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Influence of Sample Preparation on Assay of Phenolic Acids from Eggplant

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Sample preparation is often overlooked and is frequently considered as "a means to an end". This systematic study with a phenolic-enriched substrate, eggplant (*Solanum melongena* L.), was undertaken to evaluate the substantial variations in the extraction techniques, solvents, and parameters as described in the published literature. Direct comparison of over 10 extraction procedures or conditions was performed to show the importance and influence of sample preparation on the assay of phenolic compounds. Chlorogenic acid (CA) was the most abundant phenolic acid accounting for >75% of the total phenolic acids content extracted from the eggplant sample. Optimum extraction of CA and total phenolics (TP) from Black Bell cultivar of eggplant were obtained when extractions were performed with a mixture of MeOH/H_2O at a ratio of 80:20% v/v using a pressurized liquid extractor (PLE) at 100 °C. The amount of CA and TP extracted from eggplant by the previously reported procedures using a wrist shaker, rotary shaker, stirring, sonication, or reflux with different extraction solvents (acetone or varying composition of MeOH/H_2O solvent mixtures) varied significantly between 5 and 95% as compared to PLE. The predominant phenolic acids in the free phenolic acid fraction of Black Beauty cultivar of eggplant were CA isomers. However, caffeic acid isomers were the major phenolic acids extracted from the base-hydrolyzed fraction. The total amount of caffeic acid extracted from the Italian Neon cultivar was more that twice that of four other eggplant cultivars (Orient Express, Calliope Zebra Stripe, Orient Charm Neon, and Black Beauty).

KEYWORDS: Eggplant; *Solanum melongena* L.; sample preparation; extraction procedures; phenolic acids; Folin−Ciocalteu; HPLC; solvent composition; hydrolysis

INTRODUCTION

Phenolic acids (PA) are aromatic secondary metabolites biosynthesized by plants and are ubiquitous throughout the plant kingdom (*1*). The chemical nature of plant phenolics may vary from a simple monomeric unit to highly polymerized structures of varying proportions of monomeric aglycon units (*2*). In recent years, phenolic acids have received considerable attention due to their health beneficial effect for protection against certain form of cancers and cardiovascular diseases (*3*, *4*). Eggplant (*Solanum melongena* L.) is ranked among the top 10 vegetables in terms of antioxidant capacity (*5*). Eggplant is a common vegetable consumed throughout the world. It is a member of the potato family, and it is known worldwide, in various synonyms as aubergine, brinjal, melanzana, garden egg, and patlican. The first report on the extraction and identification of chlorogenic acid and browning in eggplant was published by Kozukue et al. (*6*). The influence of storage conditions (temperature and time) was studied by both Kozukue et al. and Esteban et al. (*6*, *7*). Research related to the beneficial effects of the consumption of eggplant phenolics in animal subjects was carried out by Sudhesh et al. (*8*). The antioxidant activity of eggplant by different assays was reported by Huang et al. (*9*). Recently, Stommel and Whitaker carried out a detailed study on the identification and determination of PA in different cultivars of eggplants (*10*, *11*).

The procedures used by various research groups for the extraction of phenolics from eggplant varied significantly and are summarized in **Table 1**. These data clearly illustrate that sample preparation is often overlooked and is frequently considered as "a means to an end". In recent years, there have been tremendous advancements in chromatographic and spectroscopic instrumentations for the separation, detection, and identification of phenolic compounds from natural sources. However, sample preparation has received limited attention. Optimum sample preparation is critical for any analyses and is of great importance for phenolic compounds due to the oxidative and thermal labile nature of this class of compounds. In addition, >8000 naturally occurring phenolic compounds with diverse structural configurations have been isolated from natural sources (*1*). Phenolic acids may exist in multiple forms as free, esterified, glycosylated, or polymerized and may coexist as complexes with

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Table 1. Summary of Reported Procedures Used by Various Authors for Extraction of Phenolic Acids from Eggplant and Comparison with Optimized Extraction Procedures As Described in This Paper

sample matrix	study objective	sample amount	extraction technique	solvent amount	extraction solvent	extraction time	extraction temp	ref
lyophilized powdered tissue	phenolic acid content and composition	200 mg	sonication	10 mL (twice)	MeOH with 0.5% BHT	15 min (twice)	ambient	*10*, *11*
freeze-dried and ground fruit	antioxidant activity	nr[a]	rotary shaker	nr	50% MeOH	30 min	4 °C	*9*
	influence of storage condition on phenolic content	50 g	homogenized in boiling EtOH and refluxed	100 mL (twice)	EtOH	nr	78 °C	*6*
deep-frozen fruit	phenolic acid esters and glucoside analysis	nr	homogenized samples were stirred	nr	80% MeOH	30 min (twice)	ambient	*12*
fruit	variation in chemical composition during growth and ripening	25 g	reflux	100 mL	MeOH	1 h	65 °C	*7*
homogenized fruit pulp	antioxidant activity	nr	centrifuged and residue extracted with acetone on a shaker	nr	acetone	30 min	ambient	*5*
freeze-dried, ground, and sieved (particle size $\leq$ 825 μm)	optimization of sample preparation procedure	200 mg	PLE	20 mL	MeOH/H_2O (80:20% v/v)	30 min	100 °C	optimized condition described in this paper

[a] Not reported.

proteins, carbohydrates, lipids, or other plants components (*2*). Thus, the polarity of phenolic acids varies significantly, and it is difficult to develop a uniform extraction method for extracting different phenolic acids from various matrices. These multiple factors impose major challenges in the development of a uniform, efficient, and accurate sample preparation procedure. Literature reports indicate that ~60% of the analysis time is spent on sample preparation and that ~30% of analytical error stems from sample preparation step (*13*, *14*). Three recent reviews on the subject of sample preparation in the assay of phenolics from foods emphasize a critical need for the development of systematic sample preparation procedures for optimum extraction and accurate quantitation of phenolic compounds in different matrices (*2*, *15*, *16*).

This paper provides a direct comparison of different extraction procedures performed in our laboratory used for the assay of phenolic compounds from the same eggplant sample (Black Bell cultivar) as published by various research groups (**Table 1**). In addition, we systematically investigated the influence of solvent composition, temperature, and influence of butylated hydroxytoulene (BHT) on the extraction efficiency of phenolic acids using a pressurized liquid extraction (PLE) procedure. This is the first report on the extraction of phenolic acid from eggplant using PLE. As phenolic acids are known to be present in both free and conjugated forms in food, a systematic sequential hydrolysis (free phenolic acid extraction followed by base and acid hydrolysis of the same extract) was also performed. Extracts were analyzed for phenolic content by two separate independent assays, Folin−Ciocalteu (FC) and HPLC.

MATERIALS AND METHODS

Samples. For comparison of different extraction procedures, a Black Bell variety of eggplant was obtained from a USDA farm in Riverbend, CA. In addition, five cultivars of eggplant (Orient Express, Calliope Zebra Stripe, Black Beauty, Orient Charm Neon, and Italian Neon) were purchased from a local farm (Jasmine Farm, LLC, Howard County, Maryland) for optimization of extractions with PLE and hydrolysis procedure. Immediately after receipt, all eggplant samples were stored at −60 °C under inert nitrogen atmosphere. Eggplant samples were partially thawed and peeled. The flesh was chopped into small pieces, lyophilized, and ground in a coffee grinder. The dried powdered material was passed through a standard 20 mesh sieve (particle size < 0.825 mm), and the ground sample was mixed and stored under nitrogen at −60 °C until analyzed.

Chemicals. Standards of phenolic acids (caffeic and chlorogenic), Folin−Ciocalteu reagent (FC), gallic acid, sodium carbonate, and BHT were purchased from Sigma (St. Louis, MO). HPLC-grade solvents MeOH and acetonitrile and analytical-grade ethyl acetate were purchased from Fisher Chemicals (Fair Lawn, NJ). HPLC-grade acetone was purchased from Burdick & Jackson (Muskegon, MI). Denatured anhydrous EtOH was obtained from Mallinckrodt (Paris, KY). Formic acid (HPLC-grade) and sodium hydroxide were procured from Aldrich Chemical Co. (Milwaukee, WI), and analytical grade hydrochloric acid was obtained from Fisher Chemicals. Deionized (DI) water (18 Ω) was obtained in-house using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA). Poly(vinylidene difluoride) (PVDF) syringe filters with a pore size of 0. 45 μm were purchased from National Scientific Co. (Duluth, GA). Genapol X-080 was purchased from Sigma-Aldrich Chemie Gmbh (Munich, Germany).

Comparison of Extraction Procedures. Extractions with six different extraction procedures, as reported in the earlier investigations, were performed using a single eggplant (Black Bell cultivar) sample. Four replicate extractions and analyses were carried out with each procedure.

Sonication. Freeze-dried eggplant (200 mg) was separately extracted with both 10 mL of MeOH and MeOH containing 0.5% BHT in a sonicator bath (Branson 2510, Branson Ultrasonic Corp., Danbury, CT). Sonication was carried out at ambient temperature for 15 min. The mixture was centrifuged, MeOH was decanted, and the residue was re-extracted with a fresh 10 mL of solvent. The two extracts were combined, and the volume was adjusted to 25 mL in a volumetric flask with respective extraction solvents. Appropriate aliquots of extracts were filtered through a 0.45 μm PVDF syringe filter for phenolic assay by FC method and HPLC analysis.

Stirring. Freeze-dried eggplant (200 mg) was weighed in a 16 × 125 mm screw-cap glass vial fitted wit a small magnetic bar (12.7 × 3.0 mm). The mixture was stirred with 10 mL of MeOH/H_2O (80:20% v/v) at ambient temperature. The mixture was centrifuged, MeOH was decanted, and the residue was re-extracted with a fresh 10 mL of solvent. The mixture was worked up as described above and analyzed by HPLC and FC assays.

Rotary Shaker. Freeze-dried eggplant (200 mg) was weighed in a 100 mL round-bottom flask, and 20 mL of MeOH/H_2O (1:1% v/v) was added. The mixture was placed in a rotary shaker at 4 °C for 30 min. The extract was centrifuged, the supernatant was transferred to a 25 mL volumetric flask, the volume was adjusted, and the filtered extract was analyzed by HPLC and FC assays.

Reflux. Freeze-dried eggplant (200 mg) was weighed in a 100 mL round-bottom flask, and 20 mL of MeOH/H_2O (85:15% v/v) was added. The mixture was refluxed while stirring for 60 min. The extract was centrifuged, the supernatant was transferred to a 25 mL volumetric flask, the volume was adjusted, and the filtered extract was analyzed by HPLC and FC assays.

Shaker. Freeze-dried eggplant (200 mg) was weighed in a 50 mL screw-cap vial, and 20 mL of neat acetone or acetone/H_2O (85:15% v/v) was added. The vial was then placed on a Burrell wrist shaker (model 75, Burrell Corp., Pittsburgh, PA) at a high speed (setting 10) for 30 min. The extract was centrifuged, the supernatant was transferred to a 25 mL volumetric flask, the volume was adjusted, and the filtered extract was analyzed by HPLC and FC assays.

Pressurized Liquid Extraction. For the present optimization study, an accelerated solvent extractor (ASE) from Dionex Corp. (model ASE 200, Dionex Corp., Sunnyvale, CA) was used for PLE. Aliquots of 200 ± 1 mg of dried powdered eggplant samples were placed in an 11 mL stainless steel extraction cell. Two circular cellulose filters (size = 1.98 mm, Dionex Corp.) were placed at the bottom of the extraction cell 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. Extractions were carried out with solvents of different polarities (MeOH, EtOH, acetone, and solvent with different MeOH/H_2O proportions with and without 0.5% BHT or different proportions of Genapol X-080). Extractions were performed at 1000 psi, with a 5 min equilibration time, a 5 min static time and a 90 s of purge time for each extraction cycle. A total of four extraction cycles were performed for each sample. For comparison with other extraction procedures, the static time for PLE was set to 5 min to complete extraction per sample within 30 min. The extractions were carried out at 100 °C, and a total of ~20 mL of solvent was used for the four extraction cycles with flush volume set at 75%. Extracts were collected in 60 mL amber sample vials fitted with Teflon-coated rubber caps (I-CHEM, New Castle, DE). Each extract was transferred to a 25 mL volumetric flask, and the total volume was adjusted to 25 mL with appropriate solvent mixture. Aliquots of eggplant extracts were filtered through a 0.45 μm PVDF syringe filter prior to analysis of phenolic acids by HPLC. Triplicate extractions and HPLC analysis were carried out for each sample. Extractions with different concentrations of Genapol X-080 were carried out with 80% MeOH by replacing water with Genapol for appropriate concentrations of Genapol solutions.

Free Phenolic Acid Followed by Sequential Base and Acid Hydrolysis. A single eggplant sample (Black Beauty cultivar) was analyzed for free and conjugated phenolics acids. Sequential hydrolysis by base followed by the acid was carried out according to the same procedure as described by Kumpulainen and Mattila (*17*). MeOH (7 mL) containing 0.2% 2,3-*tert*-butyl-4-hydroxyanisole (TBH) and 10% aqueous acetic acid (85:15) was added to a powdered eggplant sample (0.5 g). The mixture was sonicated for 30 min, and the volume of the extract was made up to 10 mL with water. An aliquot of ~1 mL of this was filtered through a 0. 45 μm PVDF syringe filter and analyzed for free phenolic acid by HPLC analysis, and the remaining 9 mL of extract was used for hydrolysis. Approximately 10 mL of DI water and 5 mL of 10 M NaOH were added to the mixture. The mixture was flushed with nitrogen and stirred overnight, at ambient temperature. The pH of the extract was adjusted to 2 by 6 N HCl, and the liberated phenolic acids were extracted with ethyl acetate (3 × 15 mL). The combined organic layer was evaporated under nitrogen, and the residue was redissolved in 1.5 mL of MeOH and analyzed by HPLC. After base hydrolysis, the remaining aqueous phase was treated with 2.5 mL of concentrated HCl, the mixture was stirred for 30 min in a hot water bath at 85 °C, and the liberated phenolic acids were extracted with ethyl acetate (3 × 15 mL). The combined ethyl acetate layer was evaporated to dryness, and the residue was dissolved in 1.5 mL of MeOH and analyzed by HPLC.

Base Hydrolysis and Extraction of Free Phenolic Acids. Eggplant (200 mg, particle size < 0.825 mm) was saponified by stirring ground sample with 5 mL of a basic solution (2 N NaOH, 10 mM EDTA, and 1% ascorbic acid). The mixture was stirred for 30 min at 40−45 °C (*18*). The reaction mixture was acidified by adding 1.4 mL of 7.2 N HCl. The mixture was vortexed for 5−10 s. Free phenolic acids were extracted with ethyl acetate (2 × 6.4 mL) by vortexing the mixture twice for 1 min. The extraction tubes were placed on a low-speed centrifuge for 15 min, and the upper organic layer was transferred to a separate vial. The combined organic layer was evaporated to dryness under a steady stream of nitrogen. The residue was redissolved in 2 mL of 50:50 MeOH/water. The vial was placed in a sonicator for 5 min. The extract was filtered through a PVDF syringe filter (0. 45 μm) prior to HPLC analysis. Four replicate hydrolyses and phenolic acid analyses were carried out with each cultivar.

Separation and Analysis of Phenolic Acids by HPLC. An HPLC-UV method developed in our laboratory was used for the detection and quantitation of free phenolic acids (*19*). Eggplant extracts were analyzed on a HPLC system Beckman Coulter, System Gold coupled to a programmable detector (System Gold, series 166) and an autosampler (System Gold, series 508) operated by a 32 Karat software package. 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 HPLC analysis. The column and guard column were thermostatically controlled at 25 °C, and the flow rate was set to 0.7 mL/min. The mobile phase consisted of two solvents: 0.1% formic acid (A) and MeOH (B). The mobile phase gradient in volumetric ratio of solvents was as follows: 5−30% B over 50 min. The solvent gradient was held at 30% B for an additional 15 min, and at 65 min the gradient was increased to 100% B. It was maintained at 100% B for an additional 10 min to clean up the column. Dual wavelengths (270 and 325 nm) were used to detect the eluent composition. HPLC analysis at 325 nm was used for quantification of the peak areas of individual phenolic acids.

Calibration Curves. All identified phenolic acids were quantified with external standards by using HPLC analysis as described previously (*19*). Six different standard stock solutions with various phenolic acid concentrations were prepared for chlorogenic acid and caffeic acid. The concentration of phenolic acids extracted from eggplant samples was calculated using the developed calibration curve equations as reported in the earlier publication from our laboratory (*19*).

Determination of Total Phenolics (TP) by FC Assay. The TP content was determined using the FC assay with gallic acid as a standard on a Perkin-Elmer Lambda 25 spectrophotometer (Boston, MA) (*20*). FC assay was carried out by pipetting 60 μL of eggplant extract into an 8 mL amber vial. This was followed by the addition of 4.74 mL of water. This mixture was vortexed for 10−20 s, and 300 μL of FC reagent was added. The mixture was vortexed for an additional 20−30 s, and 900 μL of filtered 20% sodium carbonate solution was added after 1 min and before 8 min of addition of the FC reagent. This was recorded as time zero; the mixture was vortexed for 20−30 s after the addition of sodium carbonate. After 2 h ± 3 min at ambient temperature, the absorbance of the colored reaction product was measured at 765 nm. The calibration curve was created using standard gallic acid solutions each time an analysis was run. The level of TP in the extract was calculated from the standard calibration curve. Results were expressed in milligrams of gallic acid equivalent per gram (mg of GAE/g) of dried eggplant powder.

RESULTS AND DISCUSSION

The problems associated with the determination of phenolic acids arise from their structural complexity. The polarity of phenolic acids varies significantly as they may exist as free, esterified, glycosylated, or polymeric compounds. In addition, phenolic acids may coexist as complexes with proteins, carbohydrates, lipids, or other plant components. Therefore, it is difficult to develop a uniform extraction method for extracting different phenolic acids from various matrices. A session at the Second International Congress on Antioxidant Methods in Orlando, FL (June 2005) was devoted to addressing the complexities associated with sample preparation, and three reviews in the past four years clearly illustrate the importance and need for optimization of sample preparation procedures (*2*, *15*, *16*).

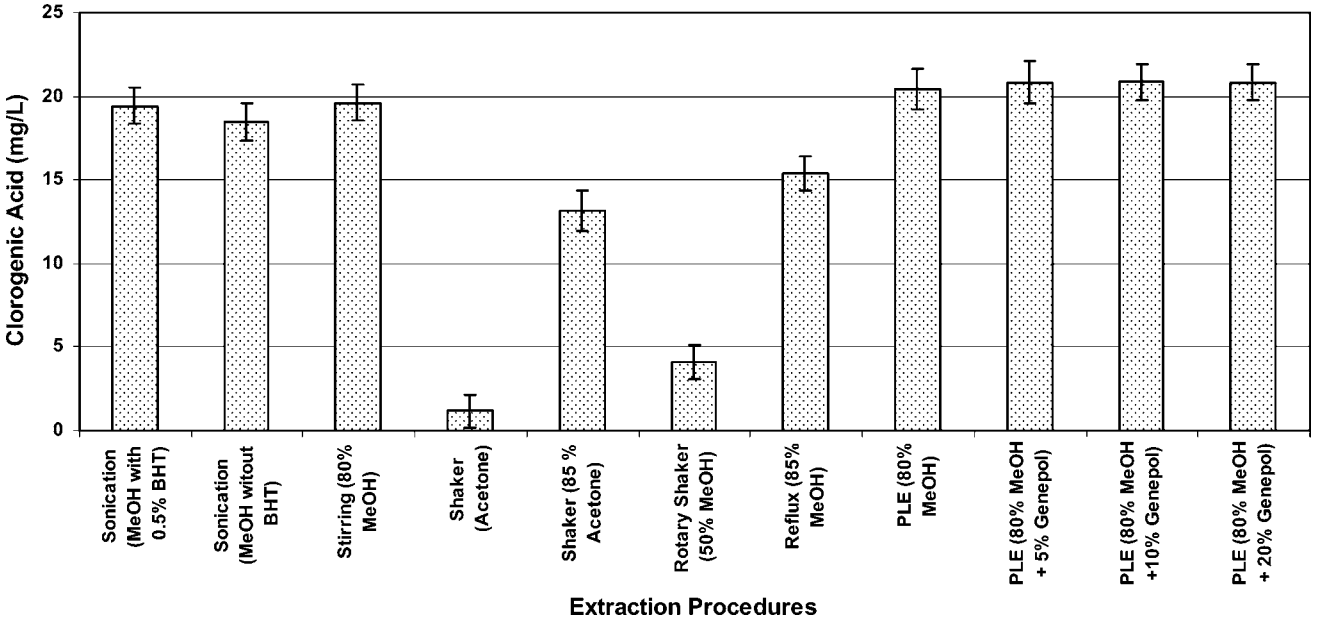

Figure 1. Comparison of extraction of chlorogenic acid from an eggplant (Black Bell cultivar) sample performed in our laboratory with 11 different reported and currently optimized extraction procedures/conditions as analyzed by HPLC with a diode array detection method.

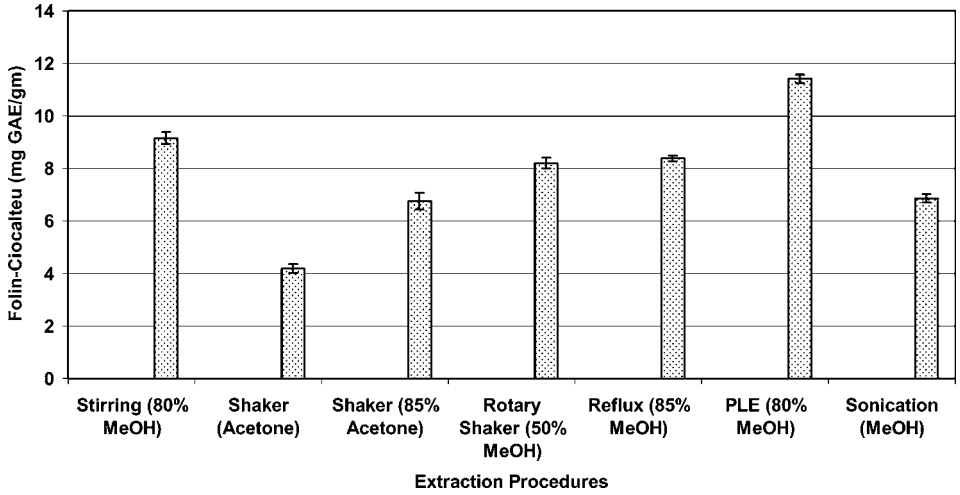

Figure 2. Comparison of extraction of total phenolics from an eggplant sample with seven different reported and currently optimized extraction procedures or conditions as assayed by the Folin–Ciocalteu method.

Direct Comparison of Reported Extraction Procedures. **Table 1** provides a summary of comparison of extraction procedures performed in our laboratory for the assay of phenolic compounds from the same eggplant sample (Black Bell cultivar) as described by various authors in the published literature. Several different solvents, namely, acetone, EtOH, MeOH, and aqueous MeOH of varying proportions, were used for the extraction of phenolic acids. In addition, different extraction techniques (reflux, sonication, mechanical stirring, wrist shaker, rotary shaker) and conditions were used for the extraction of PA from eggplants. It is clearly evident that a wide range of conditions and procedures has been followed for the extraction of phenolic acids from eggplant. The extraction efficiency of PA by different extraction procedures was estimated independently by two separate procedures HPLC analyses and a colorimetric FC assay. Chlorogenic acid was the most predominant PA extracted from eggplant (Black Bell cultivar), accounting for >75% of total PA concentration. Hence, CA concentration was used for comparison of extraction efficiency by different procedures. The results presented in **Figure 1** show that extraction efficiency varied widely with different sample preparation procedures and conditions. The lowest extraction yield (6.0%) of chlorogenic acid was obtained with a wrist shaker using acetone as an extraction solvent, whereas both sonication using MeOH with 0.5% BHT and stirring with 80% aqueous MeOH provided maximum extraction of chlorogenic acid. All other extraction procedures provided intermediate yields of chlorogenic acid. The HPLC analysis showed ~5.0% reduction in chlorogenic acid content when extraction was performed with sonication in the absence of BHT. Thus, use of 0.5% BHT during extraction with sonication provided a marginal increase in chlorogenic acid content by retarding oxidative damage during extraction and workup steps. Similar trends of extraction efficiencies were observed when extracts were analyzed by a totally independent colorimetric FC assay for the estimation of total phenolic content (**Figure 2**). Again, as shown in **Figure 2**, the wrist shaker with pure acetone as solvent provided the lowest extraction yield (36.7%). Optimum extraction of total phenolics was obtained with 80% aqueous MeOH. Only 6.0% of chlorogenic acid was extracted with a wrist shaker with acetone as extraction solvent. However, 36.7% of total phenolics were assayed for the same extract by FC assay.

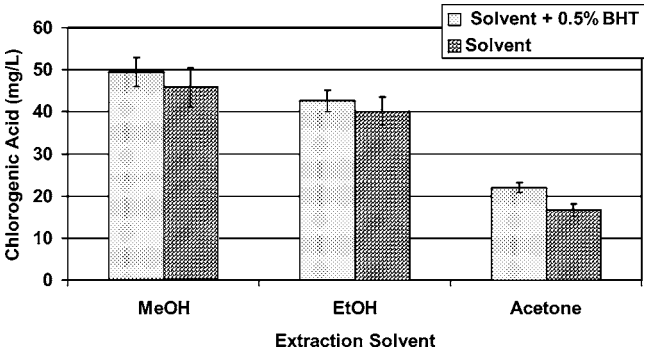

Figure 3. Influence of neat solvents with and without 0.5% butylated hydroxytoulene on extractability of chlorogenic acid from the eggplant matrix using a pressurized liquid extractor.

Similarly, noticeable differences in total phenolic content (60.0%) as assayed by the FC method and chlorogenic content (94.2%) by the HPLC analysis were observed with the sonication procedure in the absence of BHT. These differences in the phenolic content can be attributed to multiple factors such as assay procedure, total phenolics versus single major phenolic acid (chlorogenic acid) detection, and potential interferences from other components such as sugars, amino acids, and other reducing agents. In addition, FC is a nonspecific colorimetric assay. FC assays were not performed with extracts containing BHT and various concentrations of Genapol due to potential interference of the compounds with the colorimetric assay procedure.

Optimization of the PLE Procedure: Influence of Extraction Solvents with and without BHT. The results in **Figure 3** show the influence of three solvents (EtOH, MeOH, acetone) on the overall extraction efficiency of phenolic acids. All of the extraction optimization experiments were carried out with the Italian Neon cultivar, as it contained the highest levels of phenolic acids. These experiments were carried out with and without BHT to evaluate the influence of BHT on extraction efficiency. As shown, the highest levels of chlorogenic acids were obtained with MeOH as extraction solvent. The extraction efficiency with EtOH was 86.1%. The yields of chlorogenic acid with acetone were significantly lower (44.5%). These data suggest that acetone is not a suitable solvent for the extraction of phenolic acids from an eggplant matrix. On the basis of the results obtained from this experiment, MeOH was chosen as an optimum solvent for the extraction of phenolic acids from eggplant.

Influence of MeOH/H_2O Solvent Mixtures on Extraction of Phenolic Acids. Extractions with different aqueous organic solvent mixtures are commonly cited in the literature for the isolation of phenolic compounds from different matrices. Varying proportions of EtOH, MeOH, and acetone in combination with water have been commonly used (*1, 5*−*7, 9*−*12*). The addition of H_2O is known to cause swelling of the matrix, allowing efficient transfer of extraction solvent into the solid matrix, thereby increasing the extractability (*21*). In the present study, we systematically investigated the extraction of phenolic acids from eggplant matrix with six different MeOH to water ratios in the range between 0 and 100% MeOH with increments of 20% (MeOH/H_2O, % v/v, 20:80, 40:60, 60:40, 80:20, 90:10, 100). The efficiency for the extraction of phenolic acids varies significantly with different MeOH to water solvent mixtures (**Figure 4**). Optimum extraction of phenolic acids was obtained with MeOH/H_2O (80:20% v/v). Insignificant differences in extraction efficiency were observed with MeOH/H_2O (90:10% v/v). Only 36.7% of total phenolic acids were extracted

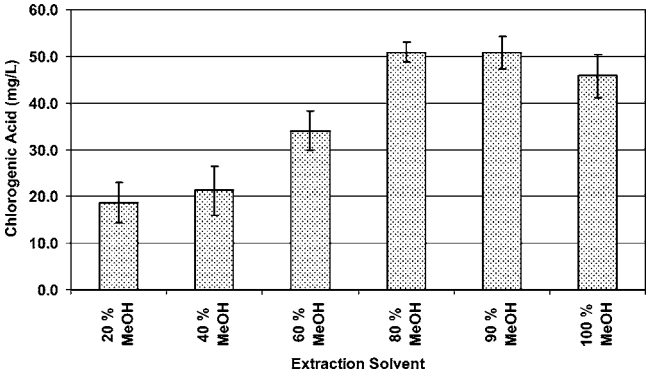

Figure 4. Influence of extraction of chlorogenic acid by HPLC analysis with six different MeOH and water solvent mixtures (MeOH/H_2O, 100:0, 90:10, 80:20, 60:40, 40:60, 20:80% v/v) using pressurized liquid extractor.

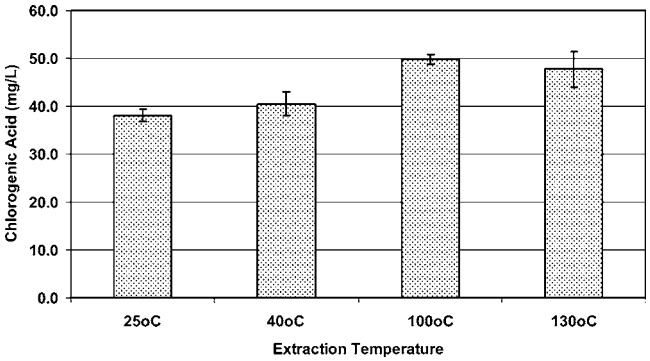

Figure 5. Influence of temperature on the extraction efficiency of cholorgenic acid from eggplant using a pressurized liquid extractor with MeOH containing 0.5% butylated hydroxytoulene as extraction solvent.

with 20% MeOH. A gradual increase in total phenolic acid extraction was observed with the increase in MeOH concentration in solvent mixture. However, a ∼2−3% reduction in extraction efficiency was detected with neat MeOH. On the basis of results obtained from this experiment 80% MeOH was chosen as an optimum solvent for the extraction of phenolic acids from eggplant.

Influence of Temperature on the Extraction Efficiency of Phenolic Acids. Figure 5 shows the influence of temperature on extraction efficiency of phenolic acid from Italian Neon cultivar using PLE using MeOH containing 0.5% BHT as extraction solvent. Optimum efficiency for the extraction of chlorogenic acid was observed at 100 °C. Approximately 76.4% of phenolic acids were extracted at ambient temperature, whereas at 40 °C the extraction efficiency increased to 81.2%. However, the extraction efficiency reduced marginally by 4.2% at 130 °C. The increase in phenolic acid concentration at 100 °C is due to several factors such as increase in diffusibility and greater solubility of analyte in hot solvent as well as weakening of the bonding between analyte and matrix at higher temperature. However, at 130 °C, the marginal reduction in phenolic acid concentration may be due to a possible degradation of phenolic compounds, internal redox reaction, or polymerization.

Comparison of Optimized PLE Method with Published Literature Procedures Used for the Extraction of Phenolic Compounds from Eggplant. Figure 1 depicts comparison of extraction efficiency of chlorogenic acid with HPLC technique using a diode array detection procedure. Optimum extraction of chlorogenic acid was obtained when extraction was carried out with PLE using MeOH/H_2O (80:20 v/v) as extraction solvent. A ∼5−95% increase in chlorogenic acid yields was obtained with PLE as compared to previously reported extraction

Table 2. Determination of Caffeic Acid Concentration in the Base-Hydrolyzed Extract of Five Different Cultivars of Eggplant Samples by HPLC Analysis

sample ID	HPLC area (caffeic acid) rep 1	rep 2	rep 3	rep 4	av	SD	% RSD	concn (μmol/g) (DMB)
Italian Neon	7227969	7155714	7256658	7239220	7219890.25	44382.3	0.61472	8.30
Calliope Zebra Stripe	2584182	2613054	2621842	2582499	2600394.25	20027.9	0.77019	2.89
Black Beauty	2023054	2014540	2030588	2003264	2017861.5	11733.8	0.5815	2.21
Orient Charm Neon	2929537	2993394	2998554	2978292	2974944.25	31468.7	1.05779	3.33
Orient Express	2257532	2278928	2273971	2247204	2264408.75	14669.0	0.64781	2.50

procedures and conditions (**Table 1**). The differences in extraction yield of chlorogenic acid were marginal (between 4 and 10%) when extraction was carried out by sonication (with and without 0.5% BHT) using MeOH or by stirring using MeOH/H_2O (80:20 v/v) as extraction solvents. However, significant differences between 40 and 80% in extraction efficiency of chlorogenic acid were observed with other reported extraction procedures using shaker or reflux as extraction techniques with varying concentrations of MeOH or acetone as extracting solvents. Extraction with PLE using 5% nonionic surfactant oligo(ethylene glycol) monoalkyl ether (Genapol X-080) showed marginal increase in chlorogenic acid yields (2%). However, further increase in Genapol concentration to 10 and 20% did not show any additional increase in chlorogenic acid yields. Similar results with Genapol X-080 were obtained by He et al. for the extraction of isoflavone diadzein from *Puerariae radix* with varying concentrations of Genapol solution (*22*).

Similar trends of extraction efficiencies were observed when extracts were analyzed for total phenolic content by FC assay (**Figure 2**). Maximum extraction of TP was obtained with PLE when extractions were performed with MeOH/H_2O (80:20 v/v) as extraction solvents. The efficiencies of extraction of TP with other procedures and conditions were between 35 and 80% as compared to PLE. Eggplant extracts containing BHT and Genapol X-080 were not assayed by FC method due to interference in the colorimetric assay procedure from BHT and Genapol X-080.

Hydrolysis of Phenolic Acid Esters. Hydrolysis of phenolic acid esters is usually carried out to provide information on the phenolic acid profiles. Although hydrolysis of phenolic acid esters can be carried out with the aid of acids, bases, or enzymes (esterases), base hydrolysis is frequently used.

To determine the total phenolic acid content, a single eggplant (Black Beauty cultivar) sample was sequentially hydrolyzed according to the procedure described by Mattila and Kumpulainen (*17*). Initially, free phenolic acids were extracted from the fraction of the sample extract. The leftover fraction was initially hydrolyzed by base, and the liberated phenolic acids were extracted with ethyl acetate and analyzed. The remainder of the aqueous extract was further hydrolyzed with hydrochloric acid to check if any additional free phenolic acids were liberated. **Figure 6** shows a typical HPLC chromatogram of the phenolic acid profiles from three extracts. The HPLC profile of the free phenolic acid fraction showed two major compounds that were identified as two chlorogenic acid isomers [on the basis of their UV spectra and comparison of the retention times with the previously published data by Whitekar and Stommel (*11*)]. Two isomers of caffeic acid were isolated from the base hydrolysis of the remaining fraction of the eggplant extract. Subsequent acid hydrolysis did not yield any additional phenolic acids. On the basis of these sequential hydrolysis experiment results, only base hydrolysis in the presence EDTA and ascorbic acid was performed with all five cultivars according to the procedure

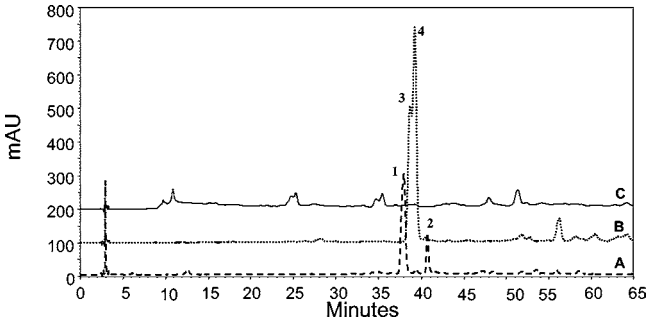

Figure 6. Sequential hydrolysis of a freeze-dried eggplant sample (Black Beauty cultivar): (**A**) free phenolic acid fraction; (**B**) base-hydrolyzed fraction; (**C**) acid-hydrolyzed fraction. Peaks 1 and 2 were characterized as chlorogenic acid isomers, and peaks 3 and 4 were identified as caffeic acid isomers.

described by Nardini et al. (*18*). The results of the base hydrolysis for all five cultivars are presented in **Table 2**. The major phenolic acid that was identified and quantified in all cultivars of eggplant was caffeic acid. The concentration of caffeic acid in Italian Neon cultivar was 8.3 μmol/g on a dry matter basis (DMB). It was more than double as compared to the other four cultivars (Orient Express, Calliope Zebra Stripe, Orient Charm Neon, and Black Beauty). The concentration of caffeic acid in all other cultivars was between 2.2 and 3.3 μmol/g (DMB).

Comparison of 11 different extraction procedures and conditions for the extraction of phenolic compound form eggplant was studied. Extraction efficiency of the chlorogenic acid and TP varied significantly with extraction procedure or conditions used. A mixture of MeOH/H_2O with a ratio of 80:20% v/v using PLE was found to be optimum for the extraction of chlorogenic acid or total phenolics as analyzed by HPLC or FC assay procedures. Maximum extraction yields of both chlorogenic acid and TP were obtained at 100 °C with PLE. Cholorgenic acid isomers were the predominant phenolic acids in the free PA fraction, and caffeic acid isomers were the major phenolic acids in the base-hydrolyzed fraction.

As >8000 different phenolic compounds have been identified and these compounds are known to exist in multiple forms (aglycon, glycosylated, acetyl/malonyl esters and polymeric forms), it is essential to systematically evaluate and optimize sample preparation procedures for accurate and reproducible analysis of phenolic compounds from different food matrices. It is vital to provide details of sample preparation and analysis procedures in the experimental details section to aid researchers in reproducing published results.

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