Phenolic Compounds Analysis in Foods and Dietary Supplements is not the Same Using Different Sample Preparation Procedures

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Abstract
Recent epidemiological studies suggest a positive correlation between diets rich in fruits and vegetables and a reduced incidence of chronic diseases. This beneficial effect is partially attributed to phenolic phytochemicals, a complex group of secondary metabolites that provide flavor and color to fruits, vegetables, and grains. More than 8,000 different phenolic phytochemicals belonging to different subgroups (polyphenols, phenolic acids, and a miscellaneous group) have been identified. The large number of phenolic compounds, their structural diversity, and their interaction with other cellular constituents present a considerable challenge in developing efficient procedures for optimum extraction and accurate analysis of different plant matrices. This paper illustrates the difficulties related to the extraction of phenolic compounds using examples from peer-reviewed literature. It discusses the importance of optimizing sample preparation procedures for accurate estimation of phenolic compounds from foods (eggplant, soybean and parsley) and dietary supplements (Black cohosh). A comparison of current and classical procedures for the extraction of phenolic phytochemicals is presented. The influence of different extraction parameters, such as solvent composition, particle size, pressure, temperature, solid-to-solvent ratio, and number of extraction cycles is also discussed. A systematic approach for optimum extraction of phenolic phytochemicals from different plant matrices has been included. Accurate quantification of bioactive phenolic phytochemicals will allow researchers to provide better guidelines on dietary intake levels necessary to achieve the optimum health.

INTRODUCTION
The importance of bioactive phytochemicals in human health and nutrition is well documented in peer-reviewed scientific literature and is also widely recognized by food, nutrition, and pharmaceutical industries, as well as by consumers worldwide (Hasler, 1998; Dufresne Farnsworth, 2001; Wildman, 2001; Mark-Herbert, 2004; Mandel et al., 2005). Phenolic phytochemicals are secondary metabolites that are widely distributed throughout the plant kingdom (fruits, vegetables, and grains) and are known to provide protection against a wide range of diseases such as coronary heart disease, stroke, and certain types of cancers. Hence, accurate quantification of phenolic phytochemicals in foods and food products is of vital importance to precisely evaluate their role in health and nutrition.

Over 8,000 phenolic phytochemicals with wide structural diversity and polarities have been isolated from plant sources, making accurate quantification of phenolic compounds a challenging task (Robbins, 2003; Luthria, 2006). This challenge is further exacerbated by the fact that phenolic compounds are not uniformly distributed in plants and can be covalently bound to other cellular components such as the cell membrane and other macromolecules (Antolovich et al., 2000; Naczk and Shahidi, 2004). Phenolic compounds can be grouped into three broad categories: phenolic acids, polyphenols, and a miscellaneous group. Phenolic acids can be further subdivided into two main subgroups, hydroxycinnamic and hydroxybenzoic acids. Similarly, polyphenols can be arranged
into two broad classes: tannins (gallic acid, catechin, or epicatechin polymers) or flavonoids (flavones, flavonols, flavanones, flavanols, anthocyanidins). The miscellaneous group is comprised of lignans, lignins, coumarins, stilbenes, and other phenolic compounds not included in the other two subgroups (Luthria, 2006).

An analytical procedure generally consists of four steps: 1. sample preparation, 2. analytical separation, 3. detection, and 4. data collection and processing. Significant advancements have been made in the final three steps; however, there has been limited progress in the first, the sample preparation step. Sample preparation is often considered as a rate limiting step and is estimated to account for approximately 30% of the analytical error (Majors, 1999). Sample preparation encompasses multiple steps such as grinding, sieving, extraction, pre-concentration, filtration, and derivatization. This manuscript focuses on the significance of the extraction step, an important part of sample preparation, on the accurate quantification of phenolic phytochemicals from different plant matrices.

MATERIALS AND METHODS

Dried parsley (Petroselinum crispum) flakes and soybean (Glycine max [L.] Merr.) samples were purchased from Giant and MOM’s (My Organic Market) and local grocery stores in Beltsville, Maryland, USA. Fresh freeze-dried powder of black cohosh (Cimicifuga racemosa) from root and rhizome was obtained from Dr. David Lytle (Eclectic Institute, Oregon, USA). Black bell variety of eggplant (Solanum melongena L.) was obtained from a USDA farm in California, USA. The flesh was chopped into small pieces and lyophilized. All samples were ground in a coffee grinder. All ground samples were passed through a standard 20 mesh sieve (particle size < 0.825 mm), mixed thoroughly, and subdivided into multiple aliquots in amber bottles. Each bottle was flushed with nitrogen, and stored in a freezer (<-60°C) until analyzed.

Extraction was carried out with different techniques, such as stirring, Soxhlet, rotary shaker, ultrasonic irradiation, and pressurized liquid extractor (PLE). The influence of various solvent compositions on the extraction of phenolic compounds from different matrices was evaluated. The impact of a variety of parameters such as solvent, particle size, pressure, temperature, solid-to-solvent ratio, and number of extraction cycles, which are often ignored or generally considered trivial, were also studied.

RESULTS AND DISCUSSION

The variability in the phenolic phytochemicals content in foods is well documented in peer-reviewed literature. These variations may be attributed to various factors namely, genotypes, cultivar, growing, storage, processing, environmental, and/or analysis conditions (Escarpa and Gonzalez, 2001; USDA Database, 2003; Ninfali and Bacchiocca, 2003; Vallejo et al., 2003; Giuntini et al., 2005; Anttonen and Karjalainenb, 2005; Luthria et al., 2006; Li et al., 2007).

Methodology of Extraction

The effect of six commonly used extraction methods on the determination of isoflavones in soybeans by six commonly used extraction techniques (PLE, sonication, Soxhlet, shaker, vortex, and stirring) was described in a recent publication (Luthria et al., 2007). Isoflavones were extracted with a single solvent mixture (dimethyl sulfoxide:acetonitrile:water, 5:58:37, v/v/v) using the same solid-to-solvent ratio. The results showed that the best yields (> 95%) of total isoflavones was achieved using the PLE and sonication procedures (Luthria et al., 2007). The efficiency of the other four classical procedures (stirring, Soxhlet, shaking and vortexing) was between 65.6 and 70.4%, as compared to PLE. Earlier, Rostagno, Araujo and Sandi (2002) had compared the extraction efficiencies of soybean isoflavones by sonication, Soxhlet, and supercritical fluid extraction. The authors reported that optimum extractions were obtained with sonication, as observed in our study. The extraction efficiency for Soxhlet was also similar to those reported in our study. The lowest yields (27.7%) of isoflavones were obtained with supercritical fluid extraction. However, in another study, Delmonte, Perry
and Rader (2006) showed similar recoveries for total isoflavones with the sonication and shaking techniques. These results differed from our present study where we observed only 75.6% efficiency with the shaking procedure, as compared to sonication. This variation in isoflavone yields may be attributed to differences in extraction solvents, solid-to-solvent ratios, and temperature used during extraction by shaker and sonication procedures. In our study, we used the same solvent mixture, solid-to-solvent ratio, and extraction conditions for both procedures (sonication and shaking).

**Extraction Solvent Composition**

Marginal variations in the solvent composition can significantly impact the quantity and quality of the extracted components (Luthria, 2008). Apiin and malonylapiin were the two most abundant phenolic compounds extracted from parsley flakes. Apiin was identified as the major phenolic compound when parsley extractions were carried out with ethanol-H2O (70:30, v/v). However, the HPLC profile showed two major compounds (apiin and malonylapiin) when the extractions solvent was changed to ethanol-H2O (50:50, v/v) (Fig. 1). These results suggest that it is essential to optimize extraction solvent composition for maximum yield of the phenolic analyte of the interest from different matrices. In other studies with soybeans, eggplant, and Black cohosh, we observed significant differences only in the extraction efficiency of the phenolic compounds, but not in the number of components extracted. (Luthria et al., 2006, 2007; Mukhopadhyay et al., 2006).

**Particle Size and Pressure**

The surface area per unit mass increases as the particle size of the plant material decreases and may influence the extraction of analytes from the plant material. The influence of particle size on extraction of phenolic compounds from parsley flakes was marginal (≤ 20%; Luthria et al., 2006) compared to Black cohosh, where a 100% increase in the amount of phenolic compounds extracted was observed with a similar decrease in the particle size (Mukhopadhyay et al., 2006). A similar increase in oil yield from soybeans was obtained when the particle size of soybeans was reduced from > 1.4 mm to < 0.3 mm (Luthria et al., 2004).

We observed no significant change in the extraction yield of the total phenolics extracted from different plant matrices (parsley, soybeans and Black cohosh) when the pressure was changed from 1,000 psi to 1,500 psi and other extraction conditions were kept constant (Luthria et al., 2006, 2008; Mukhopadhyay et al., 2006).

**Temperature**

Temperature affects both the equilibrium (solubility) and mass transfer rate (diffusion coefficient) of analyte into the extraction solvent. We used PLE to evaluate the influence of temperature on the extraction of total phenolics from Black cohosh. PLE allows the use of temperatures well above the normal boiling point of the solvent, a capability not possible with classical procedures, such as Soxhlet extraction (Mukhopadhyay et al., 2006). Total phenolics were extracted with MeOH:H2O (60:40, v/v) at six different temperatures: 40, 50, 60, 70, 80 and 100°C. The maximum extraction of TP was achieved around 90°C. The extraction efficiency increased by almost 30% as the temperature was increased from 40°C to 90°C. However, at 100°C, a 20% decline in extracted phenolics was observed. In a recent study with parsley flakes, we showed that malonylapiin was degraded as the extraction temperature increased from 40°C to 160°C (Luthria, 2008).

**Solid-to-Solvent Ratio**

The influence of the solid-to-solvent ratio is often overlooked. This is apparent from the wide range of ratios reported for the extraction of phytochemicals from different plant matrices (Luthria, 2006; Luthria et al., 2007). As extractions with PLE were carried out in fixed volume (11 ml) stainless steel cells, the influence of the solid-to-solvent ratio...
was investigated by varying the amount of powdered parsley flakes (250, 500, 750 and 1,000 mg) used (Luthria, 2008). The amount of phenolic compounds extracted increased with the increasing mass of parsley flakes. However, the yield of phenolic compounds per gram of parsley flakes was not directly proportional to the mass increase. For a set solvent volume, the extraction efficiency of phenolic compounds per unit mass of sample matrix decreased with the increasing mass of parsley flakes. For 250 mg of sample, the average integrated HPLC area for three replicate analyses of all the identified phenolics was determined as 50,444 mAU. However, with 500 mg of sample, the average area was calculated as 92,109 mAU. The efficiency of extraction with the 500 mg sample size with the identical solvent volume was 91.3%, as compared to the 250 mg sample size (100%). Extraction effectiveness declined to 78.3% and 74.8% with 750 and 1,000 mg sample sizes, respectively. Therefore, the solid-to-solvent ratio is an important factor to consider when optimizing the extraction of phytochemicals from different plant matrices. This approach will assist researchers and process operators in efficient usage of extraction solvent(s). In addition, this approach will also aid in reducing the solvent waste generated during extraction.

### Number of Extraction Cycles

Optimization of the number of extraction cycles and the flush volume (a variable parameter associated with a pressurized liquid extractor, ASE 200 from Dionex, Inc.) will not only assist in increasing the efficiency of extraction, but will also result in reducing the amount of solvent used and solvent waste generated during extractions. Percent phenolics extracted per cycle was calculated by dividing the peak areas of all identified phenolic compounds for each extraction by the sum of HPLC peak areas for all four cycles. Using parsley as a model substrate, the maximum yield of phenolic compounds (88%) in the first cycle with acetone:H2O (50:50, v/v) as extraction solvent (Luthria et al., 2006). However, when the extraction solvent composition proportion was changed to either 90:10 (v/v) or 10:90 (v/v), the extraction efficiency for the first cycle was reduced by over 30% (Luthria et al., 2006).

The flush volume is the volume of fresh solvent added to the extraction cell per cycle following the static step. This extraction parameter is related to all accelerated solvent extractors (ASE 100, ASE 200 and ASE 300) only. This determines the amount of solvent consumed per extraction. Optimization of flush volume will result in reduction of the solvent used per cycle, which in turn reduces the solvent waste generated per extraction cycle. The amount of solvents used per extraction with five different flush volume settings (10%, 25%, 50%, 75%, and 100%) were determined as 6.6, 8.8, 12.0, 14.3, and 16.9 ml, respectively (Luthria, 2008). Thus an increase of over 250% in solvents consumed per extraction was observed when the flush volume was varied from 10 to 100% when 200 mg of parsley flakes were extracted in an 11 ml cell with four extraction cycles. The result obtained with parsley flakes showed only a marginal increase (< 10%) in total phenolics extracted when flush volume was changed from 10 to 25%. Further increases in the flush volume to 50% and 100%, respectively, did not result in any significant increase in percent phenolics extracted from parsley flakes.

### CONCLUSIONS

The results reviewed in this manuscript, as well as the references cited, clearly illustrate the significance of sample preparation procedures (extraction techniques and solvent composition, temperature, particle size, pressure, solid-to-solvent ratio, number of extraction cycles, and flush volume) for accurate quantification of phenolic phytochemicals in foods, food products and dietary supplements. This will allow researchers to accurately evaluate their role as it relates to health and nutrition.

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**Literature Cited**


Fig. 1. Influence of extraction solvent on assay of phenolic compounds from parsley flakes. HPLC profile of dried parsley flakes extracted with two different solvent mixtures. Peak identification: 1) apiin; and 2) malonyl-apiin.