributed to their free radical scavenging and metal chelating properties, as well as their effects on cell signaling pathways and on gene expression (Hogan et al., 2009; Kähkönen & Heinonen, 2003; Muñoz-Espada, Wood, Bordelon, and Watkins, 2004; Yilmaz & Toledo, 2006). The mechanism of the antioxidant activity has been mainly influenced by the number of OH^- groups and their position on the ring in the molecule, which determines the antioxidant capacity of phenolic compounds (Hogan et al., 2009). Several studies have showed that the antimicrobial activity of phenolic compounds is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, more lipophilic flavonoids may also disrupt microbial membranes (Puupponen-Pimiä et al., 2001). Other mechanisms of action noted by the study of phenols in general and subclasses of phenolic acids, flavonoids and tannins involve enzyme inhibition, enzyme inactivation, formation of complexes with cell walls and metal ions (Cowan, 1999). The phenolic compounds are cyclic benzene compounds with a minimum of one hydroxyl group associated directly with the ring structure. Based on their structure two groups are distinguished flavonoids and non-flavonoid phenols (Bowyer, 2002). Flavonoid phenols are subdivided into anthocyanins, flavanols, flavonols and tannins (Kennedy, Hayasaka, Vidal, Waters, & Jones, 2001). Non-flavonoid phenols consist primarily of phenolic acids and esters (Bowyer, 2002). The differences between both groups are the number and orientation of phenolic-subunits with the molecules (Bowyer, 2002).

Flavonoids are widely distributed in grapes, especially in seeds and stems, and principally contain (+)-catechins, (−)-epicatechin. Anthocyanin mono-glucosides are pigments and mainly exist in red grape skins. Phenolic acids in grapes comprise derivatives of hydroxycinnamic acid, including caffeic acid and p-coumaric acid (Rodriguez-Montealegre, Romero-Peces, Chacon-Vozmediano, Martinez Gascuena, & Garcia Romero, 2005). Most of the research on phenolic compounds has been focused on the structural characterization of non-flavonoid/flavonoid phenols from a wide variety of plant matrices. Fruits and berries contain a variety of phenolic compounds, which are often located in the external layer of the plant, seeds and pulp and are readily extracted by organic solvents (Kähkönen & Heinonen, 2003). The external location of phenolic compounds is associated with their main natural function: protection of the plant against environmental stress and pathogens.

The extraction of phenolic compounds is primarily influenced by their sample particle size, the extraction method, and storage time (Thorsten Maier, Kammerer, Schieber, & Carle, 2008). Additional steps may be required for the removal of unwanted non-phenolic substances such as waxes, fats, terpenes and chlorophylls. Solid phase extraction (SPE) techniques and fractionation based on acidity are commonly used to remove non-phenolic substances (Rodriguez, Lombaart, & Cela, 2000). For extraction of phenolic compounds, soxhlet extraction is one of the most popular techniques for isolating non-flavonoid/flavonoid phenols from solid samples. Polar solvents, such as methanol, acetone, acetonitrile yield high extraction efficiencies. However, they also extract other undesirable polar compounds present in samples (Gao & Mazza, 1995).

Analytical techniques as reversed-phase HPLC using photodiode array detection has been extensively reported for the identification and quantification of phenolic compounds in grapes and wine (Gao & Mazza, 1995). Nevertheless, most of these methods target only certain classes of phenolic compounds (example: flavonoids and stilbenes vs. anthocyanin mono-glucosides) whereas, simultaneous determination of all compounds is fairly unusual (Lin & Harnly, 2007).

The objectives of the current study were: (a) to evaluate the presence of flavonoids (anthocyanin, mono-glucosides, flavonols and flavanols) and non-flavonoid compounds (phenolic acids and stilbenes) in Cynthiana grape (*Vitis aestivalis*) by developing a simultaneous HPLC method. (b) To identify and quantify flavonoids (anthocyanin mono-glucosides, flavonols and flavanols) and non-flavonoid compounds from the quantitative extracts of Cynthiana whole grape, juice, and pomace. (c) To identify and quantify flavonoid (anthocyanin mono-glucosides, flavonols and flavanols) and non-flavonoid compounds in Cynthiana whole, juice, and grape pomace using the following crude extraction conditions: 50% methanol–water mixture, 70% methanol–water mixture, 50% acetone–water mixture, 100% petroleum ether and 0.01% pectinase solutions.

2 Materials and methods

2.1 Solvents and reagents

Methanol, acetonitrile, acetone, petroleum ether, and phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ and were of analytical or HPLC grade. Water was from Milli-Q purification system Millipore (Millipore, Bedford, MA, USA). Ascorbic acid, ethyl acetate and β-glucosidase type HP-2 from *Helix pomatia* were purchased from sigma Aldrich (St Louis, MO, USA. Gallic acid, Ferulic acid, Caffeic acid, p-coumaric, (+) Catechin hydrate, Quercetin, Epicatechin gallate, Isorhamnetin, Myricetin, *trans*-resveratrol, 7-ethoxycoumarin and β-glucosidase were purchased from Fluka (St Louis, MO. USA). The 3-*O*-glucosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Sep-Pak C_{18} cartridges (1 g, 6 mL) were obtained from Waters Corporation (WAT051910, Waters Corp., Milford, MA, USA).

2.2 Grape collection

Thirty-five lbs of Cynthiana grape (clusters) were collected from a research station center at Oklahoma State University. Clusters were placed in 7 different bags (~5 lbs) and vacuum packed (16 × 25 inch vacuum bags, Curwood, Inc, Oshkosh,WI) under low vacuum (100 kPa to 3 KPa, Multivac C500, Multivac Inc. Kansas City, MO) and stored at –20 °C for 5 days.

2.3 Pomace preparation

Wine was made in order to collect Cynthiana pomace because of the difficulties in obtaining commercial pomace at the time the study was conducted. Cynthiana pomace was produced on pilot-scale level at Oklahoma State University by the following protocol: approximately 30 lbs of frozen grapes were weighed and thawed at 4 °C for two days. Grapes were destemmed and gently crushed on with a commercial grape destemmer-crusher (Jolly-60, St. Patrick's of Texas, Austin, TX). They were placed into a 100 L (25 gallon) stainless steel fermentation vessel for maceration and wine grade yeast (*Saccharomyces cerevisiae*) and yeast nutrient (Fermaid) was also added. The vessel was capped with adjustable height lids allowing approximately 25 cm (≈10 inches) of headspace at 20–22 °C for 5 days in order to mimic industrial process. Samples were monitored by rapid residual sugar tests (AV-RS Accuvin LLC, Napa, CA). The lid was pressed down daily to minimize headspace until fermentation was completed. After fermentation, samples were pressed using a small scale table top water-powered bladder press (Zampelli Enotech JRL, Italy), which allowed separation of wine and pomace (Jensen, Demiray, Egebo, & Meyer, 2008). Samples of pomace were collected in vacuum bags (16 × 25 inch vacuum bags, Curwood, Inc, Oshkosh, WI) and stored at –20 °C until further analysis. Wine samples were discarded.

2.4 Preparation of whole grapes, juice, and pomace for extraction

For whole grapes, woodchip and stems were removed from approximately 5 lbs of frozen clusters. In order to create a frozen powder from the grapes, whole grapes were placed in liquid nitrogen (–196 °C) using a metal strainer. Liquid nitrogen treated grapes were ground for 30 s in a 4 °C room using a Waring blender (model 51BL31) and a previously frozen blender jar. The resultant powder was placed in vacuum bags (8 × 10 inch vacuum pouches, Mid-Western Research & Supply, Inc). Prior to vacuum packaging and frozen storage, a subsample was collected for immediate extraction and analysis.

For pomace, liquid nitrogen powdering and sub-sampling was conducted as described for whole grapes.

For juice, 454 g of grapes were pressed by hand using cheese-cloth. Juice was added to an amber vial (530 mL Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc.) and kept at 4 °C. Analysis was conducted the same day.

2.5 Selection of extraction temperature

Preliminary experiments were conducted to determine optimum temperature for extraction for selected protocols (Kammerer, Claus, Carle, & Schieber, 2004; Torres, Davis, Yanez, & Andrews, 2005; Vassan, 2009). The following extraction conditions were evaluated: ice bath, room temperature and 40 °C (data not shown). Results indicated extraction on ice maximised phenolic recovery. Each extraction was carried out in triplicate and each extract was injected in duplicate.

2.6 Quantitative extraction protocols

2.6.1 Quantitative extraction I (phenolic acids, flavonols, and stilbenes)

This extraction was modified from Torres et al. (2005). Juice was first pre-treated with HCl in order to hydrolyze sugar and final concentration of HCl in juice was 0.1%. Treated juice was centrifuged for 3000*g* for 15 min prior to analysis.

Briefly, 0.5 g of sample (whole grape powder or pomace powder or pre-treated juice) was weighed (A-160, Denver Instruments Co) and transferred to a 30 mL brown bottle (Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc.) and 25 μL of 25 ppm 7-ethoxycoumarin (internal standard) and 4 mL of 50% v/v methanol–water mixture were added. The bottle was placed in an ice bath and the mixture was stirred for 1 h. The mixture was subsequently centrifuged (Clinical 50-82013-800 centrifuge VWR International, Chicago) at 3000*g* for 20 min and decanted through Whatman filter paper #41 into a 10 mL volumetric flask. Samples were then re-extracted under the same conditions and the combined filtrates were brought to volume with 10 mL of 50% methanol–water mixture. An aliquot of 2 mL was placed into a brown vial (3 mL Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc), to which 110 μL of 0.78 M acetate buffer (pH 4.8), 100 μL of ascorbic acid and 50 μL of β-glucosidase were added. Vials were capped, vortexed and incubated at 37 °C for 17 h (overnight). Samples were centrifuged at 4000*g* for 25 min and analysed by RP-HPLC (Thimothe, Bonsi, Padilla-Zakour, & Kooh, 2007).

2.6.2 Quantitative extraction II (flavonoid-anthocyanin mono-glucosides)

The extraction was adapted from Kammerer (Kammerer et al., 2004) with some modifications. Briefly, 5 g of sample, 200 μL of the internal standard (25 ppm 7-ethoxycoumarin) and 100 mL of methanol/0.1% HCl (v/v) were combined and mixed in a brown bottle for 1 h under stirring and flushing with nitrogen in order to prevent oxidation during extraction at room temperature. The extract was centrifuged at 4000*g* for 10 min, and the material was re-extracted with 100 mL of the organic solvent under the same conditions for 15 min. A 5 mL aliquot of the combined supernatants was evaporated to dryness under nitrogen water bath (Zymark TurboVap, Zymark centre, Hopkinton, MA) at 30 °C to remove the organic solvent and the residue was dissolved with 2 mL of acidified water (pH 3.0, acetic acid). Anthocyanin mono-glucosides were analysed by RP-HPLC (Thimothe et al., 2007). Heat was not applied for the actual extraction.

2.7 Preparation of crude extracts

The extraction conditions (time, solvent-to-solid ratio, temperature) were based upon literature data (Ju & Howard, 2003) and previous extraction experiences of the research group (Vassan, 2009). Conditions during all crude extraction experiments were: solvent-to-solid ratio of 40 mL solvent per 20 gr extraction material and extraction time 1 h. The solvents were removed by nitrogen evaporation at 35 °C. The extraction solvents used were: 70% methanol–water, 50% acetone–water, 0.01%

pectinase–water mixture and petroleum ether. Samples were not hydrolyzed.

Crude extraction of the phenolic compounds from whole grape or pomace powder was conducted by weighing 20 g of sample into 125 mL Erlenmeyer flasks. The appropriate solvent, 40 mL, was added. The flasks were placed in a shaker (Classic C76, New Brunswick Scientific, Edison, NJ) maintained at 18 °C and 250 rpm for 1 h. After shaking, samples were filtered under vacuum using a Buchner funnel with 5.5 cm diameter and Whatman filter #1 (55 mm, Whatman Inc. Ltd., Mainstone, England). Samples were filtered until no visible dripping and then rinsed twice with approximately 10 mL of solvent for two subsequent filtrations. The final filtrates (except petroleum ether extracts) were transferred to 100 mL volumetric flask and brought up to volume with the corresponding solvent. The petroleum ether extracts were allowed to evaporate and were re-suspended in 100% acetone. A 10 mL aliquot was subsequently evaporated to dryness with nitrogen in a water bath at 30 °C, dissolved in 7 mL of Milli-Q water, and applied to solid phase extraction cartridges (WAT051910, Waters Corp., Milford, MA). Aliquots of 5 mL were applied to the cartridges, which were activated with 5 mL of methanol, rinsed with 5 mL of deionized water and 3 mL of 0.01% HCl (v/v). Samples were eluted with 5 mL of methanol and filtered through 0.45 µm nylon filters (Fisherbrand, PTFE, Fisher Scientific, Denver, CO) and used for RP-HPLC analysis (Thimothe et al., 2007).

2.8 Identification of individual phenolic compounds by RP-HPLC

The RP-HPLC procedure utilised was modified from Thimothe et al. (2007). The method was designed to separate 17 phenolic compounds. Phenolic acids, flavanols, flavonols, and stilbenes (PAFFS) standards were received as individual compounds. Flavanoid-anthocyanin mono-glucosides (FA) standards were received as a mixture. A 100 ppm standard solution containing all (11) of the individual PAFFS, in addition to the FA mixture (6) was prepared. This standard solution also contained 25 ppm internal standard (7-ethoxycoumarin). The standard curve was prepared by serially diluting (1:1) to a final concentration of 0.78 ppm. Separation, identification and quantification of individual phenolic compounds were performed on a reversed phase chromatography system (Alliance Waters 2690, Waters, Ireland) with a photodiode array detector (PDA, Waters 2996) and Empower 2 software (Kennedy). Compounds were separated by a gradient elution system on a Sun Fire™ C18 column (5 µm particle size, 4.6 × 250 mm i.d.) including a guard column (5 µm particle size, 4.6 × 30 mm) at 25 °C. The flow rate was set to 1.0 mL/min. For gradient elution, mobile phases A and B were employed. Solution A contained 0.1% H_3PO_4 in MilliQ water (pH: 1.2), and solution B contained 0.1% H_3PO_4 in acetonitrile (HPLC grade). For mobile phase B: 0.1% phosphoric acid/acetonitrile) the pH cannot be measured using a pH metre or pH paper (not accurate), there are no free protons to measure.

Data acquisition was applied for 45 min with a total run of 65 min. Gradient elution was as follows: 92% A/8% B, at 0 min; 85% A/15% B at 5 min; 40% A/60% B at 45 min; 40% A/60% B at 55 min; and back to initial conditions 92% A/8% B at 60 min. The PDA was set at 210–600 nm and chromatograms were extracted at 280 nm for monomers of flavanols, 320 nm for hydroxinamic acids, flavonols, stilbenes and 520 nm for anthocyanin mono-glucosides with a pressure of 3000 psi.

2.9 Chemical profile of individual and tentative phenolic compounds by electron spray ionization–mass spectrometry

The mass spectrometry system was a linear triple quad (LTQ) ion trap mass spectrometer (Thermo Scientific) equipped with an electro spray ionization (ESI) source. The negative ion mode (*m*/*z* $M–H^-$) was used for detection of phenolic acids, flavonols flavanols and stilbenes. In addition, positive ion mode (*m*/*z* $M+H^+$) was used for detection of anthocyanin mono-glucosides. The mass scan range was from 100 to 700 *m*/*z*. The MS/MS fragmentation was carried out to determine the charge of state of the phenolic compounds. The identities of the compounds were obtained by matching their molecular ions (*m*/*z*) obtained by ESI-MS/MS with the standards. Nitrogen was used as a drying gas at flow rates of 11 L/mi and pressure was sat 70 psi. Helium was used as collision gas for the high collision dissociation (HCD) at pressure of 3.0×10^{-6}. Mass spectrometry was used only for the quantitative extraction I in whole grape and for 50% acetone for grape pomace.

2.10 Dry weight matter calculations

The equations used to calculate the dry matter were:

% Moisture content

$$\frac{\text{weight of wet sample} - \text{weight of dry sample}}{\text{weight of wet sample}} \times 100 \quad (1)$$

% Total solids

$$\frac{\text{weight of dry sample}}{\text{weight of wet sample}} \times 100 \quad (2)$$

Wet matter

$$\frac{\text{final concentration of phenolic X volume of the solvent extraction used}}{\text{Initial weight}} \quad (3)$$

Dry matter

$$\frac{\text{wet matter}}{\text{total solids}}. \quad (4)$$

2.11 Statistical analysis

Data were analysed using ANOVA to determine differences among solvent means using PROC GLM of Statistical Analysis SAS 9.2 (Cary, NC) version 9.2. (SAS Inst. 2003). Experimental designed was 5 × 2 × 2 factorial in a completely randomised design comparing five organic solvents (50% Methanol, 70% Methanol, 50% Acetone, 0.01% Pectinase and petroleum ether) evaluated in two different group (whole grape and pomace) each with two analytical replicates. Juice sample was not analysed using the experimental design described above because the extraction with the different solvents was not prepared. Means were separated by Tukey' ($P < 0.05$). All experiments were conducted in triplicate.

3 Results and discussion

3.1 Selection of quantitative extraction protocol

Cynthiana grapes analysed in this study were selected because they represent an important cultivar for red winemaking in Oklahoma. This study chose a red wine grape due to their higher phenolic content compared to white/table grapes and because of the availability of pomace as an inexpensive source of extractable material. Phenolic compounds were extracted from grapes and pomace by using different organic solvents.

3.2 Chemical profile of phenolic compounds by RP-HPLC

Several factors such as maximum absorbance, retention time, mobile phases and concentration were studied to develop a method capable of resolving a large (Cowan, 1999) number of the phenolic compounds that are present in grape. This method differed from Thimothe et al. (2007) by increasing the amount of the organic mobile phase (B) from 11% to 15% at 5 min and maintaining that amount of the organic mobile phase in isocratic elution for 40 min. This change allowed all the phenolic compounds investigated in this study to be eluted using a single HPLC method. Fig. 1 represents a typical separation chromatogram of the standards at 280 nm.

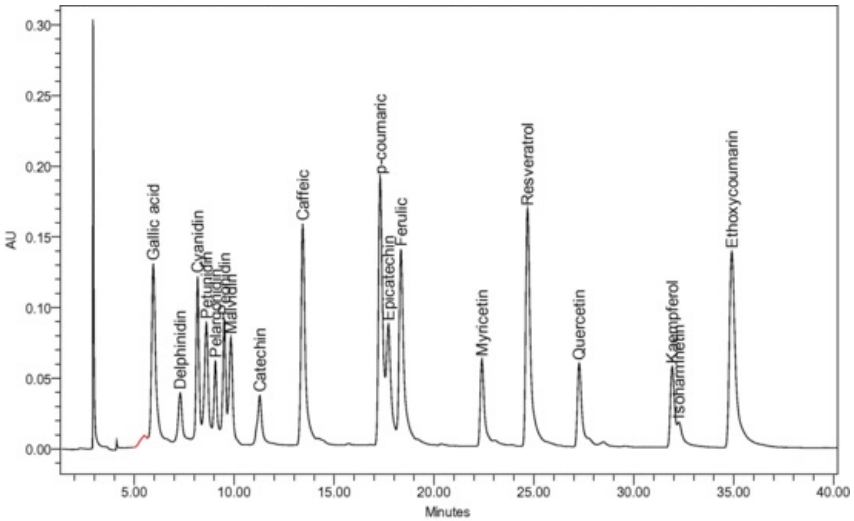

Fig. 1 Typical separation chromatogram of standards mixture at 280 nm (50 ppm).

3.3 Quantitative extraction I (phenolic acids, flavanols, flavonols and stilbenes)

In order to evaluate the effectiveness of the crude extractions on whole grape, juice and pomace, data was collected from a "quantitative" extraction. Quantitative extraction of phenolic compounds from grape has been previously reported (Kammerer et al., 2004; Thimothe et al., 2007). The extractions typically include the utilisation of enzymatic hydrolysis to simplify chromatographic data. The enzyme β-glucosidase from *H. pomatia* Type-HP-2 was used to cleave the sugar moiety off of phenolic glycosides. In addition, it was reported that β-glucosidase contained arylsulfatase activity and can also effectively deconjugate flavonoid glucosides in red fruits (Kähkönen & Heinonen, 2003). Some of the advantages for using enzymatic hydrolysis are: (a) phenolic

compounds may bind to other sample elements such as carbohydrates and proteins. These binds can be hydrolyzed by addition of enzymes, thereby promoting the release of bound phenolics; (b) the addition of enzymes might disintegrate the phenolic-cell wall matrix bonds and enhance phenolic extraction. However, enzymatic hydrolysis requires a specific incubation time and temperature, which delays the extraction procedure.

The phenolic content was based on a dry weight basis. The levels of individual and total PAFFS (phenolic acid, flavanols, flavonols and stilbenes) and FA (flavonoid-anthocyanin mono-glucosides) measured in grape juice; whole grape and pomace are displayed in Tables 1–3, respectively. In juice, 6 of 11 PAFFS were recovered. However, for whole grape only 3 PAFFS were recovered. These results are explained by the amount of soluble solids (8%) in juice than the whole grape (29%) per solid.

Table 1 Content of phenolic compounds identified in grape juice (mg/kg ± RSD) when extracted quantitatively. Results are reported on a dry matter basis. PAFFS = phenolic acids, flavonols, flavanols and stilbenes. FA = Flavonoid-anthocyanin mono-glycosides. Data are the mean for three replications.

	Analyte	Juice
PAFFS	Epicatechin gallate	0.34 ± 0.09
	(+) Catechin hydrate	6.06 ± 0.17
	Caffeic acid	0.07 ± 0.01
	Ferulic acid	0.35 ± 0.15
	Gallic Acid	0.44 ± 0.26
	Isorhamnetin	<0.1
	Kaempferol	<0.1
	Myricetin	<0.1
	p-coumaric acid	0.15 ± 0.09
	Quercetin	<0.1
	trans-resveratrol	<0.1
	Totals	7.41 ± 0.97
FA	Cy3G	910.09 ± 0.34
	Dp3G	17.22 ± 0.15
	Mv3G	194.82 ± 0.06
	Pe3G	118.52 ± 0.41
	Pg3G	67.88 ± 0.26
	Pt3G	499.91 ± 0.17
	Totals	1808.45 ± 140.35

<0.1 Lower than the detection limit 0.1 mg/kg.

Table 2 Content of phenolic compounds identified in whole grape (mg/kg ± RSD) when extracted quantitatively. Results are reported on a dry matter basis. PAFFS = phenolic acids, flavonols, flavanols and stilbenes. FA = Flavonoid-anthocyanin mono-glycosides. Data are the mean for three replications.

	Compound	50% Acetone	70% Methanol	0.01% Pectinase	Petroleum ether	Quantitative extraction I
PAFFS	Epicatechin gallate	12.52 ± 0.92	<0.1	<0.1	<0.1	<0.1
	(+) Catechin hydrate	18.88 ± 0.76	20.79 ± 0.07	55.32 ± 0.27	53.80 ± 0.06	875.83 ± 0.21
	Caffeic acid	2.28 ± 0.46	7.89 ± 1.02	<0.1	3.18 ± 0.14	<0.1
	Ferulic acid	13.06 ± 1.56	8.11 ± 1.09	5.00 ± 0.64	15.26 ± 0.02	<0.1

	Gallic Acid	<0.1	7.20 ± 0.06	5.46 ± 0.28	<0.1	115.90 ± 0.12
	Isorhamnetin	<0.1	<0.1	<0.1	<0.1	<0.1
	Kaempferol	1.50 ± 0.01	1.73 ± 0.02	<0.1	1.78 ± 0.08	<0.1
	Myricetin	0.88 ± 0.01	1.43 ± 0.15	<0.1	0.98 ± 0.18	<0.1
	p-coumaric acid	8.18 ± 0.58	15.18 ± 1.27	2.66 ± 0.83	10.40 ± 0.02	<0.1
	Quercetin	2.63 ± 0.06	<0.1	1.04 ± 0.09	0.67 ± 0.09	33.74 ± 0.18
	trans-resveratrol	<0.1	<0.1	<0.1	<0.1	<0.1
	Totals	59.76 ± 2.24[b]	62.35 ± 2.64[b]	69.48 ± 9.48[b]	86.09 ± 0.37[b]	1025.49 ± 268.05[a]
FA	Cy3G	29.88 ± 0.0	23.50 ± 0.34	28.25 ± 1.26	2.74 ± 0.39	68.58 ± 0.43
	Dp3G	8.04 ± 0.09	<0.1	8.09 ± 0.33	1.69 ± 0.50	46.88 ± 0.86
	Mv3G	2.56 ± 1.07	3.43 ± 0.27	9.16 ± 1.28	3.27 ± 0.73	3.69 ± 0.56
	Pe3G	<0.1	<0.1	<0.1	<0.1	45.67 ± 0.32
	Pg3G	26.08 ± 0.16	<0.1	<0.1	3.55 ± 0.04	4.07 ± 0.11
	Pt3G	53.60 ± 0.63	88.99 ± 0.96	228.36 ± 0.66	273.93 ± 0.03	192.73 ± 0.48
	Totals	120 ± 8.32[b]	115.93 ± 25.83[b]	273.88 ± 53.50[b]	285.19 ± 54.22[b]	362.48 ± 28.49[a]

[a,b]Means with similar letter are not significantly different (Tukey, $P > 0.05$).

<0.1 Lower than the detection limit 0.1 mg/kg.

Table 3 Content of phenolic compounds identified in grape pomace (mg/kg ± RSD) when extracted quantitatively. Results are reported on a dry matter basis. PAFFS = phenolic acids, flavonols, flavanols and stilbenes. FA = Flavonoid-anthocyanin mono-glycosides. Data are the mean for three replications.

	Analyte	50% Acetone	70% Methanol	0.01% Pectinase	Petroleum Ether	Quantitative extraction I
PAFFS	Epicatechin gallate	378.14 ± 0.76	80.94 ± 0.54	44.92 ± 0.27	103.05 ± 0.15	<0.1
	(+) Catechin hydrate	1738.89 ± 0.87	356.01 ± 1.56	2293.45 ± 0.40	98.87 ± 0.21	198.84 ± 0.07
	Caffeic acid	438.43 ± 0.54	33.40 ± 1.05	198.50 ± 0.66	36.68 ± 0.47	<0.1
	Ferulic acid	1.33 ± 0.87	<0.1	0.27 ± 0.19	<0.1	2.72 ± 0.03
	Gallic Acid	95.36 ± 0.61	7.38 ± 0.48	118.66 ± 0.28	93.87 ± 0.18	115.94 ± 0.13
	Isorhamnetin	131.51 ± 1.20	2.79 ± 0.34	54.21 ± 0.71	67.36 ± 0.84	14.02 ± 1.64
	Kaempferol	28.53 ± 1.02	3.63 ± 0.49	52.23 ± 0.51	53.25 ± 0.56	27.50 ± 0.08
	Myricetin	36.77 ± 1.02	23.40 ± 0.96	14.64 ± 0.28	34.76 ± 1.18	8.43 ± 0.01
	p-coumaric acid	214.55 ± 0.73	24.11 ± 0.59	22.19 ± 0.45	42.77 ± 0.50	3.20 ± 0.18
	Quercetin	26.25 ± 0.86	3.06 ± 1.10	30.54 ± 0.38	60.08 ± 0.76	18.55 ± 0.64
	trans-resveratrol	20.66 ± 0.89	5.60 ± 0.37	11.27 ± 0.27	36.58 ± 0.64	2.05 ± 0.06
	Totals	3110.43 ± 152.41[a]	540.31 ± 34.39[b]	2840.89 ± 204.2[a]	627.29 ± 8.54[b]	391.26 ± 22.78[c]
FA	Cy3G	10013.29 ± 1.44	489.63 ± 0.95	8510.22 ± 0.29	84.22 ± 0.23	14.34 ± 0.11
	Dp3G	2648.69 ± 0.98	324.46 ± 1.25	422.51 ± 0.68	189.00 ± 0.58	25.45 ± 0.09
	Mv3G	7289.32 ± 1.05	502.94 ± 1.46	2055.25 ± 0.25	101.68 ± 0.69	21.55 ± 0.03

	Pe3G	922.74 ± 1.08	97.76 ± 1.25	333.04 ± 0.42	120.71 ± 0.54	5.37 ± 0.18
	Pg3G	219.16 ± 0.97	38.70 ± 1.34	114.80 ± 0.21	36.33 ± 0.41	2.35 ± 0.01
	Pt3G	1610.64 ± 1.06	105.90 ± 1.42	10640.45 ± 0.28	93.95 ± 0.45	6.56 ± 0.08
	Totals	22703.85 ± 9.68[a]	1559.42 ± 84.64[b]	22076.28 ± 1.90[a]	625.89 ± 20.47[b]	80.90 ± 4.03[c]

[a,b,c]Means with similar letter are not significantly different (Tukey, $P > 0.05$).

<0.1 Lower than the detection limit 0.1 mg/kg.

For pomace, 9 of 11 PAFFS were recovered. The highest recovered PAFFS compound in whole grape was (+)-catechin hydrate. It was also the highest recovered in pomace, but at a level that was more than 4 times lower than in whole grape sample. The PAFFS concentration of the analytes measured in juice was 7 mg/kg dry matter. The results obtained in the present study were comparable with previous studies by Stalmach, Edwards, Wightman, and Crozier (2011), who found concentrations of PAFFS and FA in grape juice of 8.4 mg/g and 680 mg/g. PAFFS concentration of the analytes measured in whole grape was 1025 mg/kg dry matter, which they are in agreement with previous results found in Norton grape (Goodman, Martens & Weeden, 1993; Rivera-Dominguez, Yahia, Wlodarchak, & Kushad, 2010). In the grape pomace and pomace PAFFS were the major phenolic compounds accounting for 74% and 86%, respectively all phenolic compounds measured. In contrast, PAFFS only comprised a minor proportion (<1%) of total phenolic compounds in grape juice. The PAFFS concentration of the analytes measured in pomace was 391 mg/kg dry matter, which is in agreement with the results found by Hogan, Canning, Sun, Sun, and Zhou (2010), who reported total phenolic composition of 475.5 mg/g dry matter in Norton grape pomace extract.

3.4 Quantitative extraction II (flavonoid-anthocyanin mono-glucosides)

Anthocyanin mono-glucosides are the most common pigments in nature and can be extracted with acidified solvents like water, acetone, ethanol, methanol or mixtures of aqueous solvents. They are found in nature as anthocyanidin glycosides. The acid in the solvents acts to rupture cell membranes and release anthocyanins; however, this harsh chemical treatment may break down the innate anthocyanin structure. It is therefore important to acidify solvents with organic acids (formic or acetic acid) rather than mineral acids such as 0.1% HCl.

In the past, several studies have classified more than fifteen anthocyanidin glycones (Harborne & Williams, 2000). In this study only anthocyanin mono-glucosides were identified and corresponded to cyanidin 3-*O*-glucoside (Cy3G), delphinidin 3-*O*-glucoside (Dp3G), malvidin 3-*O*-glucoside (Mv3G), pelargonidin 3-*O*-glucoside (Pg3G), peonidin 3-*O*-glucoside (Pe3G), and petunidin 3-*O*-glucoside (Pt3G).

In juice, FA monoglucosides were the most abundant phenolic compounds recovered by this extraction technique. The main FA quantified in juice sample were: Mv3G, Pe3G, De3G, Pg3G and Pt3G. The FA concentration of the analytes measured in juice was 1808 mg/kg dry matter. Fig. 3 shows a typical separation of anthocyanin mono-glycosides in juice (A) at 520 nm.

The recovery of FA was higher than for PAFFS in whole grape. Thus, the use of 50% methanol/0.1% HCl as a solvent for whole grape resulted in a significantly higher extraction of FA than for any other solvent evaluated ($P < 0.05$) (Table 2). The concentration of FA using this is extraction method was 362 mg/kg dry matter. The results of FA concentration were similar to studies using Norton grape by Cho (Cho, Howard, Prior, & Clark, 2004), who reported to have 358 mg/kg of total anthocyanin mono-glucosides in Cynthiana grape. The higher efficiency of methanol/0.1% HCl is a consequence of the association of phenolic compounds with cell wall polymers and could be partially explained by the capacity to degrade cell walls and seeds, which have unpolar character and cause phenolic compounds to be released from cells (O'Neil, 2006). As can be noticed in grape pomace, the recovery of FA was lower than for PAFFS. Hogan found that total anthocyanin mono-glucosides were lower than total phenolics in Norton (*Vitis aestivalis*) with values of 0.93 mg/g C3GE (Cyanidin 3-glucoside equivalent) and 1.82 mg/g GAE (Gallic acid equivalent), respectively. The concentration of FA was 67.35 mg/g dry matter (Hogan et al., 2010).

The whole grape was detected to have the highest recovery of FA (362 mg/kg) than grape pomace (80 mg/kg dry matter) ($P > 0.05$). These differences suggest a potential impact of winemaking process on FA content.

3.5 Chemical profile of individual and tentative phenolic compounds by electron spray ionization–mass spectrometry

The main objective of this study was to identify and quantify the main phenolic compounds in Cynthiana grape including flavonoids and non-flavonoids under the same HPLC. These compounds are found in any red grape and their by-products. This study only focused in 16 main phenolic standards (monomers of flavanols, flavonols, hydroxynamic acids, stilbenes and anthcoyanins monoglucosides) because they are quite expensive and the budget needed it to be distributed for other materials in the research.

Table 4 shows the mass data of PAFFS and FA compounds extracted from whole grape using quantitative extractions (I and II). Additionally, the presence of other main phenolic compounds was determined using quantitative extraction I. These compounds included: hydroxybenzoic acids (quinic acid, syringic acid, vanillic acid, p-hydroxybenzoyl glucoside, 3,4 dihydroxyphenylacetic acid); hydroxycinnamic acids (caffeoylshikimic acid, caftaric acid, cinnamic acid, fertaric acid); flavanones (naringenin). These findings are similar to the non-flavonoid content found by previous studies (Hogan et al., 2009). Hogan reported that phenolic composition of Norton (Vitis aestivalis) grape was dominated by hydroxybenzoic acids (gallic acid, syringic acid

and vanillic acid) and hydroxycinnamic acids (caftaric and p-coumaric acid). For identification of FA not only mono-glucosides were detected in Norton (Vitis aestivalis) grape but also additional flavonoid compounds were identified using quantitative extraction II (Table 4). These findings were in agreement with previous studies conducted on Norton grape. Ali reported that the presence of major anthocyanin di-glucosides, acylated anthocyanins and coumaroylglucoside anthocyanins are characteristic of the methanol acidified HCl in Norton grape (Ali et al., 2011).

Table 4 Identification of phenolic compounds in whole grape extracts (quantitative extractions I, II) by mass spectrometry.

	Tentative Identification	MS (*m/z*)	MS/MS ions	MW
PAFFS $[M]^-$	(+) Catechin hydrate[a]	303	169	304
	Gallic acid[a]	169	125	170
	Quercetin[a]	301	273/179/151/107	302
	p-hydroxybenzoic acid	137	93	138
	Coniferyl aldehyde	177	149/133/105/89/77	178
	Vanillic acid	167	123/107	168
	3,4 dihydroxyphenylacetic acid	167	125/123/107/99/89	168
	Cinnamic acid	147		148
	Syringic acid	197	153/182	198
	p-hydroxybenzoyl glucoside	299	239/179/137	300
	Quinic acid	191	173/127/111/85	192
	Naringenin	271	177/151/119	272
	Vanillic acid glucoside	329	167	330
	p-coumaric acid glucoside	325	265/205/163/145/119	326
	Fertaric acid	325	193/133/87	326
	Caftaric acid	311	179	312
	Caffeoylshikimic acid	335	179/161/135	226
	Bis-HHDP-hexose	391	481/301/257	392
	Casuricti/Potentillin like ellagitannin	467	633/467/391/301	468
	Quercetin 3-*O*-glucoside	463	301	464
FA $[M]^+$	Cyanidin 3-*O*-glucoside[a]	449	287	448
	Delphinidin 3-*O*-glucoside[a]	465	303	464
	Malvidin 3-*O*-glucoside[a]	493	331	492
	Pelargonidin 3-*O*-glucoside[a]	433	271	432
	Petunidin 3-*O*-glucoside[a]	479	317	478
	Peonidin 3-*O*-glucoside[a]	462	301	461
	Peonidin 3-*O*-*cis*-*p*-coumarylglucoside	609		608
	Petunidin 3-(6″-acetylglucoside)	521	317	520
	Delphidin 3,5-diglucoside	627	465/303	626
	Delphinidin 3-*O*-acetylglucoside	507	303	506

	Cyanidin 3,5-diglucoside	611	449/287	610
	Peonidin 3,5-diglucoside	625	463/301	624
	Malvidin 3,5-diglucoside	655	493/331	654
	Delphinidin 3-arabinose	435	303	434
	Malvidin 3-*(*acetylglucoside)	535	331/316	534
	Cyanidin 3-(acetylglucoside)	491	287	490
	Cyanidin 3-*O*-*p*-coumarylglucoside	595	287	594
	Petunidin 3-sophoroside	641		640
	Delphinidin 3-*O*-(6-*O*-*p*-coumaroyl) glucoside glucosideglucosidegglglucosideglucoside	611	303	640
	Peonidin 3-*O*-(6-O-*p*-coumaroyl) glucoside	625	317	624
	Peonidin-malonylglycoside	549	463/301	548
	Malvidin 3-*O*-(6-acetylglucoside)-5-glucoside(glglucosideglglucosideglucosideglucoside *cis*	697	535/493/331	696
	Malvidin 3-*O*-rutinoside	639	331	638

PAFFS $[M]^-$ Negative-ion mode for phenolic acids, flavanols, flavonols and stilbenes.

FA $[M]^+$ Positive-ion mode for anthocyanins.

[a] Identified using the corresponding authentic standards.

3.6 Crude extracts

Extraction of phenolic compounds from grape using acetone: water, methanol: water and water has been previously reported (Lapornik, Prošek, & Wondra, 2005). The effectiveness of crude extractions in recovering PAFFS and FA from whole grape and pomace was measured by comparing results to the previously described quantitative extractions. Significant differences were found among the solvents used for extraction of phenolic compounds in whole grape ($P < 0.05$) (Table 2). In whole grape, all crude extracts produced a higher recovery of FA than for PAFFS. Statistically, there were not significant differences among the crude solvents for whole grape ($P < 0.05$). The concentration of FA using petroleum ether showed the highest recovery 285 mg/kg dry matter and PAFFS 86 mg/kg dry matter. However, there was not significant difference in mean recoveries using petroleum ether ($P < 0.05$).

As can be noticed in grape pomace, significant differences were found among the solvents ($P < 0.05$) (Table 3). The highest recovery of FA and PAFFS were observed using 50% acetone and 0.01% pectinase. The highest concentration of FA and PAFFS using acetone was 22,703 mg/kg and 3110 mg/kg dry matter, respectively. Yilmaz and Toledo (2006) compared methanol, ethanol and acetone water mixtures for extracting phenolic compounds from grape pomace, and they found recoveries of phenolic compounds were higher using acetone. Extractions of FA and PAFFS using 0.01% pectinase yielded to concentration of 22,076 mg/kg and 2840 mg/kg dry matter, respectively. Fig. 3 shows a typical separation of anthocyanin mono-glucosides in grape pomace (C) at 520 nm. Table 5 shows the mass data of PAFFS and FA compounds extracted from grape pomace using crude extraction (50% acetone). Additional PAFFS determined in grape pomace included: hydroxybenzoic acids (quinic acid, syringic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde acid); hydroxycinnamic acids (caftaric acid); phloterin, procyanidin B1, phloridzin. These results were compared to previous studies. Hogan reported similar composition of phenolic acid of Norton (*Vitis aestivalis*) grape pomace (Hogan et al., 2010). The content of FA in grape pomace were less than PAFFS. These findings are similar to the HPLC results presented in this study. However, the anthocyanin di-glucosides and acylated anthocyanins were not found in the grape pomace. This inconsistency might be attributed to the extraction solvent and again to the potential impact of winemaking process on FA.

Table 5 Identification of phenolic compounds in grape pomace extracts (50% acetone) by mass spectrometry.

	Tentative Identification	MS (*m*/*z*)	MS/MS ions	MW
PAFFS $[M]^-$	(+) Catechin hydrate[a]	303	169	304
	Gallic acid[a]	169	125	170
	Quercetin[a]	301	273/179/151/107	302
	p-coumaric acid[a]	163	119	164

	Ferulic acid[a]	193	134	194
	Laricitrin 3-*O*-glucoside	493	331/330/179	494
	Myricetin[a]			
	317	179/151/121	318	
	Kaempferol[a]	285	151/107	286
	Isoharmnetin[a]	315	300/165	316
	p-hydroxybenzoic acid	137	107/93/79/53	138
	Quinic acid	191	173/127/111/85	192
	p-hydroxybenzaldehyde	121	92	122
	Syringic acid	197	169/125/97/81	198
	Phloretin	273	167	164
	Isoquercitrin	463	301/179/151	464
	Caftaric acid	311	179	312
	Kaempferol acetyl hexoside	489	447/327/285/255	490
	Phloridzin	435	273	436
	Casuricitin/Potentillin like ellagitannin	467	633/467/391/301	936
	Procyanidin B1	577	451/425/407/289	578
FA $[M]^+$	Cyanidin 3-*O*-glucoside[a]	449	287	448
	Delphinidin 3-*O*-glucoside[a]	465	303	464
	Malvidin 3-*O*-glucoside[a]	493	331	492
	Pelargonidin 3-*O*-glucoside[a]	433	271	432
	Petunidin 3-*O*-glucoside[a]	479	317	478
	Peonidin 3-*O*-glucoside[a]	462	301	461
	Acetone derivative of peonidin 3-*O*-glucoside	501		500
	Type B visitin of petunidin 3-*O*-glucoside	503	369/353	502
	Petunidin 3-(6-*O*-p-coumaryl) glucoside	641	331	640
	Malvidin 3-*O*-rutinoside	639	331	638

PAFFS $[M]^-$ Negative-ion mode for phenolic acids, flavanols, flavonols and stilbenes

FA $[M]^+$ Positive-ion mode for anthocyanins.

[a] Identified using the corresponding authentic standards.

3.7 Comparison of solvent recovery of phenolic compounds on whole grape and pomace

Solvent extraction is traditional and preferred method for the recovery of phenolic compounds from plant matrices. Regarding to extraction solvents, there are two other important parameters that affect the yield of phenolics extracted from plant foods: time and temperature. Most of the time, increasing time and temperature promote analyte solubility; however, plant phenolics are generally degraded or undergo undesirable reactions such as enzymatic oxidation by extended extraction times and high temperatures. Increasing the solvent-to-sample ratio promotes phenolic extraction from plant samples but determining the optimum ratio is advisable so that solvent input and saturation effects of solvent by the phenolics are minimized.

Organic solvents are indeed the most common and effective solvents for extracting phenolic compounds, however, they are an environmental pollutant and more toxic than other solvents.

Limited information exists on the phenolic composition of whole grape and grape pomace from Cythiana grape. To our knowledge this is the first study that compare the original plant (whole grape) with its by-products (juice and pomace) in this variety of grape. However, when sample is fermented and dried (grape pomace), with less water in the system, the crude solvents appear to be much more effective. It is possible the fermentation process changes the susceptibility of the original fibre in the grape to pectinase. It is also possible that the phenolic compounds are bound in the whole grape system, making the crude extracts ineffective, but more accessible for extraction after fermentation.

Several researchers have used organic solvent–water mixture for the extraction of the phenolic compounds in red grapes (Martinez Vidal, Belmonte Vega, Garrido Frenich, Egea Gonzalez, & Arrebola Liebanas, 2004; Negro, Tommasi, & Miceli, 2003) but only few of them have directly compared the ability of different solvents to recover phenolic compounds (Ju & Howard, 2003; Lapornik et al., 2005). Lapornik, Prošek and Wondra compared 70% methanol and water for extracts prepared by plant by-products (Lapornik et al., 2005) and they found that 70% methanol was the most effective on recovery of by-products from natural matrices. Vatai compared different concentrations of acetone, ethyl acetate, and ethanol for extracting phenolic compounds from grape and they found that 50% acetone was the most effective on recovery (Vatai et al., 2008). In this study, there was not significant difference between 50% acetone and 70% methanol $P > 0.05$ (Table 1) for whole grape sample. However, in grape pomace sample using 50% acetone was significant different from the others treatments $P < 0.05$. This behaviour can be explained by the mixture acetone–water (50–50), which can make more hydrogen bonds.

In this study, mass spectrometry extractions with methanol/enzymatic/acid hydrolysis were found to generate extracts with more phenolic compounds compared to acetone–water.

The Quantitative extractions I & II (of whole grapes recovered more PAFFS and FA than all other solvents evaluated (Tables 2 and 3; $P < 0.05$). However, this was not the case for the pomace extraction. In fact, both the QI and QII extractions of pomace performed poorly in the recovery of phenolic compounds. There was no difference in the efficiency of the crude extracts to recover phenolic compounds from whole grapes and their poor recoveries suggest they are not good alternatives to QI and QII for this particular application. However, this is not the case for their application on pomace. With pomace, QI and QII showed very low recoveries of phenolic compounds. On the other hand, all crude extract solvents were superior in recovering phenolic compounds from pomace than QI and QII. The recovery of PAFFS and FA from pomace (Table 3), 50% acetone and 0.01% pectinase were most effective ($P < 0.05$).

There is a lot of controversy in the efficiency of recovery of phenolic compounds using aqueous acetone vs aqueous methanol in grapes. The majority of the studies for extraction of anthocyanins use acidic/methanol (references) because the acid in the solvent acts to rupture cell membranes and release anthocyanins; single aglycone can free the aglycone after acid hydrolysis facilitying a single HPLC peak.

The hypothesis to explain our results is referred to the Hansen solubility parameter values, which are based on δD (dispersion bonds). However, the results in this study cannot be explained only by difference in solubility. Many variables are involved using plants and their respective by-products. Temperature, pH, agriculture practises are some of the variables that we did not measure in this study.

Acetone and water are known for having high δD both with values of 15.5. Polar solvents like water tends to charge negatively solutes via hydrogen bonding and acetone tends to have a large dipole moment (separation of partial positive and partial negative charges within the same molecule. The value of the dipole moment for water and acetone are 1.85D and 2.88D, respectively; and the values for methanol and petroleum ether are 1.70D and 1.15D, respectively (Hansen, 2000). Neither methanol nor petroleum ether were as effective as the 50% acetone and 0.01% pectinase. The PAFFS and FA values of extracts when using 50% acetone solvent was the highest (3110 mg/kg and 22,703 mg/kg, dry matter). Other researchers have reported that grape pomace not only has a high content of FA compared to the other samples, but also appears to have a higher content of unknown compounds (Thimothe et al., 2007). Similar observations were made in the current study (Fig. 3). These unknown peaks were also detected on whole grape sample using acid hydrolysis, which can be attributed to anthocyanin 3, 5-diglucosides, which are present in abundance in grapes of *Vitis* species (Hogan et al., 2010). The mass spectrometry results showed the presence of these compounds in quantitative extraction II.

4 Conclusions

In summary, a simultaneous method was developed for the identification and quantification of flavonoid compounds (anthocyanin mono-glucosides) and non-flavonoid compounds (phenolic acids, flavonols, flavanols, and stilbenes) in Cynthiana juice, whole grape and pomace using different solvent extraction methods. It appears that the quantitative extractions (I and II) work well for a high water sample (whole grape) than a dried sample (grape pomace). The crude solvents do not seem to work very well for a high water, unfermented product. The advantages of quantitative extraction (I and II) in terms of simplicity of interpretation and quantification are apparent as seen in HPLC of grape pomace, where simplified the complex phenolic profiles dramatically. There is a considerable variation of the glycosidic bond under hydrolytic conditions. The rate of hydrolysis of glycosides depends on acid/base strength, the nature of the sugar and the position of attachment to the flavonoid nucleus. On the other hand, enzymatic hydrolysis is made from commercial pectolytic enzymes, which are commonly prepared from the extracellular material of *Aspergillus* or *Trichoderma* species. These enzymes preparations are rather crude, containing several pectinase activates as well as containing several side activities that may attack both the phenolic glycoside bonds and other bonds in the plant material.

For grape pomace polar solvents were superior in recovering phenolic compounds compared to quantitative extractions and it is explained by the polarity and contact time after fermentation process where this two factors promote analyte solubility. The tentative identification of the unknown molecules in quantitative extractions (Hogan et al., 2009) for whole grape and crude extraction (50% acetone) for grape pomace was determined by mass spectrometry. It was generally observed in whole grape that more flavonoid compounds (Cho et al., 2004), were recovered using acid hydrolysis than the crude extraction. In addition, extraction protocols for determination of the proanthocyanidins are needed in order to determine the concentration of these compounds in Cynthiana grape.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.10.078.

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Appendix A. Supplementary data

Supplementary data 1 This document file contains Supplementary figures.

Multimedia Component 1

Highlights

- Phenolic compounds were isolated from juice, whole grape and pomace of Cynthiana.
- Comparison of enzymatic, acid hydrolysis, and polar solvents for extraction.
- The identification and quantification of phenolic compounds by HPLC.
- ESI–MS was used to confirm the presence of phenolic/unknown compounds.
- Separation of non-flavonoid and flavonoid compounds under simultaneous method.

Queries and Answers

Query: Please confirm that given names and surnames have been identified correctly.

Answer: Yes. The names and surnames are correct.

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Query: The country name has been inserted for all the affiliations. Please check, and correct if necessary.

Answer: The country is correct.

Query: The affiliations ‘a’ and ‘c’ have been split into two different affiliations. Please check, and correct if necessary.

Answer: The split for the affiliations is correct.

Query: Reference “Muñoz-Espada, Wood, Bordelon, and Watkins, 2004” is cited in the text but not provided in the reference list. Please provide it in the reference list or delete this citation from the text.

Answer: Muñoz-Espada, A. C.; Wood, K. V.; Bordelon, B.; Watkins, B. A. Anthocyanin quantification and radical scavenging capacity of Concord, Norton, and Marechal Foch grapes and wines. *J. Agric. Food Chem.* **2004**, *52*, 6779-6786.