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Effect of slow-release amylase on extraction of peach peel phenolics and their antioxidant activities

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ABSTRACT

The present manuscript demonstrates the work undertaken to optimise and validate a slow-release amylase-assisted extraction of polyphenols from peach fruit peel. A careful investigation and optimisation revealed that peach peel when hydrolysed with 1.50% (w/w) of SRA containing enzyme formulation at 40 °C and 6.1 pH, for 35 min significantly ($P < 0.05$) increased the extraction yield, levels of polyphenol contents (242.89 ± 1.56 mg gallic acid equivalents – GAE), and coumaric, chlorogenic, ferulic acids or their conjugate esters in extracts. Moreover, the extracts produced through SRA-assisted extraction exhibited ample level of free radical scavenging capacity (DPPH IC_{50} 2.67 ± 0.03 μg mL^{-1}), Trolox equivalent (TE) antioxidant capacity (450.52 ± 24.58 μmol of TE g^{-1}), inhibition of peroxides in linoleic acid (85.68 ± 0.21%), and ferric reducing power of 3.11 ± 0.20 ppm gallic acid equivalents. The results suggested that the incorporation of SRA containing enzyme formulation may enhance the recovery of peach peel polyphenols while hydrolysing the glycosidic linkages without deteriorating their antioxidant character.

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KEYWORDS

enzyme cocktail, slow-release amylase (SRA), peach peel phenolics, antioxidant activities, RP-HPLC-DAD

1. INTRODUCTION

Peach (*Prunus persica* L.), a member of the family *Rosacea,* falls among the fruits frequently consumed for their extraordinary delicious taste and established health benefits against certain types of cancers and cardiovascular diseases (Medina-Meza and Barbosa-Cánovas, 2015; Bahrin et al., 2022). Many researchers and folk healers have graded the peach as the best fruit for the patients suffering diabetes mellitus, asthma, anemia, and digestive system disorders for nutritional attributes, phenolic constituents, vitamins, and minerals (Bahrin et al., 2022; Durazzo et al., 2022). Actually, the plant polyphenols may entangle inside the vacuole and cell wall microfibirils or bind with cellulosic structures through glycosidic linkages (Stavikova et al., 2011). The former class phenolics readily distribute in a compatible solvent like water, ethanol, methanol, ethyl acetate, or their mixtures (Acosta-Estrada et al., 2014), while the polyphenols of the second category, known as non-extractable polyphenols (NEPs), need some hydrolysing or macerating agents (Mukhtar et al., 2018). Besides, the presence of insolouble fibres (≥65% w/w) in addition to protein and pectin obstructs the liberation of phenolic bioactives during the normal liquid-liquid extraction (Bolanho et al., 2015).

The enzymes can selectively hydrolyse glycosidic covalent bonds and liberate these NEPs (Meinlschmidt et al., 2016; Mushtaq et al., 2017). Moreover, the simultaneous release of enzymes not only makes the process economically unviable but also reduces the efficiency of enzyme formulation (Deng et al., 2014; Granato et al., 2022). Our understanding regarding the behavior of plant phenolics suggested an enzyme cocktail containing reasonable amounts of glycosidases along with slow-release amylase (SRA) would be more useful to hydrolyse and extract peach peel phenolic antioxidants.

2. MATERIALS AND METHODS

2.1. Procurement of samples and supplies

Healthy and mature peach fruits (*P. persica* L.) purchased from the local fruit market in Lahore, Pakistans were washed with distilled water, identified as the variety "clingstone", peeled using a sharp knife, and kept frozen (4.0 ± 1.5 °C) in airtight polythene bags. The HPLC grade phenolic standards and reagents including 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (≥98%), 1,1-diphenyl-2-picrylhydrazyl radical (≥95%), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (≥98%), and Folin–Ciocalteu's reagent (2M) were supplied by Merck KGaA (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, USA). Other laboratory chemicals like sodium carbonate (Na_2CO_3), ammonium thiocyanate (NH_4SCN), potassium persulphate ($K_2S_2O_8$), potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]3H_2O$), ethanol (C_2H_5OH), and water (H_2O) were procured from RCI Labscan Chemicals (Karachi, Pakistan).

2.2. SRA enzyme formulation

The SRA containing enzyme formulation was prepared by mixing 1.0% of commercially available amylase (*Aspergillus oryzae*) with a carrier comprising calcium carbonate (78%), bentonite (5%), dried yeast (11.5%), and vegetable oil (1.0%). The resultant α-SRA was mixed with an equal volume of 0.5% cellulase (*Trichoderma* species), 1.0% xylanase (*Trichoderma viride*), 1.5% glucanase (*Aspergillus niger*), and 0.5% protease (*Streptomyces* species). The final formulation can offer enzymatic activities up to 19,000 International Units (IU)/g comprising endo-1,4-β-xylanase (5,000 I.U/g), endo-1,4-β-glucanase (2,000 IU/g), endo-1,3-β-glucanase (500 IU/g), and α-amylase (500 IU/g).

2.3. Extraction experiments layout

In all experiments, 50 g of peach peel was transferred to a 250 mL Erlenmeyer flask containing 50 mL buffer of pre-decided pH and mixed with a certain amount of SRA containing enzyme formulation observing the conditions given in Table 1. At the end of the given incubation time, the enzyme was de-activated by keeping the flask at 90.0 °C for 5 min, and the liberated phenolics were collected by shaking the aliquout with 20 mL of ethanol. The extracts obtained for each experiment were further concentrated under vacuum using a Rotary type of Evaporator (EYELA, N1300, Tokyo, Japan) thermostated at 35 °C and 90 r.p.m., and weighed to calculate extraction yield (g/100 g of fresh peach peel).

2.4. Analysis of peach peel phenolics

2.4.1. TPC in peach peel extracts. The TPC in extracts of peach peel were assessed using a Folin–Ciocalteu reagent (FCR) based colorimetric assay with slight modifications (Saeed et al., 2012). In short, different concentrations of standard gallic acids and peach peel extracts were added to 100 µL of 10% FCR, extra diluted with 800 µL of deionised water, and neutralised with 150 µL of 2.1 M sodium carbonate. The reaction vials were left in the dark at 40 °C for 20 min, chilled for 5 min, and recorded at 755 nm using a 96-well plate reader (BioTek, High Land Park, USA).

2.4.2. RP-HPLC-DAD analysis. The presence of phenolics in peach peel extracts obtained under optimum conditions was authenticated by RP-HPLC-DAD (Aaby et al., 2007).

Table 1. The range of experimental conditions (actual and conded) investigated for the SRA-assisted extraction of peach peel phenolics

	−α	−1	0	+1	+α
Enzyme concentration (A, %)	0.5	1.5	3	4	5
Incubation time (B, min)	20	50	75	100	120
pH (C)	3.5	4.5	5.5	6.0	7.5
Temperature (D, °C)	25	30	45	50	65

where (+α and –α), (+1 and −1), and (0) denote axial, factorial, and center points of the experimental design.

The extracts of peach peel (50 mg) were dissolved in a (1:1) mixture of 1% (v/v) acidified methanol and butylated hydroxytoluene and refluxed at 90 °C for 60 min using a magnetic stirrer. The resultant solution was centrifuged at 5,000 *g*, and the upper layer was filtered and injected (20 µL) into Shim-Pack CLC-ODS (Merck KGaA, 64,271 Darmstadt, Germany) C-18 column (dimensions 250 × 4.6 mm and 5-µm particle size) equipped in Shimadzu LC-10A HPLC system. The diode array detector (280 nm) response (peaks) with mobile phase (1.0 mL min^{-1}) comprising A (H_2O:CH_3COOH in a ratio of 94:6 mL) and B (acetonitrile 100%) under gradient mode, i.e. 85% A: 0–10 min, 55% A: 10–15 min, and 100% B: 15–25 min, was processed using CSW32 (datapex) Chromatography Station/data handling software to calculate the concentration of phenolic acids as per our previous analysis (Mukhtar et al., 2018).

2.5. Antioxidant evaluation of peach peel extracts

2.5.1. Radical scavenging capacity (RSC). The RSC of peach peel extracts produced under various enzymatic maceration conditions were appraised in terms of their ability to neutralise freshly prepared 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) free radicals (Kedare and Singh, 2011). The results were expressed as half maximal inhibitory concentration (IC_{50}, µg mL^{-1}).

2.5.2.* In-vitro *antioxidant capacity of peach peel phenolics. For Trolox Equivalent Antioxidant Capacity (TEAC), 200 µL of 7 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) having optical density of 0.700 ± 0.050 at 734 nm, was separately incubated with 50 µL extract solution containing 50 mg peach peel extract/mL and different concentration of Trolox standard. Absorbance was measured after 6 min to calculate TEAC as µM of Trolox Equivalents/g of peach extract.

2.5.3. Lipid peroxidation inhibition capacity (PIC). This assay involves the incubation of linoleic acid with extracts, which eventually inhibits the formation of peroxides that can be determined by a previously reported protocol by Kristl et al. (2011) with some modifications. Shortly, 50 µL of the above solution was mixed with 200 µL of 20 M $FeCl_2$ in 3.5% HCl, 1 mL of 75% ethanol, and 30% aqueous solution of ammonium thiocyanate and kept for incubation at 40 °C with continuous stirring for 3 min. Absorbance was recorded at 500 nm to calculate PIC (%) according to Eq. (1).

$$PIC\ (\%) = \left(1 - \frac{\Delta_{Control} - \Delta_{sample}}{\Delta_{Control}}\right) \times 100 \quad (1)$$

2.5.4. Ferric reducing power (FRP). In this assay, 1.0 mL of six different concentrations of the gallic acid (2.5–100 µg mL^{-1}) and 50.0 mg of peach peel extract were mixed separately with 1.0 mL of (0.2 M) 6.6 pH phosphate buffer and 500 µL of 1.0% potassium hexacyanoferrate (II) trihydrate. After 20 min of incubation at 50 °C, all solutions were treated with 1.0 mL of 10% trichloroacetic acid (Cl_3CCOOH) and centrifuged at 980 *g* for 10 min in a chilled centrifuge. Finally, 75 µL of the upper layer were separated, diluted with the same volume of de-ionised water, mixed with 50 µL of 0.1% Fe^{+3} ions, and Fe^{+2}-phenolic complex was monitored at 700 nm (Medina-Meza and Barbosa-Cánovas, 2015).

2.6. Statistical analysis

In order to modulate each response, a second-order polynomial equation (Eq. (2)) was applied.

$$R = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X^2 + \sum_{i>1}^{k} \sum_{j}^{k} b_{ij} X_i X_j + \varepsilon \quad (2)$$

where "R" denotes the output or response, b_0, ε, $\sum_{i=1}^{k} b_i X_i$, $\sum_{i=1}^{k} b_{ii} X^2$, and $\sum_{i>1}^{k} \sum_{j}^{k} b_{ij} X_i X_j$ stand for intercept, pure-error, linear, quadratic, interactions effects if extraction variables, respectively. All the responses including peach peel extract yield (Y), TPC, RSC, TEAC, LPIC, and FRP observed under each experimental condition were evaluated for mean and variance using the 11th version statistical software "Design-Expert" of Statease Incorporation (Minneapolis, United States of America) at 95% confidence level. The responses were transformed into a dimensional quantity "Desirability" following the conditions suggested by Derringer and Suich (1980). The individual desirability obtained above are finally transformed into unitless quantity following Eq. (3), which value indicates Acceptable solution ←1 ≥ d ≥ 0→Unsuitable solution.

$$\text{desitability} = \sqrt[R]{d_1 d_2 d_3 \ldots d_R} \quad (3)$$

where R denotes the number of responses to be optimised.

3. RESULTS AND DISCUSSION

3.1. Enzymatic maceration and liberation of bioactives

The initial experiments (five replicates) involving the treatment of peach peel matrix with 3.0% (w/w) of SRA containing enzyme formulation at 5.5 pH and 45.0 °C for 75.0 min offered an average recovery of crude bioactives equal to 35.01 ± 1.07 g/100 g of the fresh peel. The extraction yields of bioactive compounds in the case of enzymatically pre-treated peach peels were significantly higher ($P < 0.05$) than the control (11.18 ± 0.07 g/100 g) sample, which was processed through conventional solvent extraction, i.e. 80% aqueous methanol. In the experimental layout, when extended to factorial (eight runs) and axial (eight runs) points, the extraction yield varied from 20.48 to 39.04 g/100 g, which establishes that variation in maceration conditions influences the liberation of crude bioactives. According to Fig. 1 (b, c, and f), a small concentration of SRA enzyme formulation offered higher yields. Similarly, prolonged incubation of peach peel with a smaller concentration of SRA-containing formulation was more effective than applying higher concentration for short intervals (Fig 1 a and e). The data in Fig. 1 (c and f) indicate that incubation of peach peel with SRA-containing enzyme formulation at elevated temperatures (above 40 °C) and under mild acidic to neutral conditions (pH 5.5–6.5) would liberate a higher quantity of crude bioactives. Similarly, Fig. 1 (d and e) discloses that any deviation from ideal conditions of pH and temperature sharply reduces the extract yield.

3.2. Total phenolic contents (TPC) in peach peel extracts

It was interesting to note that prolonged incubation reduced TPC in the final extracts. Similarly, maceration of peach peel at higher temperatures produced extracts of higher phenolic constituents. Likewise, any shift of pH from 5.5 to 8.0 reduced the liberation of phenolic bioactives

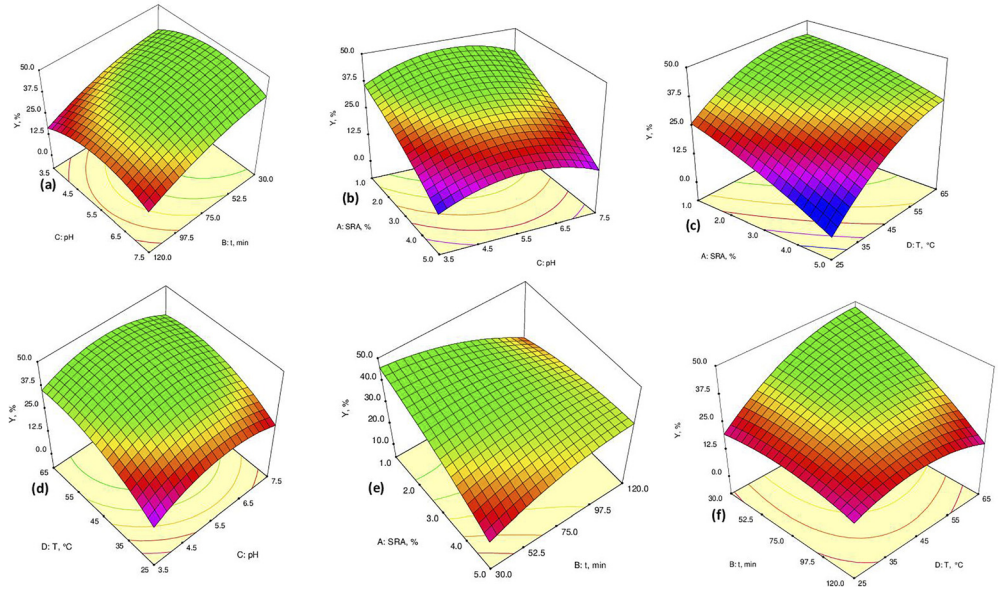

Fig. 1. Three-dimensional representation of interaction among various maceration parameters regarding the liberation of antioxidant phenolic from peach

from the peach peel. A similar kind of behavior was established by analysis of the variance of TPC in peach peel extracts produced under various conditions. The statistical analysis further elaborates that linear effects A, C, and D factors were positive, while incubation time adversely contributed toward TPC. In contrast to extract yield, the highest TPC (242.89 ± 1.56 mg GAE/g) of extracts was found in the peach fruit extracts produced with 2.0% SRA containing enzyme at 6.50 pH and 45.0 °C. The available literature hardly contains any report regarding the use of SRA-containing enzyme formulation for the recovery of peach polyphenols. However, Liu et al. (2018) used an aqueous-methanol solvent (50:50) of very acidic pH (pH = 2) and acidified methanol/H_2SO_4 (90/10) for the recovery of extractable and non-extractable polyphenols from the peach peel, and Kurtulbaş et al. (2022) recovered 19.35 mg GAE/g of phenolics from peach peel waste by applying microwave 500 W for 90 s (Table 2).

3.3. Experimental model adequacy check

In order to follow the conditions predicted by the applied experimental model, it is necessary to check whether the applied model provides an adequate approximation of response or not. The analysis of variance (ANOVA) results assembled in Table 3 impliy that the applied model is significant and there is at least one independent variable among the tested parameters that contribute significantly ($P < 0.05$) to responses. A sufficient number of replicates (at least five) were used to evaluate the design's lack of fit. The non-significant ($P > 0.05$) "Lack of Fit" probability indicates that the selected model has a good fit. Similarly, higher values of R^2 (>0.50) indicate that the chosen quadratic expression estimates well all responses under the selected experimental conditions. Finally, the observed values of the coefficient of variation (CV) in all

Table 2. The summary of validation experiments for SRA assisted extraction of peach peel phenolics

EC (A)	*t* (min) (B)	T (°C) (C)	pH (D)	Responses observed					
				Yield[L]	TPC[M]	TEAC[N]	DPPH[O]	PIC[P]	RP[Q]
1.50	35	40	6.1	42.71 ± 0.85	242.89 ± 1.56	450.52 ± 24.58	2.67 ± 0.03	85.68 ± 0.21	3.11 ± 0.2
Results predicted with (0.869) desirability				39.8	241.60	480.40	2.56	84.7	2.30
Conventional solvent extraction (80% ethanol)				11.18 ± 0.07	48.86	236.46	64.74	61.16	1.54
Microwave assisted (500 W for 90 s) extraction (Kurtulbaş et al., 2022)				–	19.35	–	–	–	–
Sum of EPs and NEPs in Dahonghua cv extracted by Liu et al. (2018)				–	157.79	5.5–10	20–65	–	–

L, M, N, O, P, and Q: represent the values in g/100g of fruit peel, milligram of Gallic Acid Equivalents/g of extract, μmol of TE g^{-1} of extracts, DPPH radical scavenging capacity (IC_{50}) μg mL^{-1}, percent inhibition of linoleic acid peroxidation, and ferric reducing power (absorbance at 700 nm), respectively.

Table 3. The analysis of variance (ANOVA) for SRA-assisted extraction of peach peel phenolics

Variable	Yield *P*-value	TPC *P*-value	TEAC *P*-value	DPPH *P*-value	FRP *P*-value	PIC *P*-value
Model	<0.0001**	<0.0001**	0.003*	<0.0001*	<0.0001**	<0.0001**
A-EC	<0.0001**	0.014*	0.004*	0.0360*	0.0167**	0.0096*
B-time	0.034*	0.008*	0.004*	0.0002*	<0.0001**	<0.0001**
C-pH	0.0007*	<0.0001**	0.09*	<0.0001**	<0.0001**	<0.0001**
D-T	<0.0001**	<0.0001**	0.66	<0.0001**	<0.0001**	0.0003*
AB	<0.0001**	<0.0001**	0.04*	<0.0001**	<0.0001**	<0.0001*
AC	0.2399	0.0980	0.93	<0.0001**	0.0826**	0.1267
AD	0.002*	0.0010*	0.04*	<0.0001**	<0.0001**	0.0001**
BC	0.0602	0.0111*	0.49	<0.0001**	<0.0001**	0.0001**
BD	<0.0001**	0.0001**	0.01*	<0.0001**	0.0008*	0.0004*
CD	<0.0001**	<0.0001**	0.47	<0.0001**	0.0026*	<0.0001**
A^2	0.0008*	<0.0001**	0.26	0.0008*	0.87	<0.0001**
B^2	<0.0001**	0.3813	0.09	0.0003*	0.38	<0.0001**
C^2	<0.0001**	0.0003*	0.14	<0.0001**	0.78	0.0002*
D^2	<0.0001**	0.0172*	0.26	<0.0001**	0.792	0.1819
Lack of fit	0.4633	0.7962	0.23	0.4888	0.7962	0.2971
R^2	0.99	0.99	0.97	0.99	0.98	0.98
Adjusted R^2	0.95	0.98	0.70	0.87	0.96	0.97
Coefficient of variation (CV)	2.10	3.57	4.25	3.34	4.03	2.21

The values were statistically different at *: $P \leq 0.05$ and **: $P \leq 0.01$; where stated otherwise, considered to be non-significant.

the cases (2.21–4.25) established that the results obtained during laboratory experiments are reliable.

3.4. Multi-response optimisation and validation

In the present study, all the responses observed were further transformed into dimensionless quantity "desirability" setting criteria shown in Eq. (3). Desirability (d) value of zero indicates a totally mismatched solution and those having $d \geq$ unity indicate the most favorable conditions. The solution with maximum d (0.807) recommended maceration of the peach peel with 1.30% SRA containing enzyme (A) for 43 min (B) at 40 °C (C) and pH 6.1 (Table 2). Moreover, desirability ramps show that these conditions can liberate 39.8 g/100 g of a crude extract containing TPC 243.11 mg GAE/g of extract exhibiting 480.4 mmol TE g^{-1} of extract TEAC, 2.56 μg mL^{-1} of DPPH radical scavenging (IC_{50}), and 84.2% PIC and reducing power 2.3 ppm GAE/g of extract.

A new set of experiments was conducted to validate these results against given maceration conditions (Table 3), and peach fruit peel, when treated with SRA, produced crude extract (42.71 ± 0.85 g/100 g) about four times higher than conventional solvent extraction (11.18 g/100 g of peach peel). The SRA liberated phenolics exhibited an ample level of antioxidant activities in terms of TEAC, DPPH RSC, PIC, and FRP with mean values of 450.52 ± 24.58 μmol TE g^{-1}, 2.67 ± 0.03 μg mL^{-1}, 85.68 ± 0.21%, and 3.11 ± 0.3, respectively. Previosly, Liu et al. (2018) reported that EPs and NEPs obtained via acid hydrolysis can exhibit TEAC of 100.9–434.7 and 24.5–40.2 μmol TE/100 g of fresh samples, respectively. Likewise, the peach polyphenols produced

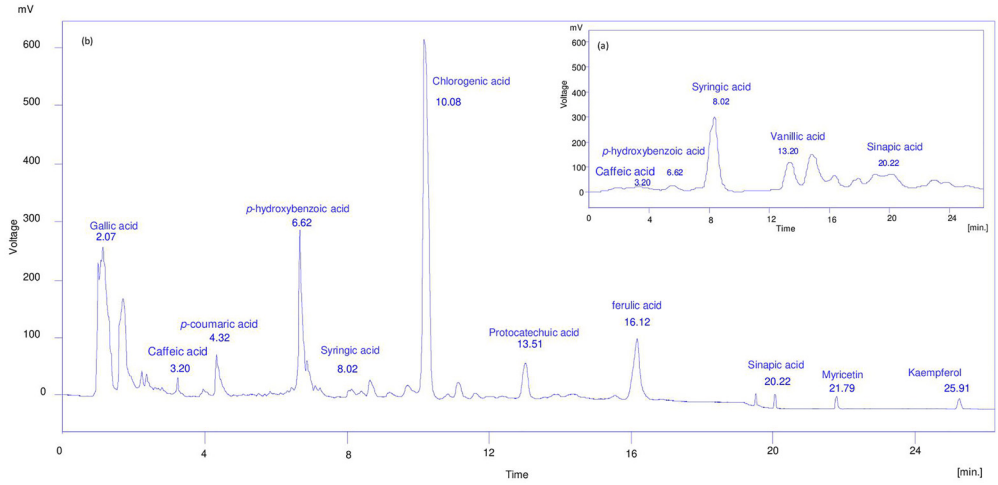

Fig. 2. Typical chromatograms of peach peel extracts obtain by (a) conventional solvent and (b) SRA-assisted extraction

via acid hydrolysis offered smaller DPPH radical scavenging in terms of IC_{50} 11.7–34.0 mg of extract/mL as compared to extract produced via current techniques *i.e.*, 2.56 mg of extract/mL. Figure 2 provides typical RP-HPLC-DAD chromatogram for extracts produced by SRA-assisted (a) and conventional extraction (b) to show that peach peel extracts obtained by SRA-assisted extraction were rich in chlorogenic (185.60–827.04 $\mu g\, g^{-1}$ of extract), *p*-hydroxybenzoic (6.32–81.45 $\mu g\, g^{-1}$ of extract), and caffeic acid (1.01–13.54 $\mu g\, g^{-1}$ of extract) derivatives.

4. CONCLUSIONS

The results of the present investigation disclosed that slow-release amylase (SRA) containing enzymes can work as an attractive choice to liberate the bound phenolics from peach fruit during their industrial processing. It was observed that enzyme concentration, pH, temperature, and incubation time equally affected the liberation of polyphenols and their subsequent antioxidant activities. Overall, incubation of peach fruit with a smaller concentration of SRA-containing enzyme at elevated temperature (35–40 °C) and pH of 6.0–6.5 for shorter time intervals was more effective to liberate peach phenolics without compromising their antioxidant character. The results of the present experiments further revealed that multi-response optimisation is more critical for the enzyme-assisted extraction of plant bioactives without compromising their antioxidant activities.

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