Optimization of β -cyclodextrin-based flavonol extraction from apple pomace using response surface methodology

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Revised: 18 January 2014 / Accepted: 4 February 2014 / Published online: 15 February 2014
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Abstract The present study investigated five cyclodextrins (CDs) for the extraction of flavonols from apple pomace powder and optimized β -CD based extraction of total flavonols using response surface methodology. A 2^3 central composite design with β -CD concentration (0–5 g 100 mL⁻¹), extraction temperature (20–72 °C), extraction time (6–48 h) and second-order quadratic model for the total flavonol yield (mg 100 g⁻¹ DM) was selected to generate the response surface curves. The optimal conditions obtained were: β -CD concentration, 2.8 g 100 mL⁻¹; extraction temperature, 45 °C and extraction time, 25.6 h that predicted the extraction of 166.6 mg total flavonols 100 g⁻¹ DM. The predicted amount was comparable to the experimental amount of 151.5 mg total flavonols 100 g⁻¹ DM obtained from optimal β -CD based parameters, thereby giving a low absolute error and adequacy of fitted model. In addition, the results from optimized extraction conditions showed values similar to those obtained through previously established solvent based sonication assisted flavonol extraction procedure. To the best of our knowledge, this is the first study to optimize aqueous β -CD based flavonol extraction which presents an environmentally safe method for value-addition to under-utilized bio resources.

Keywords Cyclodextrin · Flavonols · Apple pomace · Response surface method · Value-addition · Flavonoid

Introduction

There have been numerous studies demonstrating the health benefits of flavonoids, which are mostly known for their antioxidant and anti-inflammatory properties, as well as the benefits they provide for cardiovascular health (Alejandro et al. 2013; Izzi et al. 2012; Macedo et al. 2013; Perez-Vizcaino and Duarte 2010). Due to their immense diversity, they are divided into subgroups including flavonols, flavanols, flavones, flavanones, isoflavones, anthocyanidins and chalcones. Among them, flavonols and flavan-3-ols are the most abundant and most widely distributed flavonoids in nature (Perez-Vizcaino and Duarte 2010). The chemical structures of flavonols play a large part in their antioxidant activities and a study by Mercader-Ros et al. (2010) shows that among the flavonols, there is an inverse relationship to the number of hydroxyl groups in the B ring and their antioxidant activity.

More than 5,500 t of apple processing by-products are produced every year in Nova Scotia, Canada (Rupasinghe 2003). Among these by-products, apple pomace (consisting of a mixture of peel, core, seed, calyx, stem and soft tissue) is the major processing waste. Due to the presence of high amounts of bioactive polyphenols with health-promoting properties, apple pomace has received considerable attention in the last decade. The polyphenols found in pomace are primarily flavonoids and phenolic acids, specifically chlorogenic acid, epicatechin and phloridzin (Rupasinghe and Kean 2008). In addition, apple peel is also a good source of specific flavonols such as quercetin glycosides (Huber and Rupasinghe 2009). Despite these facts, large quantities of these by-products are being generated and disposed as waste, thereby posing serious environmental challenges. With an increasing global focus on more efficient utilization of natural resources, it is critical to look for alternative uses for these fruit processing by-products. Development of cost-effective

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methods to extract natural compounds having health promoting properties would add high value to these co-products.

Conventionally, extraction of phenolic compounds from plant materials including apples, employs the use of solvents such as methanol, ethanol, acetone or their combination with water (Alberti et al. 2014); however, these solvents have demonstrated serious environmental effects. Alternative “green” techniques being employed include supercritical fluid extraction, pressurized liquid extraction, emulsification, coacervation, inclusion complexation, nano-precipitation, solvent evaporation, non-thermal and microwave-assisted extraction (Ezhilarasi et al. 2013; Routray and Orsat 2012; Wijngaard et al. 2012). Nevertheless, these techniques require advanced equipment and can be costly procedures to execute. Previously, a fractionation method for the extraction of polyphenols from apple pomace using water at room temperature was demonstrated by Reis et al. (2012). The study showed that although water was an effective reagent for extraction of high amounts of polyphenols, it was ineffective in extracting the quercetin glycosides.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of several α -1,4-D-glucopyranose rings (Chen et al. 2012). The cylindrical structure is composed of a hydrophilic exterior and an internal hydrophobic cavity (Ferreira et al. 2010), a property that allows CDs to form inclusion complexes with organic molecules through host-guest interactions. The binding interactions within the CD-guest complex are most likely a summation of a variety of relatively weak forces such as Van der Waal forces, hydrophobic interactions and hydrogen bonding (Lofstsson and Brewster 1996). Emerging extraction technologies such as CD show great promise as these methods have been associated with reduced extraction time and lower solvent requirements. CDs are regarded as GRAS substances for their use as flavor carriers and/or flavor protectants and solubility enhancers in some food categories with their maximal concentrations. However, standard procedures for the extraction and stabilization of bioactive compounds using these technologies have yet to be developed in order to achieve greater yield, thereby promoting their commercial use as natural food additives and functional food ingredients. A recent study by Ratanasooriya and Rupasinghe (2012) demonstrated the extraction of polyphenols from grape pomace using cyclodextrin technology, showing a recovery of 70 % polyphenols compared to organic solvent-based extractions.

Although there have been several studies focusing on flavonoid encapsulation in different types of CDs for improving quality (Karangwa et al. 2012), physico-chemical stability (Chao et al. 2012; Mercader-Ros et al. 2010), solubility (Lucas-Abellán et al. 2011) and bio-availability (Šmidovnik et al. 2010), to the best of our knowledge, there are no reports available on the use of CDs in recovering flavonols from apple pomace. Therefore, the current study was carried out to

compare the flavonol extraction efficiencies of α -, β -, γ -CDs, two derivatives of β -CD and solvents from apple pomace and to optimize β -CD-based flavonol extraction conditions using response surface methodology.

Material and methods

Materials and reagents

Apple pomace, typically containing peel, core and seeds, was collected from a commercial apple juice manufacturer, J. W. Mason and Sons Ltd., Windsor, NS, Canada during the year 2011 and was from ‘Idared’ cultivar, which was stored at -20°C until use. The pomace was dried at 42°C for 72 h and milled to obtain a fine powder using a coffee grinder (Model 6378-33, Sunbeam Products Inc., Boca Raton, FL, USA.).

α -, β -, γ -, hydroxypropyl-(HP β), and randomly methyl-(RM β) CDs, were obtained from Cyclodextrin Technologies Development, Inc. (High Springs, FL, USA). Ethanol, methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Ottawa, ON, Canada). The liquid chromatography standards were purchased as follows: phloridzin, phloretin, chlorogenic acid and caffeic acid from Sigma Aldrich (Oakville, ON, Canada); catechin, epicatechin, quercetin and quercetin-3-*O*-glucoside from ChromaDex Inc. (Santa Ana, CA, USA); quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside and quercetin-3-*O*-galactoside from Indofine Chemical Company (Hillsborough, NJ, USA).

Determination of moisture

Dry matter content of the powder was carried out by oven drying at 105°C until a constant weight was achieved. All the results were expressed on dry matter (DM) basis.

Cyclodextrin-based extraction of flavonols

α -, β -, γ -, HP β -, and RM β -CDs were compared for the recovery of total flavonols from apple pomace. Concentration of each solution was 0.025 mol L^{-1} (α -CD = $2.4\text{ g } 100\text{ mL}^{-1}$; β -CD = $2.8\text{ g } 100\text{ mL}^{-1}$; γ -CD = $3.25\text{ g } 100\text{ mL}^{-1}$, HP β -CD = $3.5\text{ g } 100\text{ mL}^{-1}$; RM β -CD = $3.3\text{ g } 100\text{ mL}^{-1}$). CD solutions were prepared by adding their measured weight to the required volume of deionized water and kept in amber colored vials with tight fitting lids in a shaking water bath (70 rpm) at 55°C until CDs were completely dissolved. Apple pomace powder of 0.5 g was mixed with 10 mL of CD solution and vortexed before placing them in a shaking incubator at 60°C for 24 h. Deionized water without CD was used as the control. All the experiments were carried out in triplicates.

Solvent-based extraction of flavonols

To 0.5 g of powdered apple pomace sample, 10 mL of 70 % and 100 % methanol was added; the mixture was vortexed and placed in an ultrasonic bath for 15 min. durations for three times (total of 45 min.) with 10 min intervals (Rupasinghe et al. 2011). The sample was then centrifuged at $8,000\times g$ for 10 min followed by separation of supernatant. Two mL of supernatant was filtered using 0.2 μm nylon filters and placed in vials for liquid chromatography mass spectrometry (LCMS) analysis.

Optimization of total flavonol extraction using β -CD

In a 50 mL Erlenmeyer flask, 0.5 g of apple pomace powder and 10 mL of X_1 (0–5 g/100 mL) concentration β -CD solution were added. The required concentration of β -CD solution was prepared as described above. The samples were vortexed for 30 s before placing them at X_2 (20–72 °C) temperature conditions for X_3 (6–48) hours. After each experimental run, the samples were immediately centrifuged at 3,500 rpm for 10 min and supernatant was stored in amber colored vials at –20 °C for further analysis.

Phenolic characterization by liquid chromatography mass spectrometry (LC/MS)

Samples were prepared and analyzed using high performance liquid chromatography coupled to mass spectrometry (uHPLC-MS/MS) as described elsewhere (Ratnasooriya et al. 2010; Ratanasooriya and Rupasinghe 2012). Briefly, samples were prepared by thawing at room temperature, until a complete liquid state had been reached. They were then vortexed for 30 s. each to ensure complete mixing. A sample of 1 mL was added to 1 mL of 100 % methanol, into eppendorf tubes. Samples were vortexed once again prior to centrifugation at 3,000 rpm for 10 min. The supernatant was filtered through 0.45 μm nylon filters into HPLC vials and processed through a Waters Aquity uHPLC system containing a Waters Model code CHA Separations Module and an Aquity UPLC BEH C18 column (2.1 $\times$ 100 mm, 1.7 μm ; Waters, Milford, MA, USA). A gradient elution was carried out with 0.1 % formic acid in water (Solvent A) and 0.1 % formic acid in acetonitrile (Solvent B) at a flow rate of 0.2 mL/min. A linear gradient profile was used with the following proportions of Solvent A applied at time t (min); (t , A%): (0, 90 %), (6, 50 %), (8, 35 %), (10, 10 %), (15, 90 %), (20, 90 %). For each sample, an injection volume of 2 μL was used with a run time of 20 min. MS-MS analysis was performed with a Micromass Quattro micro API MS/MS system, which is controlled by MassLynx V4.1 data analysis system (Micromass, Cary, NC, USA). Electrospray ionization in negative ion mode (ESI-) was used for the ionization of the flavonol and flavan-3-ol

compounds. The mass spectrometry conditions included capillary voltage of 3,000 V with nebulizing gas (N_2) at a temperature of 375 °C. The total quantified polyphenols were expressed as $\text{mg } 100 \text{ g}^{-1} \text{ DM}$. Multiple reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for identification and quantification of each phenolic compound using external calibration curves generated individually for each compound measured. The limit of detection for all the compounds quantified were between 0.01 and 1 $\mu\text{g/mL}$.

Experimental design

Response surface methodology (RSM) was used to determine the optimal settings of β -CD concentration, incubation time and temperature to extract maximum amount of total flavonols from apple pomace powder. RSM involves mathematical and statistical techniques for developing functional relationship between a response variable and a number of associated controls. A 2^3 central composite design (CCD) was used to evaluate main effects, interaction effects, and quadratic effects of the analyzed factors. The design had 19 experimental runs, with eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68179 from the design center) and five replicates at the center point (Table 1) to measure pure error of the regression analysis. The quality of the fitted model was evaluated by ANOVA and on the percentage of explained variance (R^2 adj), which explains the variability in the observed response values could be due to the experimental factors and their interactions. The selected dependent variables were β -CD concentration (X_1 ; 0–5 g 100 mL^{-1}), temperature (X_2 ; 20–72 °C) and time (X_3 ; 6–48 h); while total flavonols ($\text{mg } 100 \text{ g}^{-1} \text{ DM}$) was used as response variables. The input range of these factors was selected as based on our previous study (Ratanasooriya and Rupasinghe 2012). The three independent variables were coded at five levels (Table 1); thereby resulting in experimental runs of 19 for each set.

The CCD was carried out to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the pure process variability. The response variable was fitted by a second order model in order to correlate it to the independent variables. The linear quadratic model was expressed as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y is the predicted response variable, β_0 is the intercept term, β_1 , β_2 and β_3 are linear coefficient of β -CD concentration, incubation temperature and incubation time, respectively, β_{11} , β_{22} , β_{33} are quadratic coefficients, β_{12} ,

Table 1 The central composite design variables and their experimental design levels

Coded value	Levels				
	−1.68	−1	0	+1	+1.68
Uncoded variables					
BCD concentration X_1 (g 100 mL ^{−1})	0	1.0	2.5	4	5
Reaction temperature X_2 (°C)	20	30.5	46	61.5	72
Reaction time X_3 (min)	6	14.5	27.0	39.5	48

β_{13} , β_{23} are interaction coefficients and X_1 , X_2 and X_3 are coded independent variables.

Verification of model

The predictive model (Eq. 1) developed for maximum flavonol extraction by RSM was further validated by direct comparison to experimental values. A β -CD concentration of 2.8 g 100 mL^{−1} was added to 0.5 g apple pomace powder followed by incubation at of 45 °C for 25.6 h under continuous shaking. The total flavonols were extracted and quantified using LCMS method using procedure described above and compared to the predicted values (*t*-test *p*-value<0.05) in order to assess the global validity of the models to explain the actual relationship among factors and responses (Granato et al. 2010).

Statistical analyses

The significance of differences between mean values for phenolic compounds during comparison of five CDs was assessed by a one-way analysis of variance (ANOVA). Before performing any statistical analysis, all dependent variables were checked for normal distribution and constant variance (Granato et al. 2014) using Anderson Darling test and residuals versus fitted values plot, respectively. Data used for optimization procedure was analyzed using response surface methodology (Montgomery 2005) in Minitab 15. Contour plots were drawn as a function of two factors when third factor held constant to study the interactions between them. Contour plots and three dimensional surface plots for response variables were obtained to determine the optimal levels of each factor. Assumptions of independence, constant variance, and normality were tested for the obtained data using Minitab 16 and relevant models were described by identifying and excluding outliers, when needed.

For all other experiments, the data were analyzed using ANOVA methods to compare the factor levels in terms of the mean response, using the general linear model (GLM) procedure of the SAS Institute, Inc. Differences among means were tested by the Tukey's studentized range test at $\alpha=0.05$.

Results and discussion

Comparison of CD and solvent-based extraction of polyphenols

Conventional extraction processes for natural bioactive compounds such as polyphenols are quite tedious, prolonged and requires organic solvents which could limit their applications in value-added food. Furthermore, environmental issues associated with the disposal of used organic solvents are increasing. The extraction process used might also lead to degradation of the bioactive molecules, thus hampering their bioavailability. CDs are known for their capability to form inclusion complexes between bioactive compounds and their characteristic hydrophobic cavity, thereby enhancing the solubility, stability and bioavailability of the bioactives (Chao et al. 2012; Lucas-Abellán et al. 2011; Mercader-Ros et al. 2010; Šmidovnik et al. 2010). The impact of number of glucose residues in three CD forms (α -, β -, γ -) and two derivatives (HP β - and RM β -CDs) at 0.025 mol L^{−1} and a comparison with sonication-assisted organic solvent-based extraction was investigated for the comparison of recovery of total polyphenols (flavonols + dihydrochalcones + phenolic acids + flavanols) from apple pomace powder. Among the five CDs tested, the highest extraction yield of 165 mg total polyphenols/100 g DM was obtained from β -CD (Table 2). This was followed by extraction yield using γ - and α -CD, which were not significantly different from β -CD. Interestingly, all five CDs produced similar amounts of dihydrochalcones between 42 and 47 mg 100 g^{−1} DM, except RM β -CD, that produced the least amount of 3.5 mg dihydrochalcones 100 g^{−1} DM. Recovered flavan-3-ols (specifically catechin and epicatechin) using β -CD were roughly 35–40 % higher than extracted by α -CD and γ -CD. Interestingly, β -CD extracted flavanols as effectively as 100 % and 70 % methanol. The other two derivatives of β -CD were found to behave differently in terms of extracting flavan-3-ols. While RM β -CD yielded higher amounts of flavanols (9.6 mg 100 g^{−1} DM); whereas HP β -CD was observed as the least effective, extracting only 10 % of amount extracted by β -CD. In addition, HP β -CD was also ineffective in the extraction of phenolic acids, representing merely 12 % of the amount recovered using β -CD, respectively.

Table 2 Comparison of cyclodextrin-based aqueous solutions for polyphenol extraction from apple pomace

Polyphenols	Water	α -CD	β -CD	γ -CD	HP β -CD	RM β -CD	Methanol (100 %)	Methanol (70 %)
Phenolic acids								
Chlorogenic acid	33.2 $\pm$ 3.3	31.5 $\pm$ 1.9	34.1 $\pm$ 6.1	31.2 $\pm$ 1.3	4.0 $\pm$ 0.1	32.2 $\pm$ 2.3	60.4 $\pm$ 2.7	66.5 $\pm$ 3.1
Caffeic acid	1.0 $\pm$ 0.0	0.8 $\pm$ 0.0	0.9 $\pm$ 0.0	0.8 $\pm$ 0.0	0.1 $\pm$ 0.0	0.7 $\pm$ 0.0	1.2 $\pm$ 0.0	1.2 $\pm$ 0.0
Total phenolic acids	34.2 ^b	32.3 ^b	35 ^b	32 ^b	4.1 ^c	32.9 ^b	61.6 ^a	67.7 ^a
Flavonols								
Quercetin	0.1 $\pm$ 0.0	0.28 $\pm$ 0.6	0.4 $\pm$ 0.0	0.2 $\pm$ 0.0	4.1 $\pm$ 0.1	0.8 $\pm$ 0.0	17.35 $\pm$ 0.1	14.3 $\pm$ 1.1
Q. galactoside	16.2 $\pm$ 0.2	35.4 $\pm$ 0.6	42.4 $\pm$ 1.8	37.1 $\pm$	21.1 $\pm$ 0.2	28.4 $\pm$ 0.3	77.1 $\pm$ 3.9	73.9 $\pm$ 2.1
Q. glucoside	3.0 $\pm$ 0.0	6.8 $\pm$ 0.3	7.9 $\pm$ 0.2	6.9 $\pm$ 0.1	4.2 $\pm$ 0.0	7.4 $\pm$ 0.2	16.1 $\pm$ 0.1	16.2 $\pm$ 0.1
Q. rutinoside	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0	2.0 $\pm$ 0.0	0.06 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	6.2 $\pm$ 0.2	6.8 $\pm$ 0.2
Q. rhamnoside	12.1 $\pm$ 0.1	17.4 $\pm$ 0.1	20.1 $\pm$ 0.2	17.1 $\pm$ 0.2	6.9 $\pm$ 0.1	15.0 $\pm$ 0.2	29.8 $\pm$ 2.7	31.2 $\pm$ 0.1
Total flavonols	31.4 ^f	60 ^c	72.8 ^b	61.4 ^c	36.3 ^c	51.6 ^d	146.6 ^a	142.3 ^a
Dihydrochalcones								
Phloridzin	33.8 $\pm$ 0.0	41.9 $\pm$ 1.7	47.2 $\pm$ 0.7	46.2 $\pm$ 1.6	42.4 $\pm$ 2.1	3.4 $\pm$ 0.1	75.6 $\pm$ 1.5	74.8 $\pm$ 1.2
Phloretin	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0	0.4 $\pm$ 0.1	0.1 $\pm$ 0.0	0.4 $\pm$ 0.0	0.1 $\pm$ 0.0	1.0 $\pm$ 0.0	0.9 $\pm$ 0.0
Total dihydrochalcones	33.8 ^c	42.0 ^{bc}	47.6 ^b	46.3 ^b	42.8 ^{bc}	3.5 ^d	76.6 ^a	75.7 ^a
Flavan-3-ols								
Catechin	0.9 $\pm$ 0.0	0.9 $\pm$ 0.1	1.9 $\pm$ 0.0	1.0 $\pm$ 0.0	0.3 $\pm$ 0.0	1.7 $\pm$ 0.0	1.6 $\pm$ 0.0	1.3 $\pm$ 0.1
Epicatechin	5.1 $\pm$ 0.0	5.3 $\pm$ 0.2	8.1 $\pm$ 0.0	5.6 $\pm$ 0.1	0.7 $\pm$ 0.0	7.9 $\pm$ 0.0	9.7 $\pm$ 0.1	8.6 $\pm$ 0.2
Total flavan-3-ols	6.0 ^b	6.2 ^b	10.0 ^a	6.6 ^b	1.0 ^c	9.6 ^a	11.3 ^a	9.9 ^a
Phenolics from LCMS	105.4 ^c	140.5 ^b	165.4 ^b	146.3 ^b	84.2 ^d	97.6 ^c	296.1 ^a	295.6 ^a

Values expressed in mg 100 g⁻¹ DM

^ψ Concentration of cyclodextrins = 0.025 mol L⁻¹, Temperature = 60 °C, Time = 24 h

[§] Values with different letters in each row are significantly different ($P < 0.05$) and letter grouping is between treatments

[¶] HP- hydroxypropyl; RM- randomly methyl

Flavonols, such as quercetin glycosides, are a subclass of flavonoids, which find importance owing to their health promoting effect such as anti-oxidant (Huber et al. 2009); anti-hypertensive (Balasuriya and Rupasinghe 2012), anti-diabetic (Panicker et al. 2010); anti-atherosclerotic (Thilakarathna et al. 2012), neuroprotective (Jones et al. 2012) and chemopreventive effects via anti-mutagenic, anti-oxidant, anti-inflammatory and anti-proliferative activities (Gerhäuser 2008; Gerhäuser et al. 2003; Murakami et al. 2008; Murphy et al. 2012). Therefore, the extraction of quercetin glycosides deserves special attention in terms of yield, eco-friendly aspects and physio-chemical properties. In terms of total flavonol recovery, β -CD yielded about 19–40 % higher amount than yielded by the other four CDs. While α -CD and γ -CD were similar in their flavonol yields of 60–61.4 mg 100 g⁻¹ DW, whereas, HP β -CD and RM β -CD were significantly different from each other. Total flavonol yield by two derivatives of β -CD was the lowest, which clearly suggests their inefficiency for flavonol extraction. Comparison of flavonol yields with a common organic solvent (methanol) revealed that β -CD recovered 50 % total flavonols of 100 % and 70 % methanol. Nevertheless, water alone was significantly ineffective in extracting flavonols from apple pomace. Among

flavonols, quercetin galactoside was observed in the highest quantities ranging between 16 and 77 mg 100 g⁻¹ DM, followed by quercetin rhamnoside (12–31 mg 100 g⁻¹ DM) using different extraction media. Owing to the valuable amounts of flavonols extracted using β -CD and its GRAS status, it is important to optimize the extraction procedures using β -CD based solvent that could eventually mitigate the harmful effects from the use of toxic organic solvents.

While the quantified flavonols using aqueous α -, β - and γ -CD (0.025 mol L⁻¹) were more than 50 % higher than the control; whereas, they were found to be half the amount yielded by organic solvent (70 % and 100 % methanol). The most probable reason for this could be that the used solvent based extraction procedure was optimized previously (Rupasinghe et al. 2011); while the CD based procedure was not optimized at this point. Quercetin galactoside represented more than 50 % of the total flavonols extracted by using different media (Table 2). The comparison between α -, β - and γ -CDs displayed that β -CD was able to extract significantly higher amount of flavonols than the other two CDs. The observed differences by current study in terms of flavonol recovery using α -CD, β -CD and γ -CD could be due to volume of cavities which were 0.174, 0.262 and 0.427 nm³,

respectively (Del Valle 2004). These findings agreed to the results reported by Ratanasooriya and Rupasinghe (2012), thereby demonstrating high yields of recovered flavonols from grape pomace using β -CD.

Ultra sonication-assisted extraction of flavonols showed no significant difference between the flavonols extracted using 70 % or 100 % methanol. This finding is in line with a previous study by Rupasinghe et al. (2011) that demonstrated similar effects. However, the values obtained in this study were higher as compared to reported by Rupasinghe et al. (2011). This could be due to inclusion of quercetin glucoside in addition to quercetin galactoside, rhamnoside, rutinoid and quercetin aglycone. Among total polyphenols quantified in the present study, the flavonols represented about 42–53 % using CDs and solvent based extraction procedures. In addition, apple peel is previously reported to contain about 40–60 mg phloridzin 100 g⁻¹ DM (Escarpa and Gonzalez 1998); while using the above procedures, the present study demonstrated the yield of phloridzin in a range of 34–76 mg 100 g⁻¹ DM. The observed difference in the data from two studies could be attributed to the difference in cultivars and methods used.

Optimization of β -CD based extraction of flavonols

β -CD based extraction of total flavonols was optimized using a 2³ factorial CCD. The extraction conditions for β -CD concentration (g 100 mL⁻¹), extraction temperature (°C) and

duration (h) were optimized. Five experimental runs were replicated at the center point of the design to confirm any differences in the estimation (Table 3). The ANOVA results showed the lack of fit test for the model. The lack of fit test describes the variation in the response around the fitted model. If the model does not fit the data well, the lack of fit will be significant. The large *p* value of 0.3 illustrated that the lack of fit was not insignificant, implying that the models adequately described the data (Table 4). Furthermore, an *R*²_{adj} close to the *R*² value insured a satisfactory adjustment of the quadratic models to the experimental data. For a good model of fit, the *R*² should be >80 %, which was true in the current study, as shown in Table 4. Moreover, the ANOVA on this model demonstrated that the model was highly significant, as evident from the very low probability of *p* > *F* values in the regression = 0.01.

The *p*-values are used to check the significance of each coefficient and to understand the interactions between the best variables. The smaller *p*-values (≤ 0.05) in the present study indicated more significance of the corresponding coefficient and its effect on extraction of total flavonols from apple pomace. Linear effect of X1 and quadratic of X1² were found to be highly significant (*p* < 0.05), as shown in Table 5. However, other variables did not show any significant effect. The Eigen values obtained were a mixture of positive and negative values, suggesting the stationary point for optimization to be a saddle point (point of maximum or minimum values; Montgomery 2005). The critical values for apple

Table 3 Central composite design (CCD) of factors for optimization of β -CD based extraction of total flavonols from apple pomace

Trial #	CCD design point	β -CD (X1) (g 100 mL ⁻¹)	Temperature (X2) (°C)	Time (X3) (h)	Experimental flavonol content (mg 100 g ⁻¹ DM)
1	Factorial	1	30.5	14.5	117.8
2	Factorial	4	30.5	14.5	152.8
3	Factorial	1	61.5	14.5	142.1
4	Factorial	4	61.5	14.5	167.1
5	Factorial	1	30.5	39.5	96.0
6	Factorial	4	30.5	39.5	109.1
7	Factorial	1	61.5	39.5	119.1
8	Factorial	4	61.5	39.5	110.9
9	Axial	0	46	27	49.1
10	Axial	5	46	27	85.0
11	Axial	2.5	20	27	193.8
12	Axial	2.5	72	27	166.3
13	Axial	2.5	46	6	118.7
14	Axial	2.5	46	48	134.6
15	Center	2.5	46	27	139.5
16	Center	2.5	46	27	161.6
17	Center	2.5	46	27	153.0
18	Center	2.5	46	27	189.1
19	Center	2.5	46	27	160.2

Table 4 Canonical analysis of the response surface for β -CD based extraction of total flavonols from apple pomace

Variable	Values	
	2 ³ CCD	2 ² CCD
P-value for model adequacy (Lack of fit)	0.3	0.1
R ² adjusted	83.2	89.0
Eigen values		
BCD concentration (g 100 mL ⁻¹)	22.7	
Temperature (°C)	−30.0	−84.6
Time (h)	−92.3	−102.4
Critical values		
BCD concentration (g 100 mL ⁻¹)	2.8	–
Reaction temperature (°C)	44.8	45.1
Reaction duration (h)	21.4	25.6
Predicted response value (mg 100 g ⁻¹ DM)	163.2	166.6
Stationery point	Saddle point	Maxima

apple pomace powder at uncoded levels of variables studied were: β -CD concentration of 2.7 g 100 mL⁻¹, extraction temperature of 44.7 °C and reaction duration of 21.4 h. The contour plots describing combined effect between pair of factors on extraction of total flavonols from apple pomace are represented in Fig. 1 by keeping the other variable constant at their middle level.

Following the steepest ascent of the ridge analysis, additional experimental runs were carried out to obtain maxima (optimal region releasing maximum flavonols) after fixing the β -CD concentration to 2.8 g 100 mL⁻¹. This time, a 2² factorial CCD was used with extraction temperature and time as independent factors, keeping the center point values same as in the 2³ factorial CCD. A maxima for total flavonol concentration was observed (Table 4; Fig. 2) at 166.6 mg

100 g⁻¹ DM the un-coded values for optimized parameters were: β -CD concentration of 2.8 g 100 mL⁻¹; extraction temperature of 45 °C and extraction time of 25 h. Linear effect of X₂, quadratic of X₂² and interaction effect of X₂ and X₃ were found to be highly significant ($p < 0.05$; Table 4), which showed the existence of the optimal value within the experimental area.

During optimization procedure, it was observed that the trial without any β -CD addition (0 g 100 mL⁻¹) extracted the least amount of flavonols. This was in agreement to a previous study, which demonstrated water as a weak solvent for

Fig. 1 Response contour for total flavonols using a 2³ central composite design (CCD) when **a**) β -CD is held constant at 2.5 g/100 mL⁻¹, **b**) when temperature is held constant at 45 °C and **c**) when time is held constant at 27 h

Table 5 Estimated regression coefficients of second order polynomial model for optimization of total flavonols from apple pomace powder

Term	2 ³ CCD <i>p</i> value	2 ² CCD <i>p</i> value
Constant	0.72	0.01
β -CD conc. (X ₁ ; g 100 mL ⁻¹)	0.00*	–
Temperature (X ₂ ; °C)	0.40	0.00*
Time (X ₃ ; h)	0.16	0.01*
β -CD conc. $\times$ β -CD conc. (X ₁ ²)	0.00*	–
Temperature $\times$ temperature (X ₂ ²)	0.60	0.00*
Time $\times$ time (X ₃ ²)	0.20	0.00*
β -CD conc. $\times$ temperature (X ₁ $\times$ X ₂)	0.40	–
β -CD conc. $\times$ time (X ₁ $\times$ X ₃)	0.81	–
Temperature $\times$ time (X ₂ $\times$ X ₃)	0.09	0.04

Significant at $\alpha = 5\%$

Fig. 2 Response surface for flavonol maxima for β -CD based extraction of total flavonols from apple pomace powder when β -CD was held constant at 2.8 g 100 mL⁻¹

flavonol extraction (Reis et al. 2012). Furthermore, it was observed that flavonol extraction was not directly proportional to the β -CD concentration as 5 g β -CD 100 mL⁻¹ did not extract the highest amount of flavonols. A possible reason to this could be the saturation of β -CD cavities at the operating conditions, thereby blocking further interactions of polyphenols. Furthermore, we also observed that solubility of β -CD above 3 g L⁻¹ in water was difficult to achieve. A maxima was achieved by the canonical analysis conducted on total flavonol concentration at the optimal extraction conditions of β -CD concentration, 2.8 g 100 mL⁻¹; extraction temperature, 45.1 °C and extraction time, 25.6 h. the predicted maxima of 166.6 mg 100 g⁻¹ DM was less than 10 % different from the experimental value of 151.1 mg 100 g⁻¹ DM, thereby validating the experimental model. The most interesting finding of this study was that the total flavonol yield obtained by using optimal β -CD parameters was at par with what was obtained using organic solvent. This clearly suggests that optimized β -CD-based extraction can provide an eco-friendly mode of obtaining high value bioactives at enhanced yields.

It is suggested that β -CD interacts with polyphenols, by penetrating the cell wall and cell membrane to reach the target sites. However, the exact mode of action of β -CD on the bioactives from plant materials requires special attention. Also, the physico-chemical, and biological properties of the guest molecules may be drastically altered (Polyakov et al. 2004); therefore, bioactives enclosed within CDs may have increased dissolution rate, membrane permeability and bio-availability. A study by Pralhad and Rajendrakumar (2004) had displayed the significant increase of quercetin's water solubility due to the formation of quercetin/ β -CD inclusion complex by freeze-drying. In addition, it has also been demonstrated that the inclusion complexes of quercetin with CDs retain their antioxidant potential (Jullian et al. 2007). Nevertheless, the physico-chemical and biological properties

of the extracted flavonols in the current study remain to be elucidated.

Validation of the mathematical model

With the experimental setup, a confirmation and validation experiment was conducted using the optimal extraction parameters calculated from the study. The optimal extraction conditions of total flavonols from apple pomace acquired using the model were: β -CD concentration, 2.8 g 100 mL⁻¹; extraction temperature, 45.1 °C and extraction time, 25.6 h. Under these optimal conditions, the model predicted a maximum response of 166.6 mg flavonols 100 g⁻¹ DM of apple pomace. A mean value of 151.5 mg 100 g⁻¹ DM of total flavonols was acquired from real experiments, generating an absolute error of 9.0 %. The adequacy of the model fitted by RSM was obtained due to the low absolute error value observed from the analysis of predicted versus observed values.

Conclusion

This study, the first of its kind, optimized β -cyclodextrin (β -CD) based flavonol extraction from apple pomace powder. The optimum parameters for β -CD based total flavonol extraction were: β -CD concentration, 2.8 g 100 mL⁻¹; extraction temperature, 45 °C and extraction time, 25.6 h, producing 166.6 mg flavonols 100 g⁻¹ DM. The fitted model was adequate due to the low absolute error value obtained by comparing predicted versus observed values. The amount of flavonols extracted using optimized β -CD procedure was almost equal to the amount obtained using a sonication-based solvent extraction procedure. This emerging “green” extraction technology using low-cost bio-resources could provide an environmentally friendly and economical alternative to traditional extraction methods for natural bioactive compounds.

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