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Influence of experimental conditions on the extraction of phenolic compounds from parsley (*Petroselinum crispum*) flakes using a pressurized liquid extractor

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Abstract

The influence of six pressurized liquid extraction parameters (temperature, pressure, particle size, flush volume, static time, and solid-to-solvent ratio) on the extraction of phenolic compounds from parsley flakes was examined. Parsley extracts were analyzed for their phenolic content by high performance liquid chromatography and Folin–Ciocalteu assay. The two major phenolic compounds extracted from parsley flakes were identified as apiin and malonyl-apiin. Particle size, solid-to-solvent ratio, and temperature influenced the amount of phenolic compounds extracted from parsley flakes. Higher extraction yields of phenolic compounds were obtained when extractions were performed with the smallest particle size (<0.425 mm) fraction. Temperature also showed a significant impact on the phenolic profile of the parsley extracts. The yield of malonyl-apiin decreased, while the amount of acetyl-apiin improved with the increase in extraction temperature. The latter compound (acetyl-apiin) is formed by decarboxylation of malonyl-apiin. The yield of the phenolic compounds did not increase proportionately with the increase in sample size, thereby suggesting that it is essential to have optimum solid-to-solvent ratio for accurate estimation of bioactive phytochemicals in foods. Flush volume did not show any major influence on phenolic yields, but it can be manipulated to reduce usage of extraction solvents, which in turn decreases solvent waste disposal costs generated after extraction. Similarly, changes in the static time (extraction time per cycle) and pressure did not result in any significant change in the yield of phenolic compounds extracted from parsley samples. However, sample throughput can be increased significantly by reducing static time.

Keywords: *Petroselinum crispum*; Sample preparation; Extraction temperature; Extraction pressure; Particle size; Flush volume; Static time; Solid-to-solvent ratio; Pressurized liquid extraction; Phenolic compounds

1. Introduction

Phenolic compounds are ubiquitously distributed throughout the plant kingdom (Antolovich, Prenzler, Robards, & Ryan, 2000; Naczk & Shahidi, 2004). Phenolic phytochemicals are known to exhibit several health beneficial activities such as antioxidant, anti-inflammatory, antihepatotoxic, antitumor, and antimicrobial (Criqui & Ringel, 1993; Hertog, 1995; Middleton, Kandaswami, & Theoharides, 2000; Renaud & De Lorgeril, 1992; Rice-Evans, Miller, & Paganga, 1996). The wide spectrum of bioactivities displayed by phenolic compounds isolated from different foods or food products has dictated a demand for accurate determination of phenolic compounds in different food matrices. Sample preparation is a primary and critical aspect of any analysis. It encompasses multiple steps ranging from sample grinding, extraction, preconcentration, hydrolysis, and derivatization (Antolovich et al., 2000; Luthria, 2006; Mitra & Brukh, 2003; Naczk & Shahidi, 2004). The ultimate goal of sample preparation is to eliminate or reduce potential matrix interferences and may involve conversion of the

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analyte of interest into a more suitable form that can be easily separated and/or detected.

The task of sample preparation for phenolic compounds is complex and challenging as over 8000 phenolic compounds have been isolated from natural sources (Antolovich et al., 2000; Luthria, 2006; Robbins, 2003). The chemical structure of the phenolic compounds varies from simple phenolic monomers to complex polymeric tannins. In addition, these compounds may possess multiple hydroxyl groups that can be conjugated to sugars, acids or alkyl groups (Antolovich et al., 2000; Luthria, 2006; Naczk & Shahidi, 2004; Robbins, 2003). Thus, the polarities of phenolic compounds vary significantly and it is difficult to develop a single method for optimum extraction of all phenolic compounds. Hence, optimization of the extraction procedure is essential for an accurate assay of phenolic compounds from different food matrices. Although, it is documented that approximately 30% of the analytical error for quantitative determination stems from sample preparation steps, optimization of sample preparation is frequently overlooked and often not well documented (Antolovich et al., 2000; Luthria, 2006; Majors, 1995).

In our previous communication, we evaluated the influence of extraction solvents, extraction techniques, and number of cycles on the quantity of phenolic compounds extracted from parsley flakes (Luthria, Mukhopadhyay, & Kwansa, 2006). In continuation of this research, we have studied the influence of six additional pressurized liquid extractor parameters (particle size, extraction temperature, extraction pressure, flush volume, static time, and solid-to-solvent ratio) on the extraction of phenolic phytochemicals from parsley flakes.

2. Materials and methods

2.1. Plant material

Dried parsley (*Petroselinum crispum*) flakes were purchased from a local grocery store (Giant in Beltsville, Maryland). Soon after receipt, the material was subdivided into multiple aliquots, flushed with nitrogen, and stored at −62 °C until analyzed.

2.2. Chemicals

HPLC-grade, methanol, HPLC-grade acetonitrile, diatomaceous earth (Celite 545) and Ottawa sand were purchased from Fisher Chemicals (Fair Lawn, New Jersey, USA), and HPLC-grade acetone was purchased from Burdick & Jackson (Muskegon, Michigan, USA). Denatured anhydrous ethanol was obtained from Mallinckrodt (Paris, Kentucky, USA). HPLC-grade formic acid was procured from Aldrich Chemical Company (Milwaukee, Wisconsin, USA) and apiin standard was obtained from Chromadex, Inc. (Santa Ana, California, USA). Deionized water (18 MΩ cm) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, Massachusetts, USA). Polyvinylidene difluoride (PVDF) syringe filters with pore size 0.45 μm were purchased from National Scientific Company (Duluth, Georgia, USA).

2.3. Extraction procedure

All extractions were carried out using a pressurized liquid extractor (PLE, Model ASE 200, Dionex Corporation, Sunnyvale, California, USA). For all extractions, except for the particle size experiment, powdered dried parsley flakes with particle size <850 μm were used. This ground material was obtained by grinding dried parsley flakes with a coffee grinder and passing the powdered material through a standard sieve number 20. All extractions were carried out with either one or two solvent mixtures (ethanol:water, 50:50, v/v and/or acetone:water, 50:50, v/v) that were previously optimized and reported in our earlier communication (Luthria et al., 2006).

Aliquots of 250 ± 1 mg of dried powdered parsley flakes were placed in an 11 ml stainless steel extraction cell. A total of four extraction cycles were performed for each sample and the purge time was set at 90 s for all extraction experiments. Two circular cellulose filters (size 1.983 mm, Dionex Corporation) were placed at the bottom of the extraction cell in order to prevent suspended particles from entering the collection vials. The remaining void volume in the cell was filled with Ottawa Sand. Both extraction cells and collection vials were arranged appropriately in the two designated carousels. The extracts were collected in 60 ml amber sample vials fitted with Teflon coated rubber caps. Each extract was transferred to a 25 ml volumetric flask and the total volume was adjusted to 25 ml with the appropriate solvent mixture. Aliquots of parsley extracts were filtered through a 0.45 μm PVDF syringe filter prior to assay for phenolics by HPLC and Folin–Ciocalteu (FC) assay. The additional details of conditions used for evaluation of the six extraction parameters are summarized in Table 1. Triplicate PLE extractions and analyses (HPLC and FC) were carried out with each experimental condition.

2.4. Determination of total phenolics (TP) by FC assay

The TP content was estimated using the FC assay with the gallic acid standard as described previously (Luthria et al., 2006; Singleton, Orthofer, & Lamuela-Raventos, 1974). The absorbance of the colored reaction product was measured at 765 nm. Different concentrations of gallic acid solution were used for calibrations. Results were expressed in mg of gallic acid equivalent per gram (mg GAE/g) of dried parsley flakes.

2.5. Separation and detection of phenolic compounds by HPLC and LC–MS analysis

Phenolic compounds extracted from parsley flakes (20 μL) were separated and analyzed by HPLC (Hewlett Packard 1050 pump) coupled with a photodiode array

Table 1
Details of the experimental parameters employed for optimization of the extraction procedure for phenolic compounds from parsley flakes using a pressurized liquid extractor

Experimental parameter changed	Temperature (°C)	Pressure (psi)	Static time (min)	Particle size (μm)	Solid-to-solvent ratio (mg/20 ml)	Flush volume (%)
Temperature (°C) (40,70,100,130,160)	Variable	1000	5	<850	250	75
Pressure (psi) (1000, 1250, 1500)	40	Variable	5	<850	250	75
Static time (min) (5, 10, 15)	40	1000	Variable	<850	250	75
Particle size (μm) (<425, >425 & <0.850, >850 & <2000, >2000)	40	1000	5	Variable	250	75
Solid-to-solvent ratio (mg/20 ml) (250, 500, 750, 1000, 1250)	40	1000	5	<850	Variable	75
Flush volume (%)(10, 25, 50, 75, 100)	40	1000	5	<850	250	Variable

detector (DAD) (series 1040M, series II) and an autosampler (series 1050) operated by HP ChemStation software (Lin & Harnly, 2007; Luthria et al., 2006). Mass spectral analysis was carried out on an Agilent 1100 LC system coupled with a diode array and MSD (SL) detectors (Agilent, Palo Alto, CA, USA) in a positive ion mode at both low and high fragmentor voltages (70 V and 250 V). The MS was set to scan from 100 to 2000 mass units. The instrument settings for the mass spectrometer were the same as reported previously (Lin & Harnly, 2007; Luthria et al., 2006). A reversed phase C_{18} Luna column (Phenomenex, 150 × 4.6 mm; particle size 5 μm), preceded by a guard column (Phenomenex, 4 × 3.0 mm) of the same stationary phase, was used for both HPLC and LC–MS analyses. The column and the guard column were thermostatically controlled at 40 °C and the flow rate was set to 0.5 ml/min. The mobile phase consisted of two solvents: acetonitrile (A) and water with 0.1% formic acid (B). The solvent gradient in volumetric ratios of solvents A and B was as follows: 0–30 min, 10–30% A; 30–50 min, 30–60% A; 50–55 min, 60–100% A; 65–70 min, 100–10%A; 75 min 10%A. Dual wavelengths (270 nm and 350 nm) were used to detect the eluent composition. HPLC chromatograms were obtained using a photo diode array UV detector. The structure of each phenolic compound was identified by LC–MS analysis as described in the earlier communication (Luthria et al., 2006).

2.6. Statistical analysis

All statistical analyses were conducted using a Statgraphics Plus 5 (Manugistics, Inc., Rockville, MD, USA) software. For each extraction parameter, a one-way ANOVA and subsequent multiple range tests were conducted on the peak areas for the two major and all identified phenolic compounds using the LSD technique to identify differences among the different extraction parameter levels.

3. Results and discussion

As described in our earlier communication (Luthria et al., 2006), an authentic purified standard of malonyl-apiin was not available from known commercial sources. Comparison of extraction efficiency was calculated on a percent basis from the HPLC peak areas of either all eight identified phenolic compounds (1. Apigenin-7-apiosylglucoside (apiin), 2. Diosmetin-apiosylglucoside, 3. Diosmetin-apiosylglucoside isomer, 4. Apigenin-malonyl-apiosylglucoside (malonyl-apiin), 5. Apigenin-malonyl-apiosylglucoside (malonyl-apiin), 6. Diosmetin-malonyl-apiosylglucoside, 7. Apigenin-malonyl-glucoside, and 8. Apigenin-acetyl-apiosylglucoside (acetyl-apiin)) and/or two major phenolic compounds (Apigenin-7-apiosylglucoside (apiin) and Apigenin-malonyl-apiosylglucoside (malonyl-apiin)) (Fig. 1). The details of the identification of all phenolic compounds by ultraviolet and mass spectral analysis have been previously reported (Luthria et al., 2006). Standard stock solutions (40 mg/l) of apigenin and apigenin-7-O-glucoside (Extrasynthese, Genay, France) were used as reference standards to monitor the performance of HPLC on a regular basis. All extractions were performed with either one and/or two best solvent systems (ethanol:water, 50:50, v/v and/or acetone:water, 50:50, v/v) with four extraction cycles that provided optimum extraction yield of phenolic compounds, as described in our earlier communication to evaluate the influence of six additional PLE parameters: particle size, extraction temperature, solid-to-solvent ratio, extraction pressure, static time, and flush volume.

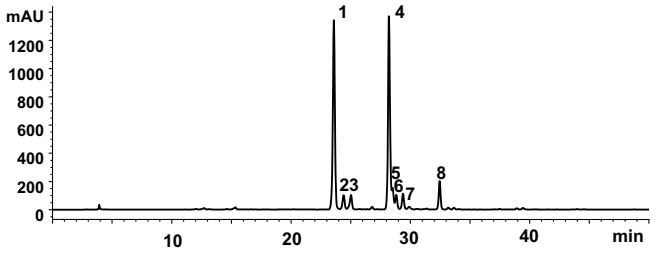

Fig. 1. HPLC profile of dried parsley flakes extracted with EtOH:H_2O, 50:50 (v/v) solvent mixtures by pressurized liquid extractor. The peaks were identified as 1. Apigenin-7-apiosylglucoside (apiin), 2. Diosmetin-apiosylglucoside, 3. Diosmetin-apiosylglucoside isomer, 4. Apigenin-malonyl-apiosylglucoside (malonyl-apiin), 5. Apigenin-malonyl-apiosylglucoside (malonyl-apiin), 6. Diosmetin-malonyl-apiosylglucoside, 7. Apigenin-malonyl-glucoside, and 8. Apigenin-acetyl-apiosylglucoside (acetyl-apiin).

3.1. Particle size

Influence of particle size on extraction efficiency was evaluated as this parameter is often overlooked by researchers and frequently not well-defined in peer-reviewed food and biological sciences journals (Canini, Alesiani, D'Arcangelo, & Tagliatesta, 2007; Dastmalchi, Dorman, Laakso, & Hiltunen, 2007; Tsai, Chang, & Chang, 2007). Selection of four different particle size fractions was based on the availability of sieves at our facility as well as on the sieves placed on different commercial grinders used in an analytical laboratory (Luthria, Noel, & Vinjamoori, 2004). Partially ground parsley flakes were passed through three different stacked standard-size sieves (numbers 10, 20, and 40) corresponding to pore sizes of 2.0, 0.85 and 0.425 mm, respectively. Four different particle size fractions were collected (>2.0, >0.85 and <2.0, <0.85 and >0.425, <0.425 mm) and extracted separately. Fig. 2 shows the influence of particle size on the extraction yield of phenolic compounds with two different assay procedures, namely HPLC and FC. Higher extraction yields of two major and all identified phenolic compounds was obtained when extractions were performed with the smallest particle size (<0.425 mm). The yields of all identified phenolic compounds extracted were reduced by approximately 5.0% and 11.2% with intermediate size particles (<0.85 and >0.425, >0.85 and <2.0). Only 82.3% of the phenolic compounds were extracted from the largest particle size fraction (PS > 2.0 mm) with the same extraction solvent under identical experimental conditions. A similar trend in the yields of TP compounds extracted from different particle size fractions was obtained from an independent colorimetric FC assay (Fig. 2B). As observed with HPLC analysis, optimum yield (22.9 mg GAE/g) was obtained with the smallest particle size fraction and the lowest amount (18.3 mg GAE/g) was obtained from the largest particle size fraction. The impact of particle size on extraction yields of phenolic compounds was not linear and similar results were also observed by Smith and Thomas (2005) during extraction of coffee solubles from different particle size coffee fractions. The increase in yields of phenolic compounds extracted from the small particle size parsley flakes fraction is due to larger surface area per unit mass that results in greater accessibility of the analyte to extraction solvent. In addition, the migration rate of the analyte through the pores of the solid matrix is also increased with the decrease in particle size. The influence of particle size on the extraction of phenolic compounds from parsley flakes was marginal (20%) as compared to black cohosh where twice the amounts of phenolic compounds were extracted with a similar decrease in particle size (Mukhopadhyay, Luthria, & Robbins, 2006). This dif-

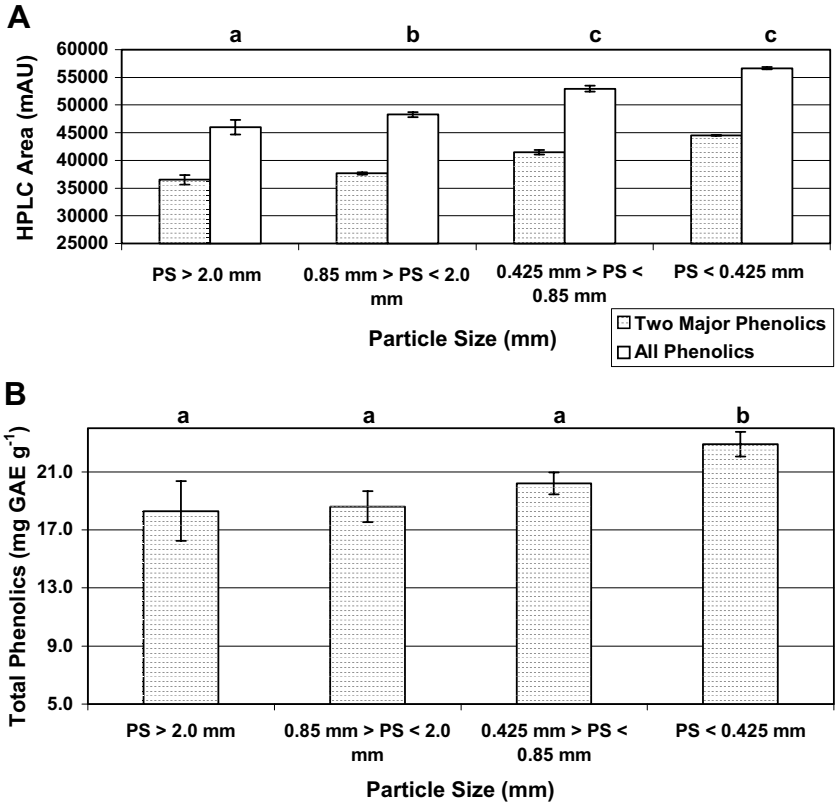

Fig. 2. Influence of particle size on extraction yields of phenolic compounds at 40 °C as assayed by: (A) high performance liquid chromatography method using diode array detection and (B) Folin–Ciocalteu assay. *Estimation of phenolics associated with different letters are significantly different at $\alpha \leqslant 0.05$.

ference in the effect of particle size in extracting phenolics is due to the difference in the type of plant matrix (dried leaf flakes vs. dried roots and rhizomes).

3.2. Temperature

The effect of temperature on extraction efficiency was investigated, since it impacts the equilibrium (solubility), mass transfer rate (diffusion coefficient), and the stability of phenolic compounds (Gertenbach, 2001; Spigno, Tramelli, & De Faveri, 2007). It is also possible to extract an analyte at temperatures well above the normal boiling point of the solvent with PLE. However, extraction beyond the boiling point of the extraction solvent is not possible with Soxhlet, sonicators, stirring, and shakers that are commonly used for extraction. Five different temperatures (40, 70, 100, 130 and 160 °C), two below and three above the boiling point of the extraction solvent were selected to evaluate the influence of temperature on the total extraction efficiency and stability of phenolic phytochemicals from dried parsley flakes. Fig. 3a depicts the influence of temperatures on the extraction efficiency of phenolic compounds from parsley flakes. Insignificant changes (<10%) in the total amount of phenolic compounds extracted were observed when the temperature was increased from 40 to 160 °C at 30 °C increments (Fig. 3a). Although the quantity of phenolic compounds extracted changed only marginally, the profile of the phenolic compounds extracted from parsley flakes changed significantly with the increase in temperature from 40 °C to 160 °C as shown in Fig. 3b. The amount of apiin and acetyl-apiin, as estimated by HPLC, increased with the increase in temperature, whereas the quantity of malonyl-apiin decreased with the increase in temperature. These results indicate that malonyl-apiin is unstable at high temperature and gets partially converted to acetyl-apiin and apiin by decarboxylation and deacetylation.

3.3. Solid-to-solvent ratio

The impact of solid-to-solvent ratio is also frequently overlooked as a wide range of solid-to-solvent ratio have been employed and reported for extraction of phytochemicals from various plant matrices (Antolovich et al., 2000; Luthria, 2006; Mukhopadhyay et al., 2006). Since fixed volume (11 ml) stainless steel cells were used for extraction with PLE, the influence of solid-to-solvent ratio was studied by varying the amount of powdered parsley flakes (250, 500, 750 and 1000 mg) used per extraction. The range of solid-to-solvent ratio selected in the present study was

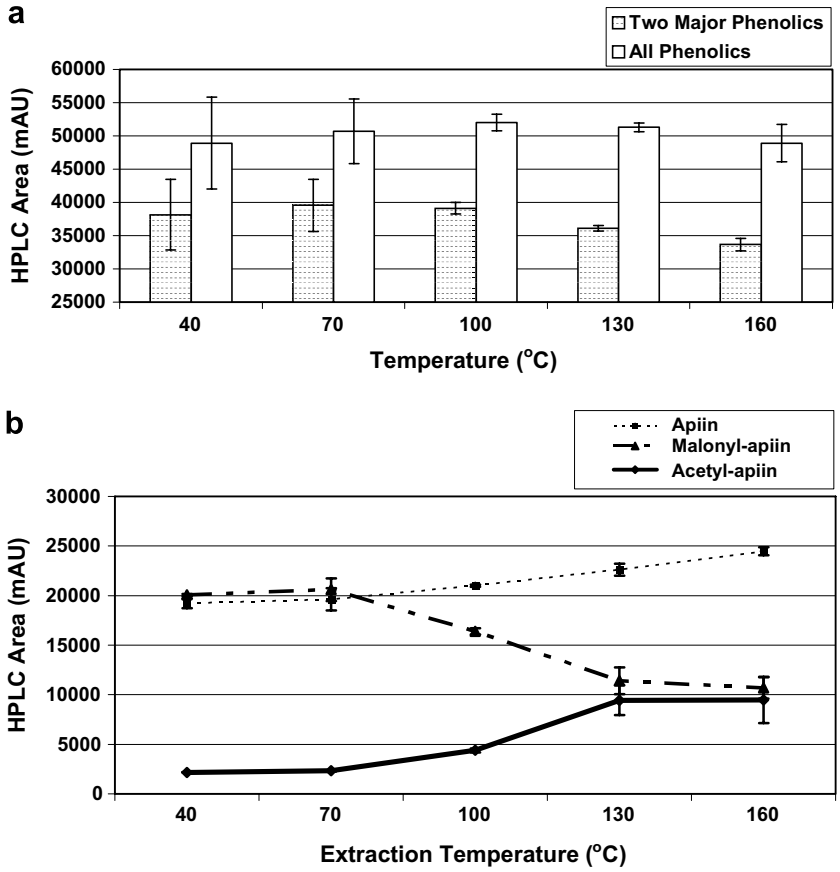

Fig. 3. Impact of temperature on extraction yields on: (a) total phenolic compounds or two major phenolics and (b) individual phenolics: apiin, malonyl-apiin, and acetyl-apiin using high performance liquid chromatography method with diode array detection.

based on frequently reported values used as sample size in an analytical laboratory as documented in the peer-reviewed published literature (Lin & Harnly, 2007; Luthria et al., 2006; Mukhopadhyay et al., 2006; Spigno et al., 2007; Tsai et al., 2007). The amount of phenolic compounds extracted from varying quantities of parsley flakes is shown in Fig. 4a. The amount of phenolic compounds increased with an increase in the quantity of parsley flakes; however, the increase in the yield of phenolic compounds was not directly proportional. The extraction efficiency of phenolic compounds per unit mass of sample matrix decreased with the increase in the amount of parsley flakes. For 250 mg of sample, the average HPLC peak area for all the identified phenolics was determined as 50,444 mAU. However, with 500 mg sample size, the average peak area was calculated as 92,109 mAU. The efficiency of extraction with the 500 mg sample size with identical solvent volume was 91.3% as compared to the 250 mg sample matrix. However, extraction effectiveness reduced to 78.3% and 74.8% with 750 and 1000 mg sample size, respectively. Thus, it is important to evaluate the influence of solid-to-solvent ratio during optimization of extraction of phytochemicals from different plant matrices. This approach will aid researchers and process operators in efficient usage of solvent or solvent mixtures for extracting phytochemicals from plant matrices. In addition, this approach will also reduce solvent waste disposal costs generated during extraction.

3.4. Pressure

Extraction of phenolic compounds from parsley flakes was carried out at three different pressure settings (1000, 1250, and 1500 psi) that are commonly applied with PLE to evaluate whether pressure influences the extractability of phenolics by increasing diffusivity of extraction solvent within the sample matrix (Carabias-Martínez, Rodrıguez-Gonzalo, Revilla-Ruiz, & Hernández-Méndez, 2005). Three replicate extractions were carried out at each pressure setting. The results presented in Fig. 4b illustrate that pressure did not influence the extraction efficiency of phenolic compounds from parsley flakes as similar yields were

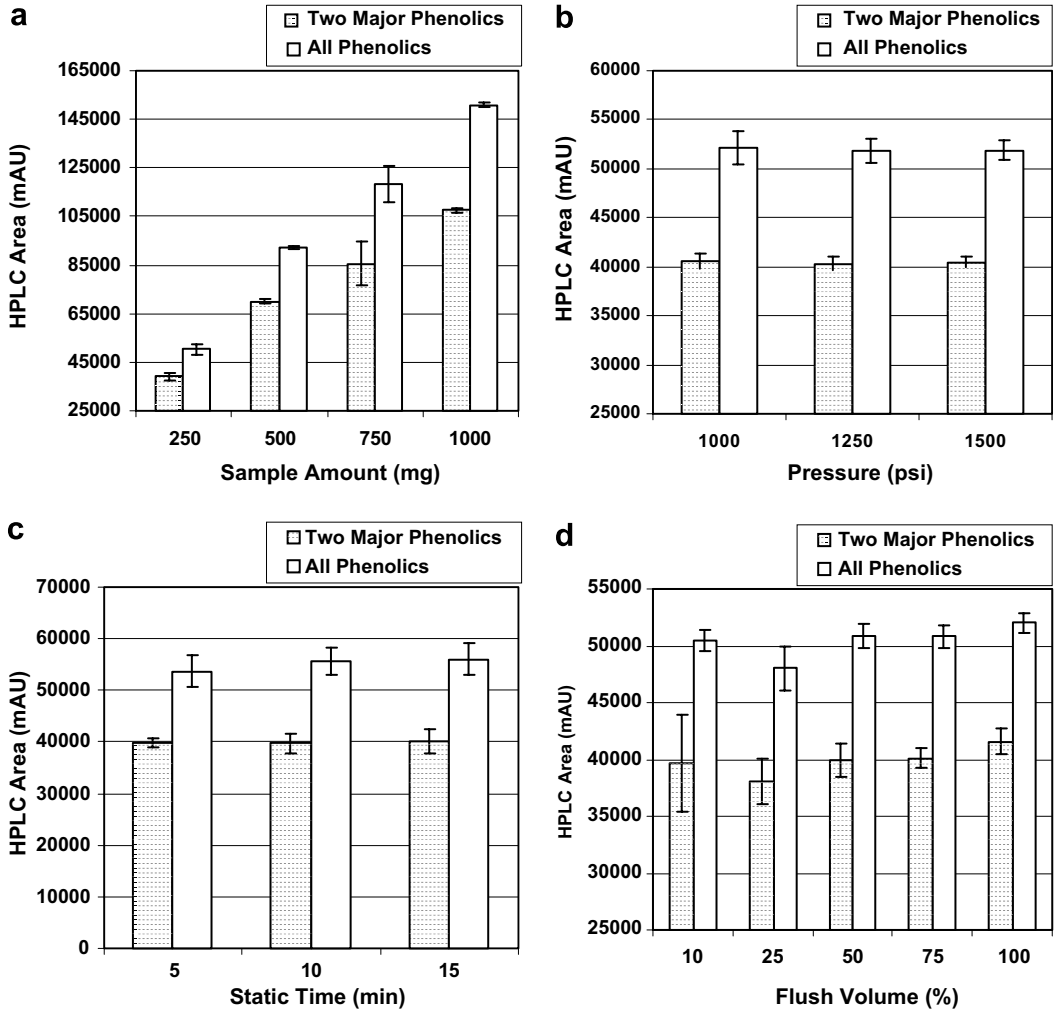

Fig. 4. Influence of pressurized liquid extraction parameters: (a) solid-to-solvent ratio, (b) pressure, (c) static time, and (d) flush volume on the assay of phenolic compounds from ground parsley flakes using a high performance liquid chromatography procedure with diode array detection.

obtained at all three pressure settings, whether monitoring the two major or all the identified phenolic compounds. Similar results were also observed during extraction of phenolic acid from black cohosh and isoflavone from soybeans (Mukhopadhyay et al., 2006; Rostagno, Palma, & Barroso, 2004). In all the above cases, an increase in pressure did not change the amount of phenolic compounds extracted from the different sample matrices.

3.5. Static time

Static time is defined as the time the sample interacts with the extraction solvent per cycle in the extraction cell under defined extraction conditions in a PLE. To study the impact of static time, parsley flakes were extracted with three different static time settings (5, 10, and 15 min) as a static time between 5 and 30 min has been frequently reported by different researchers with PLE (Carabias-Martínez et al., 2005). Three replicate analyses were carried out at each time setting. The results obtained from this experiment are presented in Fig. 4c. No significant changes in the extraction yields were observed at three different static time settings whether monitoring the two major phenolics or all the identified phenolic compounds. These results are different from those observed for isoflavones, where an increase in static time from 5 to 7 min resulted in an increase in the isoflavones yield extracted from soybean samples (Rostagno et al., 2004). Thus, by optimizing static time the PLE extraction sample throughput can be significantly improved.

3.6. Flush volume

The flush volume determines the amount of solvent used per extraction. Optimization of the flush volume results in a reduction in the amount of solvent used per extraction, which in turn determines the solvent waste generated per extraction. The amount of solvent used per extraction with five different flush volume settings (10%, 25%, 50%, 75%, and 100%) was determined as 6.6, 8.8, 12.0, 14.3, and 16.9 ml, respectively. Thus an increase of approximately 300% in solvent consumed per extraction was observed when the flush volume was changed from 10% to 100%. The five step increment was selected as these are often documented in the literature and there is significant differences in the volume of solvent consumed and the waste generated during extractions (Carabias-Martínez et al., 2005; Luthria, 2006). The data presented in Fig. 4d depict the influence of flush volume on extraction efficiency of phenolics from parsley flakes. Insignificant differences in extraction efficiency were obtained when the flush volume was changed from 10% to 100%.

4. Conclusion

The influence of six different parameters (particle size, extraction temperature, extraction pressure, flush volume, static time, and solid-to-solvent ratio) on the extraction of phenolic compounds from ground parsley flakes was studied. The phenolic compounds extracted from parsley were analyzed by two independent procedures, namely HPLC and FC assay. The extraction yield of the phenolic compounds was influenced by temperature, particle size, and solid-to-solvent ratio. Temperature had a major impact on the phenolic profile, as at higher extraction temperature, malonyl-apiin was partially degraded to acetyl-apiin and apiin. No significant impact on the amount of phenolic compounds extracted from parsley flakes was observed with the other two parameters (static time and pressure). Even though the flush volume showed marginal influence on the extraction yield of phenolic compounds from parsley flakes, its effect on the amount of solvent consumed and waste generated was significant. Additional, optimization using experimental design and interactions between different experimental parameters will be beneficial in designing industrial scale extraction of bioactive phytochemicals for different matrices. The primary objective of this manuscript is to increase awareness and importance of sample preparation that is often overlooked, not optimized, or not properly documented in peer-reviewed published manuscripts as sample preparation is often considered "as a means to an end" by most researchers. The data presented in this manuscript and some previously reported manuscripts and reviews (Antolovich et al., 2000; Carabias-Martínez et al., 2005; Luthria, 2006; Naczk & Shahidi, 2004; Robbins, 2003) unambiguously demonstrates that optimization of the sample preparation procedure is critical for accurate analysis of different phytochemicals. Precise quantitation of bioactive phytochemicals will allow researchers to reproduce published results and accurately determine dietary intake levels, safety guidelines, and also precisely evaluate the health beneficial properties of bioactive phytochemicals.

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