Journal of Food Quality

Quality of Phenolic compounds: Occurrence, Health Benefits, and Applications in Food Industry

Lead Guest Editor: Amani Taamalli
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Editorial Board

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Editorial

Quality of Phenolic Compounds: Occurrence, Health Benefits, and Applications in Food Industry

Amani Taamalli,[1] **María del Mar Contreras**,[2,3] **Ibrahim M. Abu-Reidah**,[4] **Najla Trabelsi**,[5] **and Nabil Ben Youssef**[6]

[1]*Department of Chemistry, College of Sciences, University of Hafr Al-Batin, Hafr Al-Batin, Saudi Arabia*
[2]*University of Cordoba, Cordoba, Spain*
[3]*Department of Chemical, Environmental and Materials Engineering, Universidad de Jaén, Campus Las Lagunillas, 23071 Jaén, Spain*
[4]*Arab American University, Nablus, State of Palestine*
[5]*Laboratory of Olive Biotechnology, Center of Biotechnology of Borj Cedria, Borj Cedria, Tunisia*
[6]*Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia*

Correspondence should be addressed to Amani Taamalli; amani.taamalli@cbbc.rnrt.tn

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Phenolic compounds are widespread phytochemicals in nature. This means that these compounds cannot be synthesized in the human body and are mainly taken from food and medicinal herbs. Over the past years, plant extracts rich in phenolic compounds have shown increasingly interest to enhance food quality. Moreover, their therapeutic use as functional ingredients has been the basis of numerous studies. Nonetheless, there is still much work to do. In this special issue, the published studies give new insights into how processing affects the phenolic composition of foods and the bioactivity of nanoencapsulated phenolics on cancer cells.

Olives and olive oil are a natural source of polyphenols. Their health effects are closely related to their phenolic compounds and their antioxidant activities. However, the table olive industry requires reducing the bitterness of olive phenolic compounds when looking for tasteful products. In this case, processing methods, including acid, base, and/or enzymatic hydrolysis, have been revised as well as the novel technologies that aim to face environmental sustainability challenges. In another context, centrifugation, storage, and filtration can have an impact on the content of minority compounds in olive oil, including phenolic compounds. This has been evaluated and related to olive oil quality.

Cereals, especially whole grains, are important sources of dietary polyphenols. Physicochemical processing may influence the phenolic composition of grains, and this has been summarized in this special issue in order to provide the basis for promoting the development and utilization of cereals.

In the case of blackberry fruits, the effect of some organic fertilization treatments along with harvest date and storage time has been evaluated on two blackberry cultivars. It has been shown that different physicochemical and antioxidant properties of the fruits could potentially provide different shelf-lives during their commercialization in the market as fresh products.

Nanoparticle delivery systems have successfully been used to encapsulate bioactive compounds and deliver them to intended targets. In this sense, this special issue includes a study on nanoencapsulated phenolics from *Callistemon citrinus* extract, berberine, and combination of both that enhanced their bioactivity against three breast cancer cell lines by nearly 2-fold.

We are pleased to present this special issue, which includes the aforementioned studies with interesting results concerning the relationship of the trinomial food processing, phenolic compounds, and quality.

Conflicts of Interest

The editors declare that they have no conflicts of interest.

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Amani Taamalli
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Nabil Ben Youssef

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Research Article

Effect of Different Commercial Fertilizers, Harvest Date, and Storage Time on Two Organically Grown Blackberry Cultivars: Physicochemical Properties, Antioxidant Properties, and Sugar Profiles

George Cavender,[1] **Mingyang Liu**,[2] **Javier Fernandez-Salvador**,[3] **Deborah Hobbs**,[4] **Bernadine Strik**,[3] **Balz Frei**,[4] **and Yanyun Zhao**[2]

[1]*University of Georgia, Department of Food Science and Technology, 122 Food Science Building, Athens, GA 30602-2610, USA*
[2]*Oregon State University, Department of Food Science and Technology, 100 Wiegand Hall, Corvallis, OR 97331, USA*
[3]*Oregon State University, Department of Horticulture, 4017 Agriculture and Life Sciences Building, Corvallis, OR 97331, USA*
[4]*The Linus Pauling Institute, 307 Linus Pauling Science Center, Corvallis, OR 97331, USA*

Correspondence should be addressed to George Cavender; cavender@uga.edu

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Guest Editor: Maria del Mar Contreras Gamez

Despite increased consumer interest in organic produce, little is known about how different organic production methods affect both the traditional measures of quality and the naturally occurring health promoting (bioactive) compounds of food. In this study, "Obsidian" and "Triple Crown" blackberries (both *Rubus* hybrids) were cultivated organically and fertilized with either soy meal, fish emulsion/hydrolysate blend, or processed poultry litter fertilizers at a fixed rate. Fruits were hand-harvested three times during their peak production period and stored at 4°C and 85% RH for up to 12 d. Fertilizer effects on the physicochemical properties were minor, while harvest period had a stronger effect, though that trend varied by year. Antioxidant and sugar profile data revealed an interesting pattern: "Obsidian" had ORAC and lower sugar than "Triple Crown" at harvest and also had greater differentiation due to fertilizer treatments. Fertilizer effects differed based on harvest date and cultivar, with late harvest fruit fertilized with fish emulsion fertilizer showing higher TPC and ORAC than other fertilizer treatments, while the early and middle harvest fruit showed similar or greater responses to soy meal-based fertilizer. Time of harvest and length of storage also affected the antioxidant properties and sugar profiles in different ways depending on fruit cultivar, again with the "Obsidian" fruit showing greater variability in general. This study demonstrated that the two cultivars of organically grown blackberry fruit have different physicochemical and antioxidant properties, thus potentially different shelf lives in the fresh market.

1. Introduction

Consumer interest in organic produce has resulted in the rapid growth of organic agriculture in the US, with total acreage of organic crops increasing 253% between 2000 and 2011, and the previous year data are available from USDA [1]. One of the major reasons for consumer choosing organic produce is the belief that the products are "healthier for me and my children" [2]. Meanwhile, public health experts are recommending increased consumption of fruits because they are rich in natural antioxidants that have been linked to reduced risk of various health maladies, including cancer, coronary heart disease, metabolic disorders, and inflammatory responses [3–8].

Conventional farming has been able to keep up with increased demand for blackberries through new cultivars and improved agricultural practices, and by 2005, 4,818 ha of land were able to produce 31,840 metric tons of blackberries [9]. Though many of the improved practices should translate well into organic production systems, there are some

limitations. For example, one of the most important factors that can affect plant productivity is the fertilizer regimen used, and while many studies have been undertaken to determine the ideal amounts and application times of conventional fertilizer in order to optimize blackberry production [10], the published guidelines and recommendations tend to focus solely on the rates of nitrogen application, making little or no distinction in the nitrogen source, aside from the general caveat that nitrate forms are better than amide forms [10–12].

This lack of focus on the type of nitrogen source is problematic for organic growers; while conventional/synthetic fertilizers are composed of a handful of industrial chemical compounds, organic fertilizers are derived from plant/animal wastes and as such are much more complex. Even if it were possible to extrapolate the guidelines for conventional fertilizers to organic ones, it is entirely possible that the plants will respond differently as studies which have compared conventional and organic production methods in various crops have shown marked differences in performance and fruit/vegetable quality [10, 13–17]. Even among different types of organic fertilizer, great variability was found in horticultural measures of performance, such as yield and nitrogen uptake [18, 19]. Further, it is unknown how the different organic fertilizer sources can affect the overall fruit quality, thus a question that needs to be answered, particularly given that Bulluck et al. [20] showed that different organic fertilizer sources not only affect crop yield but also modify the physical, chemical, and microbial properties of the soil itself and in turn the quality of fruit [21–24].

Being living systems, fruiting plants also show variability during the course of the growing season, and this variability can greatly affect the quality and health properties of the fruit, as has been shown in several studies. Basiouny [25] found that anthocyanin content and shelf life decreased in later harvest blackberries, and Thompson et al. [26] noted a decline in total soluble solids, total phenolic content, anthocyanin content, and pH as harvest season progressed. While these studies dealt with conventionally grown (CG) berries, more recent studies involving organically produced blackberries have shown that harvest time has a marked effect on total yield, average berry weight, and total soluble solids [24, 27].

Given the focus on perceived health benefits of organically grown produce, it is essential to quantify the bioactive compounds and antioxidant potential of organic blackberry fruit. The former allows the characterization of known compounds which may have been linked to particular health benefits, while the latter attempts to give an overall measure of how well all of the compounds scavenge specific types of free radicals compared with a set standard. Further measurements of antioxidant potential will vary based on the free radical and comparative standard used in the assay, making comparison between different methods difficult. These benefits and drawbacks lead most researchers to perform one or more assays of each type in an attempt to get a better overall grasp of the antioxidant properties, as well as allow for more avenues of comparison with previous studies. Most antioxidant studies involving blackberries have focused on total phenolic compounds and anthocyanins, as blackberries are well known to be high in both, and also often included one or two measures of antioxidant activity [3, 4, 28–30]. Despite the potential effect of fertilizer source on the antioxidant properties of blackberries, there have been no published studies on the subject.

This study aimed to examine the effect of differing organic nitrogen sources and harvest periods on the physicochemical and antioxidant properties of two different organically grown blackberry cultivars at the time of harvest and during refrigerated storage. Specifically, the rates of decay and leakage, berry firmness, sugar profile, total phenolic content, total monomeric anthocyanins, and the overall antioxidant potential were measured and compared using three different methods were measured and compared. It is important to note that this study did not compare organically and conventionally produced fruit, primarily based on the consideration that in order to properly compare organic and conventional fruit, the plants must be grown in the same planting area with replicated treatments. This is extremely difficult if not impossible, as the "organic" could never be certified organic due to the close proximity of the conventional plots and the size of the required buffer zones.

2. Materials and Methods

2.1. Materials. All chemical reagents were analytical grade, except for the ultrapure (<18.2 MΩ cm) water used as a mobile phase in HPLC analysis of sugar profile, which was prepared *in situ* using a Millipore filtration system (Millipore Corp., Bedford, MA USA).

Two blackberry cultivars, "Obsidian" and "Triple Crown," were evaluated in this study, chosen for their suitability for the Pacific Northwest fresh market. Specifically, "Obsidian" berries have an early harvest season (mid-June to mid-July), while "Triple Crown" has a later harvest season (mid-August to early September). All berries used in this study were grown on a certified organic farm (Riverbend Organic Farms, Jefferson, OR, USA) in eighteen separate plots (9 plots for each cultivar). Complete details of the growing conditions were described in the recent publication of Fernandez-Salvador et al. [27]. Briefly, all plots were grown using the same management system (e.g., irrigation, pest control scheme, and weed management technique) and were fertilized with one of the three commercial organic fertilizers: processed poultry litter (PPL-"Nutri Rich 4-3-3 Ca 7%," Stutzman Farms, Canby, OR USA), soy meal (SM-"Phyta-grow leafy green special," California Organic Fertilizers Inc., Fresno, CA USA), or a blend of fish emulsion and fish hydrolysate (FE-"True 402," True Organic Products Inc., Helm, CA USA). All fertilizers were applied at the recommended nitrogen rate (56 kg N/he), and distribution of fertilizer treatments was randomized within blocks of three plots.

Berries were hand-harvested three times during the 2012 and 2013 growing seasons (July 6–17 and June 24 to July 9, for "Obsidian" in 2012 and 2013, respectively; August 10–24 and August 1–15 for "Triple Crown" in 2012 and 2013,

respectively) for a given cultivar at approximately one week interval and named as "Early Harvest," "Middle Harvest," and "Late Harvest," respectively. Note that depending on the weather conditions (temperature and UV index), the exact harvest date varied year by year. Approximately 16 berries were placed into each hinged polyethylene terephthalate (PETE) clamshell container (Pactiv, LLC, Lake Forest, IL, USA). Individual containers were placed into open-topped cardboard boxes, stored at 4 ± 1°C and 85% RH and sampled at days 0, 2, 4, and 6 and 0, 2, 5, 8, and 10 for "Obsidian" in 2012 and 2013, respectively, and at days 0, 4, 10, and 12 (±1) and 0, 4, 8, 10, and 12 for "Triple Crown" in 2012 and 2013, respectively, with sampling discontinued when more than half of the berries in a given container were found to be decayed. On each sampling day, one randomly determined container from each of the 9 plots was removed from storage.

2.2. Fruit Decay and Leakage. Decay and leakage of fruit were evaluated following the procedures described by Civello et al. [31] with some modifications. Briefly, individual fruits were gently taken out of the clamshell containers and inspected visually for mold growth and/or extensive damage (defined as having <3 ruptured/crushed contiguous drupelets or <5 ruptured/crushed drupelets overall), either of which rendered a berry "decayed." Nondecayed fruits were tested for leakage by transferring them to a standard "letter size" (215.9 mm × 279.4 mm) sheet of white printer paper and gently rolled, so that all berry surfaces had been exposed to the paper. Juice stains on the paper rendered a fruit "leaking." Decay rate was calculated as the percentage of berries in a container which were decayed, while leakage was calculated as the percentage of nondecayed berries in a container which were leaking.

2.3. pH and Titratable Acidity. pH and titratable acidity (TA) were determined using the methods from Fisk et al. [32]. Two individual fruits from each clamshell container were used for describing measurements. The fruit was mixed with 9 times of fruit weight of distill water and blended for 1 min using a 12-speed homogenizer (Osterizer, Jarden Corp., Mexico). The mixture was filtered through qualitative filter paper to remove insoluble material. The filtrate was assayed for pH using a pH meter (Corning 125, Corning Science Products, Medfield, MA, USA), TA was determined by titration to an endpoint of pH 8.2 with a standardized 0.1 N aqueous NaOH solution, and values were calculated based on the assumption of malic acid as the predominant acid.

2.4. Fruit Firmness. Five nondecayed berries from each clamshell container were individually measured on each sampling date for firmness using methodology originally developed by Joo et al. [33] with modification to better approximate the conditions of the nondestructive subjective manual test commonly used by growers [34]. Briefly, berries were placed on their side, and the force (in N) required to compress the berry 5% of its total thickness was measured using a texture analyzer (Model TA-XT2, Texture Technologies Corp. Scarsdale, NY, USA) fitted with a 25 kg load cell and a 50 mm cylindrical probe.

2.5. Fruit Extraction for Antioxidant Assays. Four berries were taken from each clamshell container and combined according to treatment group, giving 12 berry samples from each, and rapidly frozen by immersion in liquid nitrogen. Frozen samples were then pulverized under liquid nitrogen using a one-liter blender (Waring Laboratory Science, Torrington, CT, USA) which had been fitted with a specialized lid to allow for pressure release while preventing sample loss.

Samples of pulverized berry powder (15 g) were subjected to a modified ultrasound assisted sequential extraction procedure developed in our laboratory [35]. Briefly, a given sample was extracted first using acidified acetone (0.1 mL/L HCl), then twice with a 3 : 7 of water : acidified acetone solution, with each extraction involving a fixed time ultrasound treatment (90, 300, and 300 s, respectively). After centrifuging, supernatants were decanted and pooled together for partitioning with 150 mL of chloroform, vortexing thoroughly, and centrifuging to separate the two phases for removing any lipophilic components. The aqueous phase was then decanted and evaporated to remove residual organic solvents using a rotary evaporator (Rotovap, Brinkmann Instruments, Westbury, NY, USA). Extract volume was standardized to 150 mL using deionized (DI) water, and 1.5 mL aliquots of the standardized solutions were stored at −80°C until the time of assay.

2.6. Juice Extraction for Sugar Profiling. A modified procedure from Qian [36] was used to prepare aqueous berry extracts. Briefly, ~35 g of the pulverized berry powder not used for the antioxidant assays were mixed with DI water equal to 1/2 the mass of the sample in a glass jar. Jars were fitted with lids and immersed in a boiling water bath (100°C) for 20 min to inactivate enzymes. The resultant juice/slurry was centrifuged to remove solids and decanted into clean polypropylene bottles for storage at −25°C until the time of assay. Extracts were prepared from berries harvested during the 2012 season only in order to avoid the variation inherent to the first fruiting of blackberries.

2.7. Analysis of Total Phenolic Content (TPC). TPC was determined using the Folin–Ciocalteu colorimetric method, as described by Singleton et al. [37]. Briefly, aqueous extracts were diluted until their absorbance value was less than 1.2, and 0.5 mL aliquots of this diluted sample were added to tubes containing 7.5 mL of DI water and 0.5 mL of Folin–Ciocalteu reagent. After vortexing to mix, solutions were allowed to react for 10 min before the addition of 3 mL of 20% sodium carbonate solution. The resultant mixture was vortexed and then placed into a 40°C water bath for 20 min. Following the heat treatment, samples were plunged into a 0°C ice/water bath until they were at or below room temperature. Absorbance of the samples at 765 nm was

measured using a spectrophotometer (Model UV160U, Shimadzu Corporation, Kyoto, Japan). These values were used to calculate gallic acid equivalents based upon the equation of a standard curve prepared the same day using solutions of gallic acid (0, 150, 200, and 250 ppm). Assays were performed in triplicate, and values were reported as mg gallic acid equivalents (GAE)/g fresh weight (FW).

2.8. Analysis of Radical Scavenging Activity (RSA). The refined colorimetric assay method relying on the reduction of the stable free radical 2,2-diphenyl-1-picryhydrazyl (DPPH) [38] was used to determine RSA. Briefly, 1.5 mL of freshly prepared DPPH solution in methanol (0.09 mg/mL) was added to disposable cuvettes containing 0.75 mL of diluted fruit extract, mixed, and allowed to react at room temperature for 5 min. Absorbance at 517 nm was measured and used to calculate ascorbic acid equivalents (AAE) based upon the equation of a standard curve prepared the same day using ascorbic acid solutions (0, 100, 200, 300, and 400 ppm). Assays were performed in triplicate, and values were reported as mg AAE/g FW.

2.9. Analysis of Total Monomeric Anthocyanins (TMA). The spectrophotometric method based upon pH-induced changes in absorbance (Giusti and Wrolstad [39]) was used to assay TMA. Briefly, for each sample, aliquots of extract were placed into two disposable cuvettes, diluted with either a standardized sodium acetate buffer (pH 4.5) or a standardized potassium chloride buffer (pH 1.0), and allowed to equilibrate for at least 15 min at room temperature. Optical absorbance was measured at both 510 nm and 700 nm, with the former value being selected based upon the predominant anthocyanin in blackberries, cyanidin-3-glucoside [28, 29]. Absorbance values were then used to calculate concentration of monomeric anthocyanins (expressed as mg TMA/g FW) in the fruit using the Beer–Lambert–Bouguer law according to the following equation:

$$\mathrm{TMA}\left(\frac{\mathrm{mg}}{\mathrm{g\,FW}}\right) = \frac{\left[\left(A_{510\mathrm{nm}} - A_{700\mathrm{nm}}\right)_{\mathrm{pH}\,1.0} - \left(A_{510\mathrm{nm}} - A_{700\mathrm{nm}}\right)_{\mathrm{pH}\,4.5}\right] \times 449.2\,(\mathrm{g/mol}) \times DF \times 1000\,(\mathrm{mg/g})}{26900\,(L/(\mathrm{cm}\cdot\mathrm{mol})) \times 1\,\mathrm{cm}} \times \frac{1\mathrm{L}}{100\,\mathrm{g\,FW}}, \quad (1)$$

where DF is the dilution factor and each extract was assayed three times.

2.10. Oxygen Radical Absorbance Capacity (ORAC). ORAC was measured using the fluorescent method described by Cao et al. [40] which had been adapted for use in a 96-well microplate fluorometer (SpectraMax Gemini XS, Molecular Devices, Foster City, CA, USA). Briefly, three 30 μL aliquots of each extract (diluted as necessary) were dispensed into the wells of a prewarmed microtiter plate along with 200 μL of a prewarmed β-phycoerythrin solution (6.65 μg/ml in phosphate-buffered saline, pH 7.4). Microtiter plates were incubated at 37°C for 1 h, after which 70 μL of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was added to initiate the reaction. Fluorescence of β-phycoerythrin was induced by excitation at 485 nm and was measured at 585 nm every 2 min for 2 h. Proprietary software (SoftMax Pro 5.4.5, Molecular Devices, LLC, USA) was used to calculate the antioxidant capacity based upon positive changes to the area under the curve as compared to curves generated using a series of standardized Trolox solutions (0, 10, 20, or 40 μmol/L). Results were expressed as μmol Trolox equivalent (TE)/g FW.

2.11. Ferric Reducing Antioxidant Power (FRAP). FRAP was assayed using the automated colorimetric method developed by Benzie and Strain [41]. Duplicate aliquots of 40 μL were taken from each extract and dispensed into the wells of a prewarmed microtiter plate along with 300 μL of prewarmed FRAP reagent (a mixture of 83% 300 mmol/L acetate buffer, 3.5% 10 mmol/L tri(2-pyridyl)-s-triazine, and 3.5% 20 mmol/L iron (III) chloride). Plates were incubated at 37°C for 15 min and then measured for absorbance at 550 nm using a microplate absorbance reader (SpectraMax 190, Molecular Devices, Foster City, CA). Proprietary software (SoftMax Pro 5.4.5, Molecular Devices, LLC, USA) was used to calculate antioxidant power from the measured absorbance values based upon a standard curve generated from a series of standardized Trolox solutions (0, 62.5, 125, 250, or 500 mmol/L Trolox). Values were reported as μmol Trolox equivalent (TE)/g FW.

2.12. Analysis of Sugar Profile. A high-pressure liquid chromatography (HPLC) system, consisting of a quaternary pump, solvent degasser, autosampler, column heater, and refractive index detector (Series 1200, Agilent Technologies, Santa Clara, CA, USA), was fitted with a 300 mm × 7 mm ligand exchange column (particle size 8 μm), and an appropriate guard column (Hi-PLex pB, Varian, Inc., Palo Alto, CA, USA) was used to determine the sugar profile of the fruit according to the method of Cavender et al. [42]. All samples were assayed in triplicate, and the concentrations of the three major sugars (fructose, sucrose, and glucose) were calculated based upon standard curves constructed using a series of pure sugar solutions (0.9375, 1.875, 3.75, and 7.5 g/100 mL of each).

2.13. Experimental Design and Statistical Analysis. A completely randomized design was employed in this study with the principle effects being fertilizer treatment and harvest date. Data were analyzed for statistical significance via multiway analysis of variance (ANOVA) with least significant

difference (LSD) *post hoc* testing as appropriate, using statistical software (SAS v9.2, the SAS Institute, Cary, NC, USA). Results were considered to be different if p value < 0.05.

3. Results and Discussion

3.1. Fruit Decay and Leakage. For both decay and leakage, fertilizer types had no significant influence on the measures, but harvest date did. Figures 1 and 2 present the mean measures across all three fertilizer treatments for the 2012 and 2013 harvest, respectively. Decay rates for the two cultivars ranged 5.56–54.86% in 2012 and 0.74–46.89% in 2013, with "Triple Crown" having higher decay rate in 2012 and "Obsidian" having higher decay rate in 2013; though in the former case, it should be noted that the "Triple Crown" storage time was twice as long as the "Obsidian." Examining the effect of storage time on decay showed decay rates tending to increase with prolonged storage, which was fairly predictable, given that the decay of blackberries is usually caused by fungal growth, and *Botrytis cinerea*, the primary fungal disease of ripe blackberry fruit, can continuously grow at low temperatures [33, 43].

Leakage rates remained more flat, ranging 27.08–72.34% in 2012, and 40.77–89.63% in 2013, with the data for a given storage time/harvest date showing great variability, and "Triple Crown" tending to have slightly higher leakage rates in both years. Harvest date also contributed to the differences in the two measures, though its effect varied greatly between cultivars and harvest years. This variability can be explained by differences in weather conditions at the time of fruit harvest which can directly impact fruit ripeness, mold growth, and some other physicochemical properties. In particular, rain during or immediately prior to harvest can result in significant fruit decay due to favorable mold growth conditions, and high temperature and high UV index may result in advanced ripening which leads to loss of firmness and greater fruit leakage [44, 45].

"Obsidian" and "Triple Crown" have about one month harvest period in June-July and August-September, respectively [46]. In order to maintain the uniform quality of fruit for fresh market, the growers usually picked up fruit at 2~3 different times during the harvest season [32]. Based on the results obtained in this study, according to USDA standards of grades on blackberries (United States Department of Agriculture (1928)), organically grown "Obsidian" berries are marketable for roughly 2 d, while "Triple Crown" berries remain so for about 4 d. These values are reasonable compared with other commercial varieties, as Perkins-Veazie et al. [45] reported decay rate exceeded 35% after 7 days in the "Cheyenne" and "Shawnee" cultivars. However, later work by the same group highlights the natural year-to-year variability of such measures, showing decay rates less than 12% decay after 7 d of refrigerated storage for "Shawnee" [47].

3.2. pH and Titratable Acidity of Fruit. Overall, the pH values of both cultivars were within the range reported for eleven conventionally grown blackberry cultivars [48]. Still, within these values, there was variability based on harvest year, cultivar, harvest time, and storage. In 2012, while no significant difference in pH between the two cultivars was observed and no significant change was seen due to storage, TA values showed more variation. Specifically, in the initial (day 0) TA values, "Obsidian" had higher levels than "Triple Crown," and the TA values of early and late harvest fruit of both cultivars decreased during storage. Further, for "Obsidian," harvest date affected both TA and pH in 2012, with early harvest fruits showing significantly lower pH and higher TA than middle harvest fruits. "Triple Crown" showed higher initial pH in the late harvest fruits compared with the early harvest, but these differences vanished during storage, while the pH of early and middle harvest fruits increased 5.72% and 6.85%, respectively, during the 12 d of storage. Similar trends were seen in the TA values for "Triple Crown" for that year.

The fruit from the 2013 harvest followed a different pattern of trends, with "Obsidian" having higher initial (day 0) pH and TA than "Triple Crown." Unlike the 2012 harvest year, in 2013, "Obsidian" berries from the three harvests showed no significant difference in either initial pH or TA, but after 10 d of storage, TA of early harvest "Obsidian" fruits was slightly higher than those of the late harvest (1.30% vs. 1.06%). Within a given harvest, storage time had a limited effect as well, with pH of early harvest fruit decreasing and TA increasing after 10 d of storage, and the late harvest fruit undergoing a slight (~2.97%) increase in TA during the first 5 d of storage, but not thereafter. TA of the middle harvest fruit also decreased significantly at 10 d of storage, going from 0.96 to 0.67%. For the "Triple Crown" fruit, the initial pH of the middle harvest was significantly higher than that early and late harvest one at the harvest, but the late harvest fruit showed significantly higher initial TA than the fruit from other two harvest dates. Storage also had some effects, with the late and middle harvest fruit undergoing significant increases (16% and 17.1%, respectively) over the 12 d of storage, which was also accompanied by a more profound decrease in TA (29.38% and 23.02%, respectively).

As it is well known that fruit metabolism consumes starch and acid during postharvest storage, leading to increases in pH and decreases in TA [49, 50], our results are hardly surprising. Both "Obsidian" and "Triple Crown" cultivars showed decrease in TA during storage, which was also reported in other conventionally grown blackberry cultivars [33, 47]. Perkins-Veazie et al. [45] reported that TA decreases 60% between mottled and shiny black stage and decreases 40% between shiny and dull black stage. We might conclude that the high TA value in our study for the early harvest fruit is due to the less ripe of the fruit that the acids inside the fruit have not been converted to sugar compounds yet. Woods et al. [51] reported pH increase of conventionally grown "Triple Crown" and other cultivars during storage, probably owning to the binding of pectin from the fruit cell wall to polyphenols [52].

3.3. Fruit Firmness. No significant differences in firmness were seen between berries grown with different fertilizer

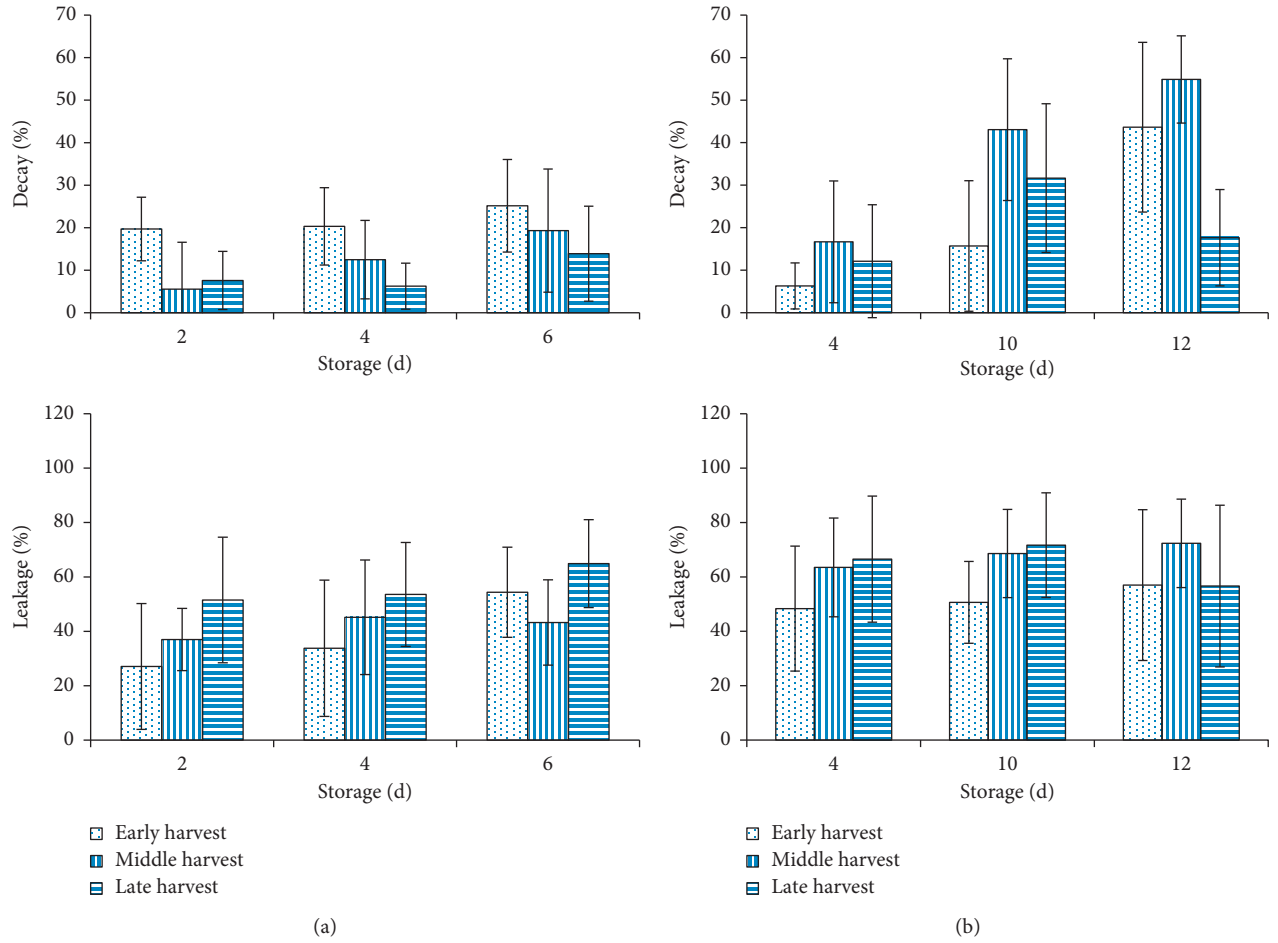

Figure 1: Effects of harvest date and refrigerated storage on the decay and leakage rate of two blackberry cultivars: "Obsidian" (a) and "Triple Crown" (b), 2012 harvest.

types, but differences were seen among the different harvest dates in both years. Figure 3 presents the mean values across all three fertilizer types. In general, "Obsidian" fruits were firmer than "Triple crown" during both harvest years, with initial values of the former ranging 1.28–2.23 N in 2012 and 0.84–0.99 N in 2013, while the initial values of the latter ranged 1.06–1.90 N and 0.65–0.87 N in the same years. In general, these values were consistent with those reported in previous works on conventional berries [33, 53, 54]. Further, the differences between the two were not surprising, given that trailing cultivars like "Obsidian" typically produce firmer fruit than semierect cultivars like "Triple Crown" [46].

Comparing harvest years, in both cultivars, the fruits from the 2012 harvest were firmer than those harvested in 2013, and the effect of storage showed variation among the two cultivars and harvest years, with most experiencing the expected stability or decline during storage, but some, specifically early and middle harvest "Obsidian," seeing increases in firmness by 4 d of storage. While this effect was present in both harvest years, it was more pronounced (but also had greater variation) in 2012. These variations in firmness were likely due to the variance of ripeness between individual berries, as the less ripe fruit is typically firmer than that of the ripe or overripe fruit [45].

Fruit softening, one of the more important factors of postharvest deterioration, involves a series of physiological and biochemical changes resulting in cell wall hydrolysis and pectin degradation [49, 55 56]. While these changes tend to result in softer fruit, it is possible that enzymatic transformations of cell wall pectin and the bonding of calcium to pectic acid polymers could potentially lead to some amount of fruit hardening during storage [53]. While this might explain the increase in hardness observed in the 2012 early and middle harvests of the "Obsidian" fruit, which increased reached peak firmness after 2–4 d of storage, it also may have a simpler explanation, namely, that berries become more fragile as they ripen, increasing their risk of microbial spoilage. Thus, the berries which were the softest at harvest may have become decayed by day 2 or day 4, excluding them from firmness testing.

The variations are seen in the 2012 "Triple Crown" harvest, specifically the lack of significant change in firmness for the middle harvest fruit and greater firmness in the late harvest fruit compared with the early and middle harvest is likely the result of environmental/weather concerns, as the fruits developed during a period of elevated temperature and UV index. These conditions likely lead to physical damage to the fruits. The effects of the environment appear again

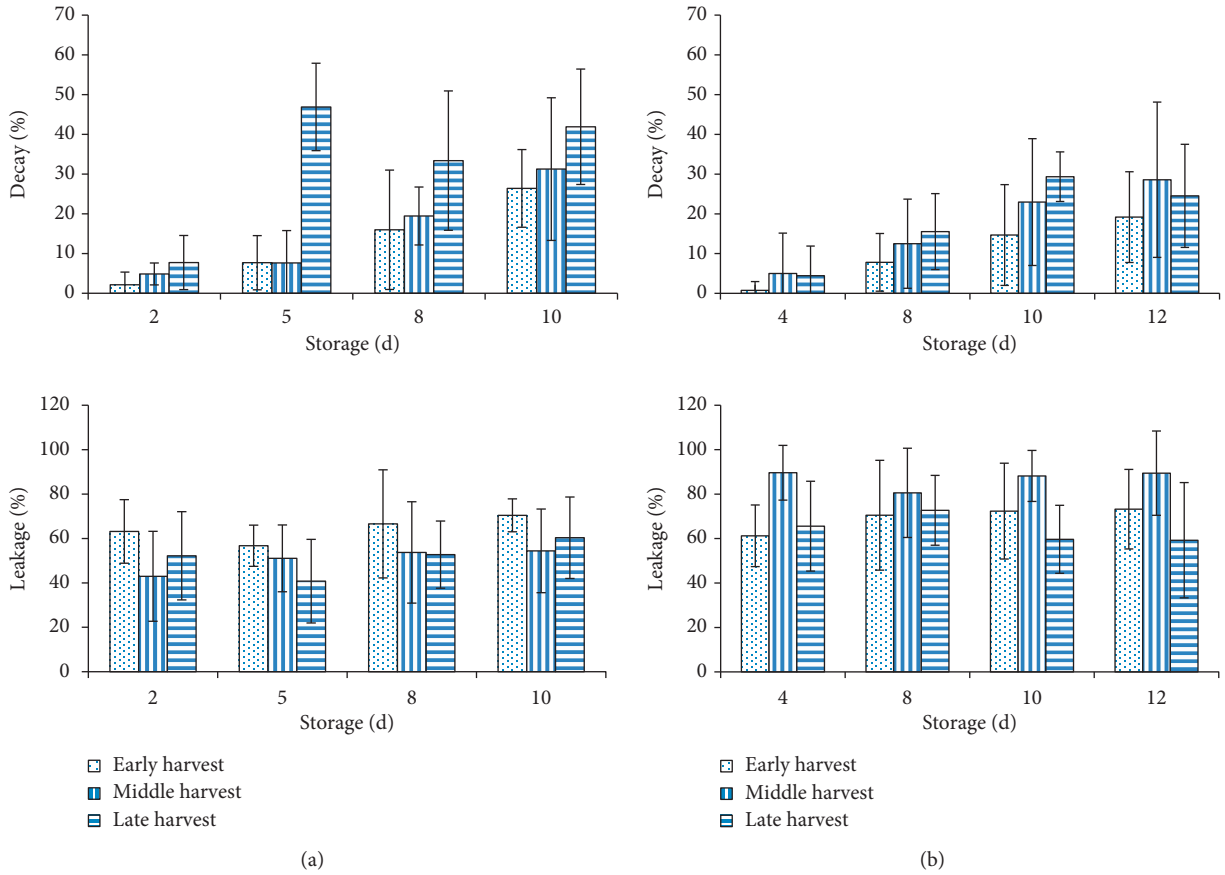

Figure 2: Effects of harvest date and refrigerated storage on the decay and leakage rate of two blackberry cultivars: "Obsidian" (a) and "Triple Crown" (b), 2013 harvest.

during the 2013 harvest, where a period of elevated temperatures likely resulted in the lower firmness levels seen in the late harvest "Obsidian" fruit, compared with the early and middle harvest.

Unlike the 2013 "Obsidian" cultivar, the "Triple Crown" fruit from the 2013 early harvest showed significantly higher initial firmness than the late harvest fruit, again, likely due to the degree of berry ripeness. Firmness also decreased significantly during storage, with the firmness of the middle harvest fruit decreasing by 23.87% and that of the early harvest by 22.92% after 10–12 d of storage. These decreases in firmness generally agreed with the findings of Perkins-Veazie et al. [54] that commercially grown (CG) "Navaho" blackberries lost 36% firmness during refrigerated storage and Joo et al. [33] that CG "Chester" blackberries underwent a 35% decrease in firmness after 12 d of refrigerated storage.

3.4. Sugar Profiles. Figures 4 and 5 illustrate the sugar profiles of fruit from the 2012 harvest during refrigerated storage for "Obsidian" and "Triple Crown," respectively. The observed effects of fertilizer on total sugar content varied depending on cultivar, with "Obsidian," initial total sugar content ranged from 14.7 to 18.7 g/100 g FW and "Obsidian" fertilized with SM having the highest initial values in the early and middle harvests, and those fertilized with PPL having the highest in the late harvest, with SM fertilized berries having the highest values in the early and middle harvests, and PPL ones having the highest in the late harvest. Overall, the "Triple Crown" fruit had higher initial total sugar content than that of "Obsidian," ranging from 22.14 to 28.41 g/100g FW, with SM fertilized berries again having the highest values in the middle harvest, and PPL and FE fertilized fruits having the highest values in the early and late harvest, respectively. These fertilizer effects were hardly surprising, given that the three regimens likely have different rates of nitrogen release/absorption, and multiple studies have shown a relationship between available nitrogen and fruit sugars in diverse fruits such as strawberries, tomatoes, chokeberries, dates, and grapes, as well as potential influence of other trace minerals [57–61].

Examining individual sugars, overall, sucrose comprises a minor fraction (2-3% for "Obsidian" and 0–7.4% for "Triple Crown") of the total sugars in all harvests, while fructose represented the major fraction in all harvests of the "Triple Crown" fruit, as well as the early harvest of the "Obsidian" fruit, but not the middle and late harvests, where glucose dominated. During storage, the ratio of sucrose to fructose remained fairly consistent throughout storage across all treatments and harvests in both cultivars, while the amount of sucrose showed a definite cultivar-specific response. The initial relative values agreed with previously

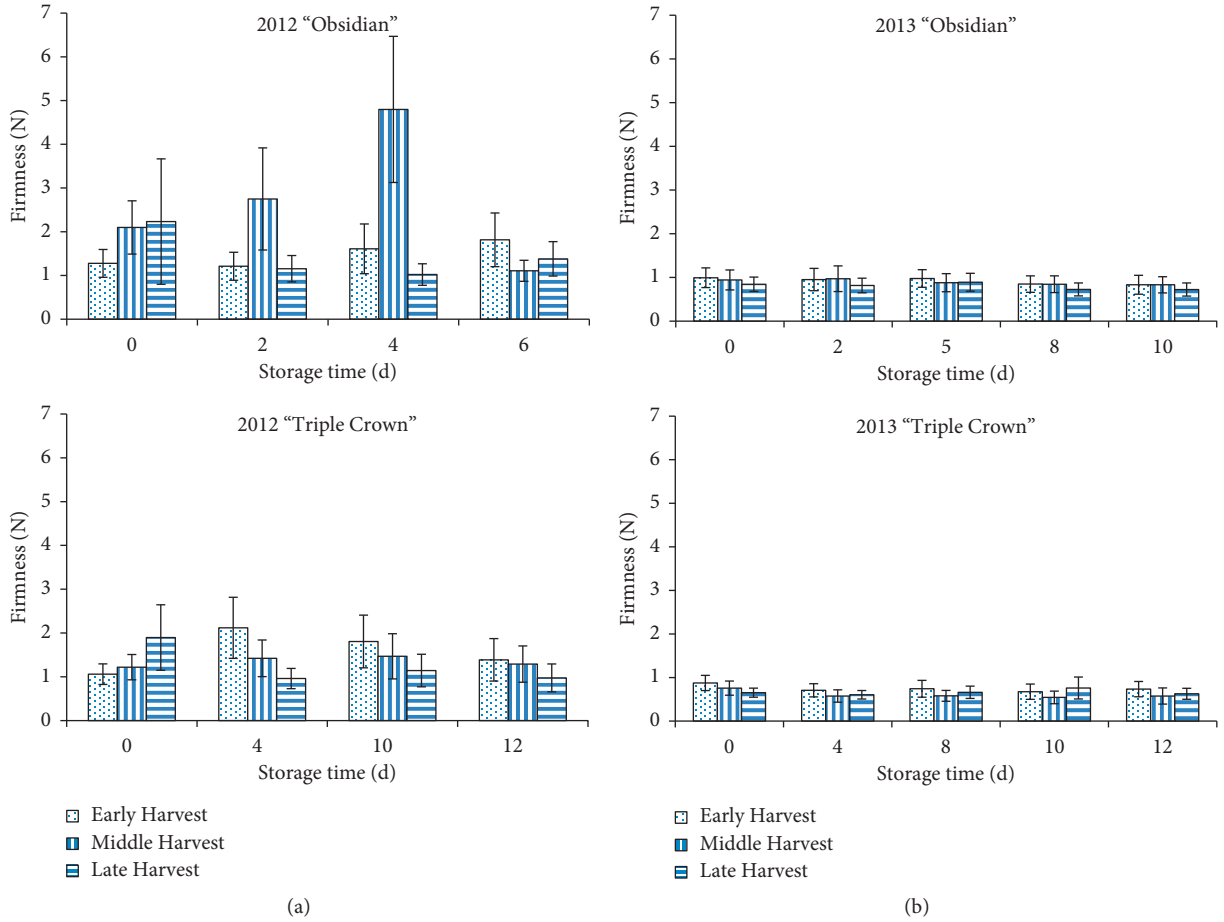

Figure 3: Effect of different harvest date and refrigerated storage on the firmness of two blackberry cultivars: "Obsidian" and "Triple Crown" at 2012 and 2013 harvest.

published data by Fan-Chiang [62] and Kafkas et al. [63] for other CG blackberry varieties, and the observed shift in predominant sugar during the "Obsidian" harvest might be explained as fruit harvested early in the season would be more likely to be underripe, thus less sweet. However, not only did the early harvest "Obsidian" fruit generally contained greater overall amount of sugar but also they showed higher proportions of fructose, which strongly implied that they would be perceived as sweeter, given that fructose is roughly twice as sweet as glucose and 1.7 times as sweet as sucrose [64, 65]. By contrast, the "Triple Crown" fruit followed a more predictable pattern, with the middle and late harvests having higher initial values of both total sugars and fructose.

The effect of storage on the sugar profile tended to follow a trend of increasing slightly shortly after storage and then either leveling off, or declining slightly. Of the five harvest/treatment combinations that did not follow this trend, four were fertilized with PPL (early and late harvest "Obsidian" and middle and late harvest "Triple Crown") and one was fertilized with FE (late harvest "Triple Crown"). These harvest/treatment combinations showed marked (as much as 30%) increases in total sugars, which could be explained by a combination of postharvest ripening and the degradation of anthocyanins during storage, the latter of which released previously bound sugars [39], while the differences in fertilizer effect were likely related to the effects of different rates of nitrogen release on the average maturity of the harvested berries which would in turn show different trends in postharvest ripening.

Looking at individual sugars, the cultivar-specific response to storage is quite noticeable. Except in the early harvest, "Obsidian" berries had decreased sucrose levels as storage progressed, most likely due to sucrose hydrolysis due to metabolic processes in the fruit. "Triple Crown" berries, on the other hand, showed the opposite relationship, except for the SM-fertilized middle harvest berries, with the early harvest showing undetectable levels of sucrose across all storage periods, while the middle and late harvests actually showed significant increases in sucrose levels. While few studies have examined the effects of cold storage on the sugar profile of blackberries, other CG fruits were shown to undergo reductions in relative sucrose levels, including pears [66], strawberries [67], and peaches [68], and the downward trend observed in "Obsidian" was similar to the findings for raspberries and blackberries from Ali et al. [50]. Explaining the increases in sucrose seen in the early harvest "Obsidian" and middle/late harvest "Triple Crown" fruit was more

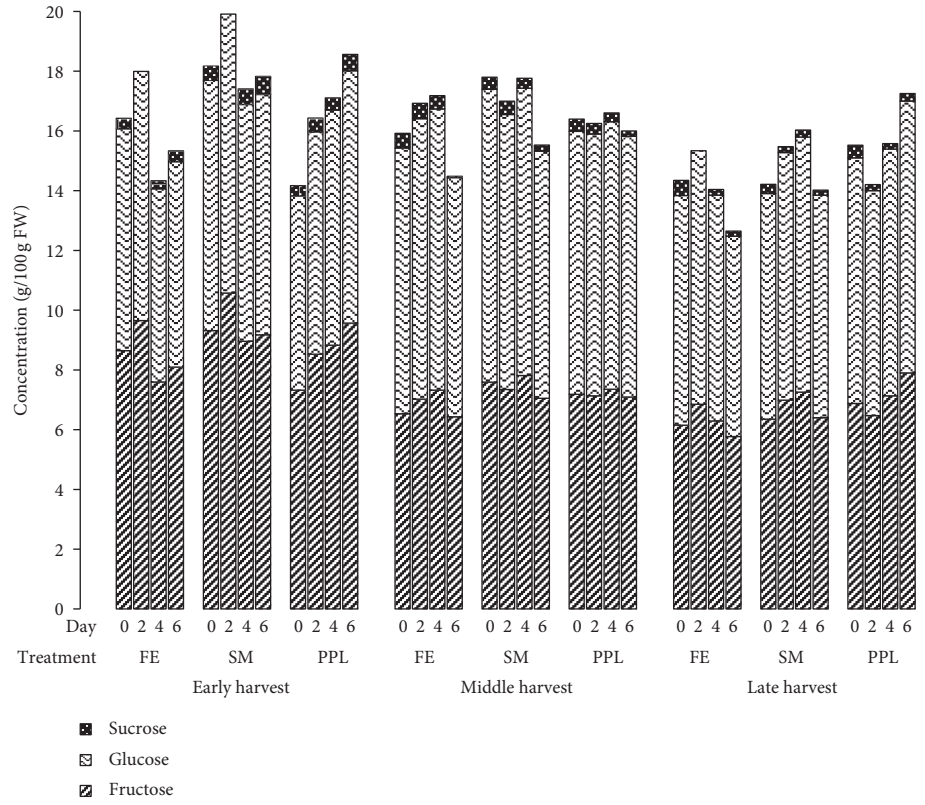

Figure 4: Sugar profile of "Obsidian," expressed in total concentration, 2012 harvest. FE: blend of fish emulsion and fish hydrolysate; SM: soy meal; PPL: processed poultry litter.

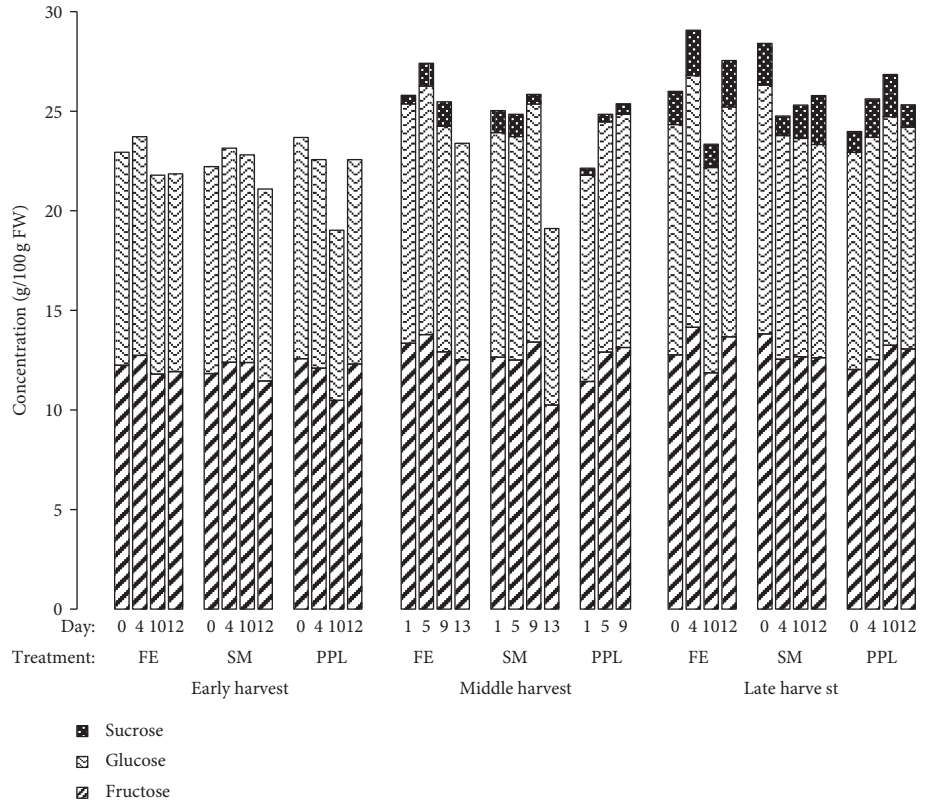

Figure 5: Sugar profile of "Triple Crown," expressed in total concentration, 2012 harvest. FE: blend of fish emulsion and fish hydrolysate; SM: soy meal; PPL: processed poultry litter.

difficult, particularly in the latter as it was so pronounced. One possibility was that these fruits were slightly undermature when picked, and underwent ripening during storage, as was explained for the changes in antioxidant measures. This could have resulted in an increase in sucrose metabolism, as was observed in strawberries [65] and peaches [66], with the latter showing differences in behavior based upon degree of fruit ripeness.

3.5. Phenolic Content and Antioxidant Capacity. As previous studies have indicated significant year-to-year variability in antioxidant content and capacity [29, 69], antioxidant analysis was only performed on fruits from the 2012 harvest. Table 1 presents the initial (day of harvest) TPC and TMA values for both cultivars, while the three measures of antioxidant capacity (DPPH, ORAC, and FRAP) are presented in Table 2. In general, "Obsidian" fruits had higher antioxidant content, with values ranging 3.31–4.85 mg GAE/g for TPC and 2.28–3.51 mg/g for TMA, than "Triple Crown" fruits, which ranged 2.71–4.39 mg GAE/g and 1.89–2.65 mg/g, and a similar trend was seen in the measures of antioxidant capacity, with "Obsidian" having higher values than "Triple Crown" in DPPH (8.44–10.84 mg AAE/g vs. 6.61–7.89 mg AAE/g), ORAC (289.52–763.73 μMol TE/g vs. 253.09–467.42 μMol TE/g), and FRAP (596.05–791.71 μMol Fe^{2+}/g vs. 489.67–646.68 μMol Fe^{2+}/g)).

Examining the measures of a given harvest date and fertilizer combination found that the "Triple Crown" fruit had lower measures of both content and capacity than the corresponding combination for "Obsidian," with the sole exception being the ORAC values for the early harvest which were 4.4–61.4% higher in "Triple Crown," depending on the fertilizer source.

The effect of refrigerated storage was quite erratic, with some samples showing increases in both amount of antioxidant contents (TPC and TMA) as well as antioxidant capacity (DPPH, ORAC, and FRAP), while others showed marked decreases in the same measurements. Figure 6 presents the relative changes in TPC and TMA, and Figure 7 reports the relative change in DPPH, ORAC, and FRAP. In all cases, the relative change was calculated using

$$\text{relative value}_{\text{day } X}(\%) = \frac{\text{mean measurement at day } X}{\text{initial mean measurement}}. \quad (2)$$

3.6. Initial Measures of Antioxidants. The values generally fall within the ranges reported for CG berries from the same two cultivars by Siriwoharn et al. [29] and Moyer et al. [69]. While the purpose of the current work was not to compare the two cultivars directly, it is important to note that, for all TPC, TMA, and DPPH measurements, the "Triple Crown" fruit showed lower values overall compared with "Obsidian" berries. Further, with the exception of the early harvest ORAC values (Table 2), the measures of a given harvest date and fertilizer combination for "Triple Crown" berries were also lower than the corresponding combination for "Obsidian" except the ORAC value in the early harvest "Triple Crown." The overall trend agreed with the previous findings from Moyer et al. [69], which showed similar results for CG berries of the two cultivars. Regarding the different behaviors seen in the early harvest ORAC, the explanation could be the differences in berry maturity between the samples taken during the two early harvests, as previous studies on CG berries have shown that ORAC values increase over 40% during the transition of fruits from underripe to overripe [29].

The data also revealed a complex interplay between fertilizer type and harvest date. As it was expected, the harvest date had a significant effect on the antioxidant properties of the fruit, and the effect varied depending on the fertilizer applied. In general, the middle harvest fruit showed the highest TPC, TMA, and DPPH values in both cultivars, excepting the FE samples and the TMA of "Triple Crown" which both showed increased values during the late harvest date. These results were hardly a surprising, as harvests from later in the season were more likely to include ripe or overripe fruit than earlier harvest, and an increase in anthocyanins, the primary red/blue/purple pigments in fruits [70], have been observed in a variety of CG berry and nonberry fruits as they become more mature [29, 71, 72]. Furthermore, the observed differences in FE fertilized samples were likely due to the differences in application regimen mentioned above. By contrast, the effect of harvest date on FRAP and ORAC values varied depending on the cultivar (Table 2), with the late harvest fruit showing lower values compared to the early harvest among the "Triple Crown" berries (except for the FRAP of SM samples which showed the highest value in the late harvest), while the "Obsidian" berries showed the lowest ORAC values, but the highest FRAP values in the early harvest. This could be caused by a variety of factors, including the aforementioned nutrient stress, as well as climate/weather conditions, as the middle/late harvest of "Obsidian" and the early/middle harvest of "Triple Crown" both experienced higher temperatures and greater sunlight. This increase in sunlight exposure was important, as one of the principle reasons hypothesized for the existence of plant phenolics is protection from photooxidation; i.e., the plant produces antioxidant phenolic compounds in order to quench radicals generated by exposure to UV [73]. Hence, fruits exposed to higher levels of UV would register lower overall values in assays that rely upon the quenching of radicals, as the activity of the compounds would already have been depleted quenching the ROS from UV exposure.

The trends observed among the fertilizer treatments were likely best explained by two factors: differences in application methods and differences in soil/plant responses to the fertilizers. Regarding application method, while all fertilizers were applied at the same rate, there were two different application schedules—SM and PPL, being pelletized products, were applied a single time, while FE was applied in four intervals, the final of which occurred shortly prior to the beginning of the harvest season, after the plants had bloomed [27]. This application schedule likely provided the plants receiving FE

Table 1: Total phenolic content and monomeric anthocyanins at harvest for cultivars "Obsidian" and "Triple Crown," 2012 harvest[†].

	Fish emulsion/hydrolysate blend	Soy meal	Processed poultry litter
TPC (mg GAE/g)[‡]			
Obsidian			
Early harvest	A3.31 ± 0.08[a]	B3.86 ± 0.10[a]	B4.05 ± 0.21[a]
Middle harvest	A4.21 ± 0.02[a]	B5.55 ± 0.15[b]	A4.30 ± 0.15[b]
Late harvest	A4.85 ± 0.23[c]	B4.59 ± 0.06[c]	C3.54 ± 0.11[c]
Triple crown			
Early harvest	A3.030 ± 0.07[a]	B3.76 ± 0.14[a]	C3.42 ± 0.06[a]
Middle harvest	A2.96 ± 0.07[a]	B3.81 ± 0.09[a]	C4.39 ± 0.12[b]
Late harvest	A3.55 ± 0.18[b]	B2.89 ± 0.05[b]	C2.71 ± 0.05[c]
TMA(mg/g)[‡]			
Obsidian			
Early harvest	A2.28 ± 0.11[a]	A2.51 ± 0.14[a]	A2.45 ± 0.11[a]
Middle harvest	A3.16 ± 0.20[b]	B3.51 ± 0.24[b]	C3.10 ± 0.19[b]
Late harvest	A3.17 ± 0.22[b]	B3.11 ± 0.21[c]	B2.56 ± 0.16[a]
Triple crown			
Early harvest	A1.89 ± 0.00[a]	A1.93 ± 0.08[a]	A2.08 ± 0.08[a]
Middle harvest	A2.39 ± 0.08[b]	B2.10 ± 0.03[b]	B2.14 ± 0.04[a]
Late harvest	A2.65 ± 0.02[b]	A2.57 ± 0.03[c]	B2.57 ± 0.05[b]

TPC: total phenolic content; TMA: total monomeric anthocyanins. [†]Mean values ± SD, $n = 3$. Values preceded with the same capital letters (A–C) within the same row of a given table are not statistically different ($\alpha \leq 0.05$). [‡]Within a given cultivar and phenolic determination method, values followed with the same lowercase letters (a–c) within the same column of a given table are not statistically different ($\alpha \leq 0.05$).

Table 2: Antioxidant capacity of "Obsidian" and "Triple Crown" blackberry cultivars at harvest (2012 harvest).

	Fish emulsion/hydrolysate blend	Soy meal	Processed poultry litter
DPPH (mg AAE/g)[†‡]			
Obsidian			
Early harvest	A8.44 ± 0.75[a]	A8.77 ± 0.52[a]	A7.78 ± 0.21[a]
Middle harvest	A9.94 ± 1.04[a]	A10.84 ± 0.13[b]	A9.26 ± 0.15[b]
Late harvest	A10.01 ± 0.31[a]	A 9.20 ± 0.90[a]	A 8.98 ± 0.11[c]
Triple crown			
Early harvest	A6.96 ± 0.29[a]	A6.63 ± 1.18[a]	A6.68 ± 0.06[a]
Middle harvest	A6.61 ± 0.28[a]	A7.61 ± 0.58[a]	A7.98 ± 0.12[b]
Late harvest	A7.70 ± 0.68[a]	A6.80 ± 0.47[a]	A6.63 ± 0.05[c]
ORAC (μmol TE/g)[†*#]			
Obsidian			
Early harvest	A289.52 ± 22.57[a]	B427.86 ± 27.86[a]	B392.54 ± 0.11[a]
Middle harvest	A380.48 ± 9.79[b]	B501.69 ± 0.51[b]	A416.80 ± 0.19[b]
Late harvest	A763.73 ± 18.39[c]	B717.15 ± 55.05[c]	C591.50 ± 0.16[a]
Triple crown			
Early harvest	A467.42 ± 36.00[a]	A446.88 ± 5.89[a]	A427.55 ± 0.08[a]
Middle harvest	A362.66 ± 12.78[b]	B403.50 ± 12.65[b]	A387.89 ± 0.04[a]
Late harvest	A253.09 ± 5.69[c]	A268.04 ± 24.43[c]	A253.71 ± 0.05[b]
FRAP (μmol Fe^{2+}/g)[†*#]			
Obsidian			
Early harvest	A753.51 ± 0.06[a]	A791.71 ± 0.00[a]	A767.75 ± 6.60[a]
Middle harvest	A596.05 ± 60.21[b]	B727.32 ± 11.85[b]	A649.29 ± 2.05[b]
Late harvest	A709.10 ± 6.39[a]	A680.87 ± 34.27[b]	B622.75 ± 5.66[b]
Triple crown			
Early harvest	A521.53 ± 4.35[a]	B588.15 ± 3.04[a]	A534.38 ± 1.06[a]
Middle harvest	A489.67 ± 3.06[b]	B548.21 ± 13.17[b]	C600.98 ± 9.18[b]
Late harvest	A646.48 ± 13.73[c]	B574.66 ± 10.10[a]	B559.62 ± 5.37[c]

DPPH: radical scavenging activity by the 2,2-diphenyl-1-picrylhydrazyl colorimetric method; ORAC: oxygen radical absorbance capacity; FRAP: ferric-reducing antioxidant power. [†]Mean values ± SD, n = 3. [‡]Mean values ± SD, n = 2. [*]Values preceded with the same capital letters (A–C) within the same row of a given table are not statistically different ($\alpha \leq 0.05$). [#]Within a given cultivar and antioxidant method, values followed with the same lowercase letters (a–c) within the same column of a given table are not statistically different ($\alpha \leq 0.05$).

fertilizer a more uniform amount of nutrients, allowing for greater reserves during fruit development and maturation. In addition to the application schedules, the properties of the individual fertilizer types might also play a role, as it has been shown that fertilizer source and form can have a profound effect on short-term availability of

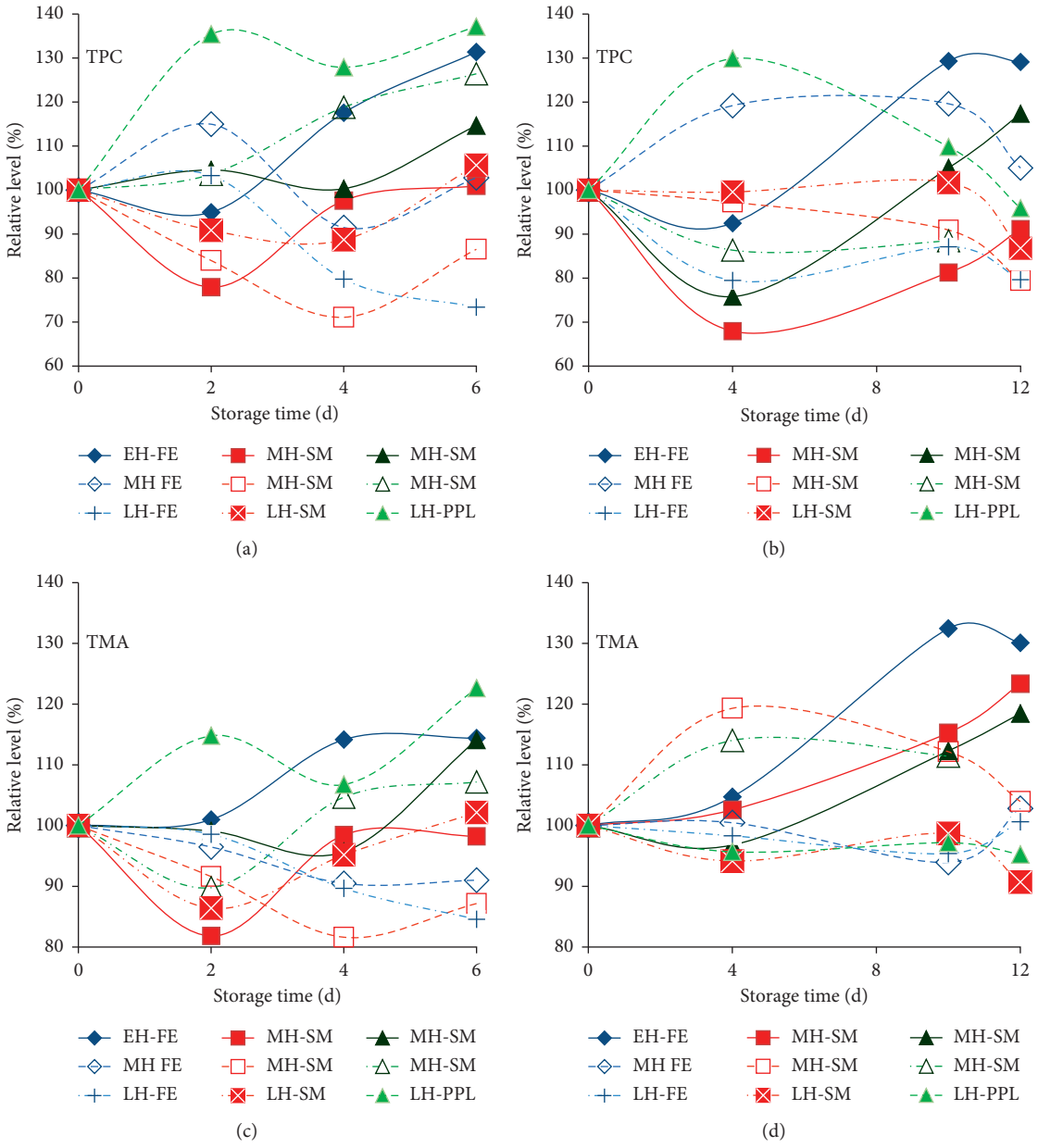

Figure 6: Relative phenolic content and monomeric anthocyanins during storage of two blackberry cultivars: "Obsidian" (a) and "Triple Crown" (b), 2012 harvest. FE: blend of fish emulsion and fish hydrolysate; SM: soy meal; PPL: processed poultry litter.

nutrients in the soil, with composted fertilizers having the smallest immediate effect, animal slurries having some of the highest, and legume meals falling somewhere in between [74]. Since it is well known that plants respond to various stresses in complex ways, typically involving the use of reactive oxygen species (ROS) as mechanisms for signaling various types of stress, from drought to pollutants to excess UV to diseases [75, 76], it is possible that if the nitrogen release rates of the various fertilizer types result in a dearth of available nutrients, this stress would be similarly signaled, affecting the antioxidant content of the resulting fruit. Such behavior has been seen in multiple plant species, with increased fertilization, particularly prior to flowering, reducing the levels of various antioxidants in fruits [73, 77, 78].

3.7. Effect of Storage on Antioxidants. Storage had a very erratic effect on both the antioxidant content and capacity, with many harvest/fertilizer treatments showing increases in either or both during storage. While these increases might be counterintuitive, this was not the first time such trends have been noticed. Wu et al. [35] found similar trends in the CG blackberry cultivars they examined, Kalt et al. [79] also observed increases in various CG small fruits, including raspberries, and Ali et al. [50] reported increases in ellagic acid in

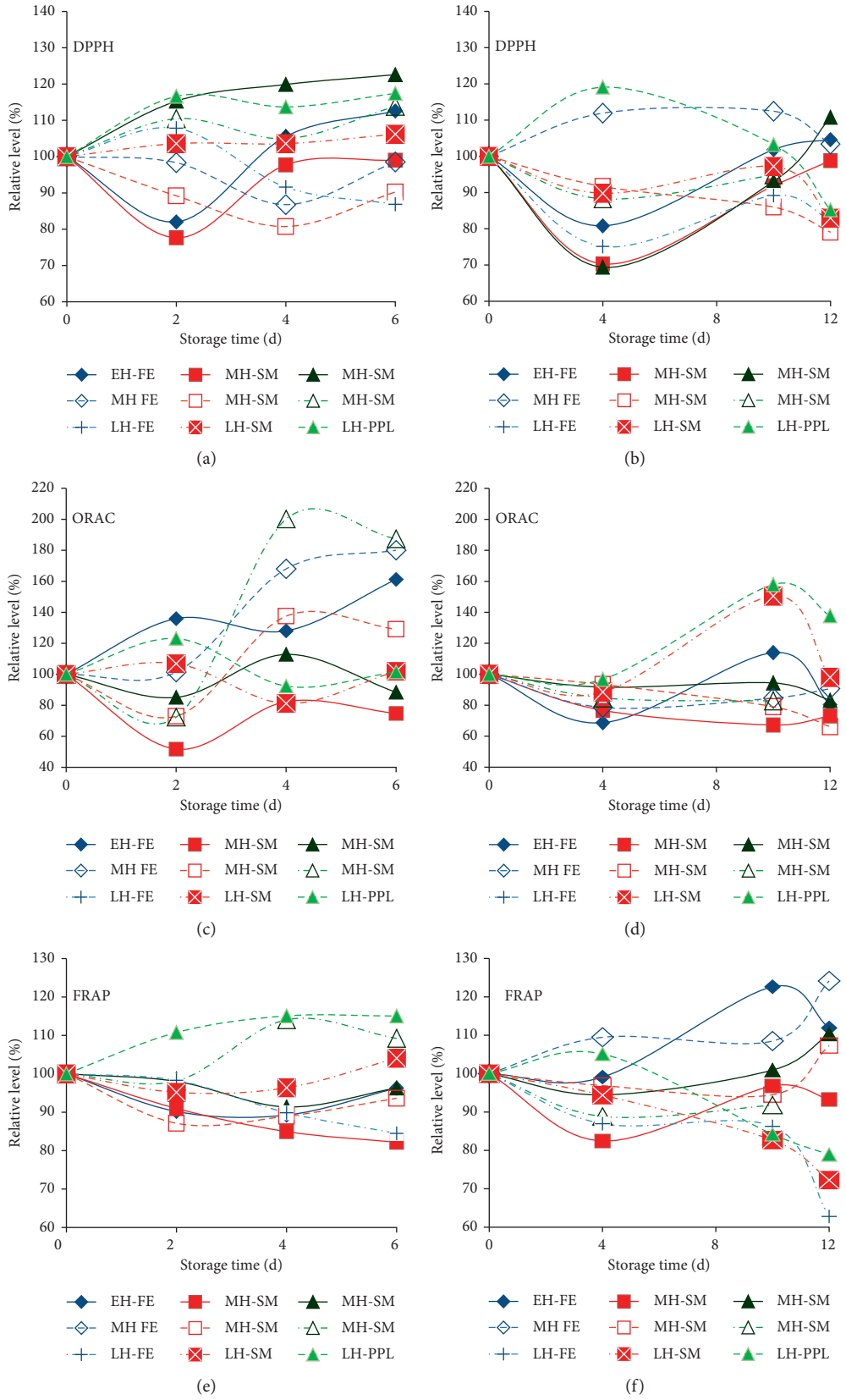

Figure 7: Relative antioxidant potential by three methods (DPPH, ORAC, and FRAP) during storage of two blackberry cultivars: "Obsidian" (a) and "Triple Crown" (b), 2012 harvest. FE: blend of fish emulsion and fish hydrolysate; SM: soy meal; PPL: processed poultry litter.

late-harvest CG blackberries and raspberries, as well as increases in anthocyanins and total phenolics in raspberries. While metabolic mechanisms were indicated in these rises, there were some debates over what initiated the process, with possibilities ranging from normal ripening of the potentially undermature fruit to the breakdown of other fruit components (notably organic acids), creating additional carbon skeletons to feed the pathways that synthesized phenolic compounds [35, 79]. In addition, it was also possible that the stimulus for the production is related to the aforementioned plant responses to stress [76], as it could be expected that refrigeration at 4°C would create temperature stresses in the summer fruit, and such behavior was noted in CG tomatoes, watermelons, apples, strawberries, and mangoes [66, 80–82].

4. Conclusion

In organically grown blackberries, the use of different fertilizers had virtually no significant effect on the physicochemical properties of "Obsidian" and "Triple Crown" blackberry fruit at the time of harvest and during the refrigerated storage, but did have a profound effect on the measures of antioxidant content, antioxidant capacity, and the composition of sugars. Furthermore, these measures were also affected by differences in blackberry cultivar, harvest date, and storage time, with the interaction between the factors showing great complexity. Despite this, several general observations could be made, namely, that with respect to both antioxidant measures and sugar profiles, fertilization with either a fish emulsion/fish hydrolyate or soy meal-based fertilizers were preferable to the use of processed poultry litter, that during storage ripening tended to increase the relative levels of fructose at the expense of decreasing sucrose content and that while the middle harvest had higher initial levels of phenolic compounds, including anthocyanins, and their antioxidant capacity followed less predictable trends, particularly in the "Triple Crown" cultivar. Further, the organically grown "Obsidian" fruit may be marketed for fresh consumption within 4 d after harvest while the "Triple Crown" fruit can be extended for 8 d due to its lower rates of decay and leakage. While further study is needed to elaborate the mechanisms involved and how well these findings can be applied over other cultivars, it is entirely likely that the fertilizer regimen might be able to be used to maximize the healthful properties of blackberries, and potentially in other fruits, while having a negligible effect on traditional physicochemical measures of quality.

Data Availability

All the data related to this article are described in Tables 1 and 2 and/or depicted in Figures 1–7. Persons interested in the raw data may contact the corresponding author to receive a copy.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Poly(lactic-*co*-glycolic acid) Nanoparticles Loaded with *Callistemon citrinus* Phenolics Exhibited Anticancer Properties against Three Breast Cancer Cell Lines

Rashid Ahmed,[1,2,3] Muhammad Tariq,[1] Irfan S. Ahmad,[2] Hanafy Fouly,[2] Fakhar-i-Abbas,[4] Anwarul Hasan,[3,5] and Mosbah Kushad[6]

[1]*Department of Biotechnology, Mirpur University of Science and Technology (MUST), Mirpur 10250, Azad Jammu and Kashmir, Pakistan*
[2]*Micro and Nanotechnology Laboratory, University of Illinois at Urbana Champaign, Urbana, IL, USA*
[3]*Department of Mechanical and Chemical Engineering, College of Engineering, Qatar University, Doha 2713, Qatar*
[4]*Bioresource Research Centre Islamabad, Islamabad, Pakistan*
[5]*Biomedical Research Centre, Qatar University, Doha, Qatar*
[6]*Department of Crop Sciences, University of Illinois at Urbana Champaign, Urbana, IL 61801, USA*

Correspondence should be addressed to Anwarul Hasan; ahasan@qu.edu.qa and Mosbah Kushad; kushad@illinois.edu

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Fruit and vegetable diets rich in phenolic compounds reduce the risk of various cancers and offer multiple other health benefits due to their bioactivity and powerful antioxidant properties. However, the human health benefits of most phenolic compounds are restricted due to their limited aqueous solubility, low absorption, restricted passive cellular efflux, and poor gastrointestinal stability. Nanotechnology has been used to deliver various therapeutic drugs to specific targets overcoming many of the limitations of direct treatments. This study was designed to develop poly(lactic-*co*-glycolic acid) (PLGA) nanoencapsulated phenolic-rich extracts from *Callistemon citrinus* and berberine and to evaluate their effectiveness against extremely invasive MDA-MB 231, moderately invasive MCF-10A, and minimally invasive MCF-7 breast cancers. We have achieved about 80% encapsulation of phenolics from *C. citrinus*. Most encapsulated nanoparticles were polygonal with particles sizes of 200 to 250 nm. Release of phenolics from encapsulation during storage was biphasic during the first week and then levelled off thereafter. Nanoencapsulated phenolics from *C. citrinus* extract, berberine, and combination of both enhanced their bioactivity against the three breast cancer cell lines by nearly 2-fold. Growth inhibition of cells was a linear curve relative to phenolic concentration, with a maximum inhibition of nearly 100% at 0.1 mg/ml compared to control.

1. Introduction

Voluminous *in vivo* and *in vitro* studies have confirmed that diets containing fruits and vegetables, which are rich in phenolic phytochemicals, reduce the risk of several types of cancers and provide multiple human health benefits [1–3]. Recent research and review articles have demonstrated that dietary phenolic compounds reduce the risk of UV-induced oxidative and free radical damage, prevent cancers of the breast, stomach, prostate, and skin, and protect against inflammation, diabetes, and neurotoxicity [2, 4, 5]. It has been suggested that dietary phenolics deliver better preventive and therapeutic options, by improving phytonutrient bioavailability and enhancing drug activity, while exerting low toxicity, compared to conventional drug treatments [6].

The genus *Callistemon* consists of 34 species widely grown in several parts of the world. Leaves, inflorescence,

and oils collected from this genus have for centuries been used in tribal medicine to treat gastrointestinal disorders, various pains, and infectious diseases [7]. Scientific evidence collected over the last few decades indicates that several *Callistemon* species contain bioactive compounds with medicinal properties against cardiovascular diseases and inflammation and anticancer and antidiabetic activities [7]. The medicinal properties of many of these species have been attributed to their rich content of polyphenolic antioxidants, flavonoids including flavanols, flavanones, terpenoids, and tannins, and several nonphenolic bioactive compounds including alkaloids, glycosides, and saponins [7, 8]. Significant differences in polyphenolic concentrations and bioactivity were observed among different species within the *Callistemon* genus and between different plant parts of the same species with leaves of *C. viminalis* containing as high as 44% per dry weight polyphenols [9, 10].

Several studies have examined the antibacterial and antifungal properties and bioactivity of oil extracts from several *Callistemon* species including *viminalis*, *comboynensis*, *lanceolatus*, *citrinus*, *rigidus*, and *linearis* [11, 12]. For example, nitisinone extracted from *C. rigidus* was reported to lower tyrosinaemia type 1, which can lead to buildup of tyrosine and its byproducts causing serious illnesses [7], while extracts from *C. lanceolatus* were reported to possess anticholinesterase activity [13]. However, limited research has been done on the anticancer properties of the nonoil bioactive compounds (primarily phenolics) found in *Callistemon* species. *C. citrinus* (Curtis) Skeels, commonly known as crimson or lemon bottlebrush, is very rich in phenolics and other bioactive compounds; however, there are very limited data on the potential of this species to reduce cancer [14, 15].

Significant advances have been reported in recent years in nanoparticle systems, especially polymeric nano/microparticles used in cancer therapy [16]. Nanoparticles have been developed from various biocompatible and biodegradable materials that may be natural and/or synthetic and therefore display putative ability as carriers for treating various kinds of diseases, especially cancer [17]. In addition, polymeric nanoparticles are nontoxic and display a prolonged circulation potential and a wide payload spectrum for therapeutic agents. Synthetic polymers/copolymers such as PLGA, PLA, and PVA are preferred materials for the development of polymeric nanoparticles, as these systems can deliver drugs for days or even weeks compared to natural polymers, such as chitosan and sodium alginate nanoparticles, which have a shorter period of drug delivery and often require potentially toxic organic solvents [18]. Polymeric nanoparticles can be degraded enzymatically or nonenzymatically in vivo and thus produce biocompatible and safe byproducts that can be easily cleared from the body. Drugs encapsulated in polymeric nanoparticles are either dispersed inside the polymer matrix or attached/conjugated to polymer molecules allowing their release from nanoparticles upon their degradation [18].

Even though there is conclusive evidence to support the positive effects of phenolics on human heath, their low solubility and bioavailability may have limited their health benefits in many of the previous studies [19]. Nanoparticle delivery systems have successfully been used to encapsulate bioactive compounds and deliver them to intended targets in order to enhance their absorption and/or bioavailability [20, 21]. The aim of this study was to utilize advances in nanotechnology to encapsulate phenolic-rich extracts from *C. citrinus*, examine stability and effectiveness of nanoencapsulated *C. citrinus* nanoparticles, and evaluate their effects on growth and proliferation of three types of breast cancer cell lines.

2. Materials and Methods

2.1. Materials. Biopolymer poly(lactic-*co*-glycolic acid) (PLGA) 50 : 50, berberine chloride hydrate (mol. wt. 371.9 Da), gallic acid, sodium carbonate, Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and polyvinyl alcohol (PVA) (99.3–100% hydrolyzed, average mol. wt. 85,000–124,000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone and methanol were purchased from Fisher Scientific Laboratory (NJ, USA), and potassium phosphate buffer was purchased from EMD Chemicals, Inc. (Gibbstown, NJ, USA). Human breast carcinoma (MCF-7, MCF-10A, and MD-MB 231) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle medium, fetal bovine serum (FBS), and penicillin-streptomycin/EDTA solution were purchased from Gibco (Carlsbad, CA, USA).

2.2. Tissue Sample Preparation. Bottlebrush *Callistemon citrinus* leaves and stems were collected from mature plants grown in Chakwal District of Punjab Province, Pakistan. Fresh tissue was frozen at −70°C, freeze-dried, and ground into fine powder. A subsample of 20 g freeze-dried tissue was mixed with 200 ml methanol and homogenized, and the homogenate was placed on an orbital shaker (VWR, Hampton, NH, USA) for 12 hours. The mixture was filtered through a Whatman (#4) filter paper (Marlborough, MA, USA), and the filtrate was concentrated in a rotary evaporator (Buchi Co., New Castle, DE, USA). Fresh extracts were prepared for each assay.

2.3. Preparation and Loading of PLGA Nanoparticles. Nanoparticles were prepared by a nanoprecipitation technique [22] with slight modifications as described by Pereira et al. [23]. An organic phase was formed when a fraction of 5.0 mL of the previously prepared methanolic tissue extracts was combined with 50 mg PLGA prepared in acetone solution and stirred at 150 rpm for 45 minutes. An aqueous phase of 1% polyvinyl alcohol (PVA) solution was prepared by dissolving 1.0 g of PVA in 100 ml of ultrapure H_2O and then heated and stirred to dissolve PVA. The organic fraction of the PLGA tissue extract was added to 20 mL PVA solution in a dropwise manner.

Similarly, a standard of 1.0 mg of berberine chloride was dissolved in 1.0 ml of methanol and 7 mg of PLGA prepared in 1.0 ml acetone. Berberine chloride solution was added to

the PLGA solution and stirred for 45 minutes. The PLGA-berberine mixture was added to another 20 mL PVA solution in a dropwise manner and stirred for 10 minutes at room temperature. Mixing of the organic phase with the aqueous phase while stirring resulted in aggregation of PLGA nanoparticles, which was prevented by adding 2.0 ml purified H_2O. The solvents (acetone and methanol) were removed by rotary evaporation. Similarly, blank samples of PLGA nanoparticles were synthesized without the tissue extracts by adding only PLGA solution following the same steps outlined previously. The PLGA nanoparticles were centrifuged (Eppendorf centrifuge 5415C, Hamburg, Germany) at 14,000 rpm for 15 minutes and then collected and purified by ultrafiltration as previously described [24, 25]. PLGA nanoparticles were lyophilized by mixing synthesized nanoparticles with trehalose (EDM Chemicals, Philadelphia, PA, USA) at 1 : 1 ratio by pouring the PLGA nanoparticles into glass vials covered with a double layer of Parafilm. The PLGA nanoparticles were stored at −80°C for 24 hours and then freeze-dried (Labconco, Kansas City, MO, USA).

2.4. Scanning Electron Microscopy (SEM) of PLGA Nanoparticles. The prepared nanoparticles were evaluated under a scanning electron microscope (Hitachi S-4800 FE-SEM, Japan) for their size, morphology, and surface properties. Nanoparticles were washed three times with distilled water, freeze-dried, and coated with gold palladium to improve electrical conductivity before imaging under SEM at 15 kV.

2.5. Nanoparticle Properties. PLGA nanoparticle diameter (Ø, size), surface property or zeta potential (ζ), and polydispersity index (PI), which determines nanoparticle penetration potential, aggregation capacity, and diffusion rate, were determined using Nano ZS90 Zetasizer (Malvern, UK) and Hitachi S-4800 scanning electron microscopy (SEM). PLGA nanoparticles were suspended in pure water in a cuvette (Malvern Panalytical, UK) and were sonicated to uniformly disperse the particles, in order to determine their size and any aggregate formation. Aggregation of PLGA nanoparticles was eliminated by filtering through a 0.2 μm nylon filter (Acrodisc, Pall Corporation, Port Washington, NY, USA). The zeta potential of nanoparticles was determined by suspending PLGA nanoparticles in pure water and then transferring them into a zeta capillary cell (Malvern, Worcestershire, UK), which was loaded into a Malvern Zetasizer. The passage of the laser beam through PLGA nanoparticles and fluctuation in scattering intensity of nanoparticles produced the signal of an electrophoretic charge present on PLGA nanoparticles. All readings were recorded at 25°C and a scattering angle of 90° after dilution of samples with pure water to prevent multiscattering. Polystyrene was used as a standard to measure light scattering at an angle of 173°. Each sample was measured in triplicate.

2.6. Total Phenolic Entrapment Efficiency within Nanoparticles. UV spectroscopy was used to determine the amount of polyphenol and polyphenol matrices of the *C. citrinus* extract, berberine, and *C. citrinus* extract-berberine mixture entrapped into PLGA nanoformulations. The amount of PVA applied as a stabilizer and surfactant were kept constant at 1% during formulation of PLGA nanoparticles. Entrapment efficiency (η%) of total phenolics of the *C. citrinus* extract within nanoparticles was determined by measuring the UV absorbance of total phenolics entrapped within PLGA nanoparticles according to Pereira et al. [23] with a slight modification. The *C. citrinus* extract and berberine loaded PLGA nanoparticles were suspended in 95% methanol solution at a concentration of 1.0 mg/ml and placed in the dark for 30 minutes at 37°C. The mixture was filtered through a 0.2 μm nylon filter. A 100 μl fraction of the filtrate was mixed with 20 μl Folin–Ciocalteu reagent, 830 μl purified water, and 50 μl sodium carbonate solution. Samples were placed in the dark at room temperature for 30 minutes, and absorbance was recorded at 725 nm using a UV160U spectrophotometer (Shimadzu Corp., Kyoto, Japan). Gallic acid was used as a standard to estimate the total phenolic content in the encapsulated PLGA nanoparticles [25, 26]:

$$\eta = \frac{\text{amount of total phenolics entrapped}}{\text{total phenolic quantity used for encapsulation}} \times 100. \quad (1)$$

2.7. In Vitro Release of Total Phenolics. The release of total phenolics from nanoparticles was estimated by combining lyophilized nanoparticles in a phosphate buffer saline (pH 7.2) at 1.0 mg/ml, which was suitable to create a sink condition. The mixtures were incubated at 37°C in a water bath for 24 hours and then filtered through a 0.2 μm Acrodisc nylon filter [25]. Later, the filtrate was collected, and total phenolics released were measured as described in Section 2.5.

2.8. Stability of PLGA Nanoparticles. The stability of PLGA nanoparticles was evaluated by measuring the amount of total phenolics released after one week of storage at 4°C. In summary, 1.0 mg/ml subsamples of refrigerated PLGA nanoparticles were centrifuged at 3,000 rpm for 3 minutes. The supernatants were collected, and the amount of total phenolics released was measured as previously described. The results were compared to total phenolics released by freshly prepared PLGA nanoparticles.

2.9. Anticancer Activity Determination. The anticancer activity of *C. citrinus* and berberine extract loaded PLGA nanoparticles was determined by a sulforhodamine B (SRB) colorimetric assay (Invitrogen, Carlsbad, California) according to a protocol outlined by Vichai and Kirtikara [27]. The protocol was used for cytotoxic screening of PLGA nanoparticles based on cell density measurement by estimation of protein content. Three cancer cell lines (MCF-7, MCF-10A, and MDA-MB 231) were used to study the effect of PLGA nanoparticles and unencapsulated form of

C. citrinus and berberine. The human hormone-dependent breast cancer cell lines MCF-7 and MCF-10A were cultured in Dulbecco's modified Eagle medium (Gibco, Carlsbad, CA, USA), and MDA-MB 231 hormone-independent breast cancer cell lines were cultured in Leibovitz's L-15 medium (Sigma-Aldrich) in T75 cell culture flasks (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin/EDTA solution (Gibco, Carlsbad, CA, USA). Cultured cells were immediately transferred to a 96-well plate (Thermo Fisher Scientific) and incubated overnight to a rate of about 1×10^4 cell density per well, and the percentage of growth inhibition of breast cancer cell lines was measured after a period of 48 hours.

Tested PLGA nanoparticles loaded with *C. citrinus* and berberine and free extracts from the same treatments ranged from 0.0008 to 0.1 mg/ml. Samples were placed in 96-well plates, incubated under 5% CO_2-enriched air and 100% relative humidity at 37°C for 48 hours, fixed by adding 50 μl of cold 10% (w/v) TCA, and then stored at 4°C for 1 hour. Each sample well was washed four times with distilled water and then dried by a purified air stream at room temperature.

The staining step was performed by adding 100 μL of 0.57% (w/v) sulforhodamine B (SRB) dye to each sample well. Treated samples were incubated at room temperature for 30 minutes and then washed four times with 100 μl of 1% (v/v) acetic acid to remove the unbound dye. Finally, 100 μl of 10 mM Tris base (pH 10.5) was added to each sample well, and the plates were placed on a shaker for 3 minutes. The absorbance of each sample was measured using a spectrophotometer set at 490–630 nm, and the growth inhibition percentage was determined using the following formula:

$$\text{growth inhibition } (\%) = \left[\frac{\text{mean OD of sample} - \text{mean OD}_b}{\text{mean OD of control} - \text{mean OD}_b}\right] \times 100, \quad (2)$$

where OD_b is the OD for a blank sample.

2.10. Statistical Analysis. Results are expressed as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance, and differences among the means of groups were analyzed by an unpaired, two-sided Student's *t*-test. Differences were significant at $P < 0.05$.

3. Results and Discussion

3.1. Preparation and Characterization of PLGA Nanoparticles. In our study, PLGA nanoparticles were synthesized by a nanoprecipitation technique, as outlined in Figure 1. Polyvinyl alcohol (PVA), a biodegradable and biocompatible synthetic biopolymer, was used as a stabilizer for PLGA nanoparticles. By using PVA as a stabilizer and PLGA as a carrier, we were able to obtain high yield of nanoparticles. PLGA has been widely used for drug delivery applications of hydrophobic drugs such as polyphenols. It is also biodegradable, biocompatible, and a highly stable polymer. It has also been approved by the USA Food and Drug Administration.

3.2. Scanning Electron Microscopy. Sizes of PLGA nanoparticles loaded by berberine, *C. citrinus* extract, and mixtures of berberine and *C. citrinus* extract were 250.7 ± 0.06, 278.8 ± 0.007, and 274.8 ± 0.028 nm, respectively (Table 1). Nanoparticle shapes observed under SEM were polygonal (hexagonal) with diverse sizes, with unique morphological features (Figures 2(a) and 2(b)), as compared to a previously reported formulation [24]. Various studies have demonstrated that tailoring of the shape of nanoparticles could improve their effectiveness. For example, it was reported that polygonal nanoparticles have more antibacterial activity against *E. coli* than triangular and spherical nanoparticles [28]. In another study, it was observed that oblong-shaped nanoparticles improved targeted delivery of antibody nanoformulations [29]. Nanorods coated with trastuzumab exhibited a 66% increase in binding and uptake potential by BT-474 breast cancer cell lines compared to an equal dose of spherical nanoparticles, resulting in a 5-fold growth inhibition potential against BT-474 cancer cells [30]. While oblate nanoparticles exhibited a better degree of adherence to mammalian cells and a higher drug-carrying capacity compared to spherical particles [31], polygonal nanoparticles were reported in an earlier study to exhibit a prolonged stay in blood stream due to uptake failure of macrophages [32]. This mechanism may lead to enhanced growth inhibition due to higher penetration through cell membranes, subsequently releasing polyphenols into the cytoplasm, which contains various organelles that could be affected by the nanoformulations [25].

The enhanced growth inhibition feature of polygonal nanoparticles was observed during their cytotoxic effect on various types of cancer cell lines as these nanoformulations penetrate more efficiently into cells due to their morphological matrix [33, 34]. In addition to their morphology, nanoparticles' surface charges also determine their potency. The effectiveness of positively charged PLGA nanoparticles was higher than that of PLGA nanoparticles bearing negative charges [35]. Nanoparticles' surface charges also affect their aggregation. Aggregation of nanoparticles developed in this study was significantly reduced by filtration followed by addition of an excess solvent. Our PLGA nanoparticles were more efficiently separated when they had higher surface charges and tended to aggregate when they had lower surface charges.

3.3. Characterization of Nanoparticles. Nanoparticle size and polydispersity index (PDI) define efficient delivery of loaded materials. Both nanoparticle size and PDI determine drug loading and drug release profiles, stability, toxicity, and targeting potential, which are main requirements for an ideal cargo carrier system. The nanoparticle size obtained in the current study ranged from 200 to 250 nm (Table 1), which is considered an optimum size range for PLGA nanoparticles [36]. Zeta potential is another important characteristic that determines cytotoxicity

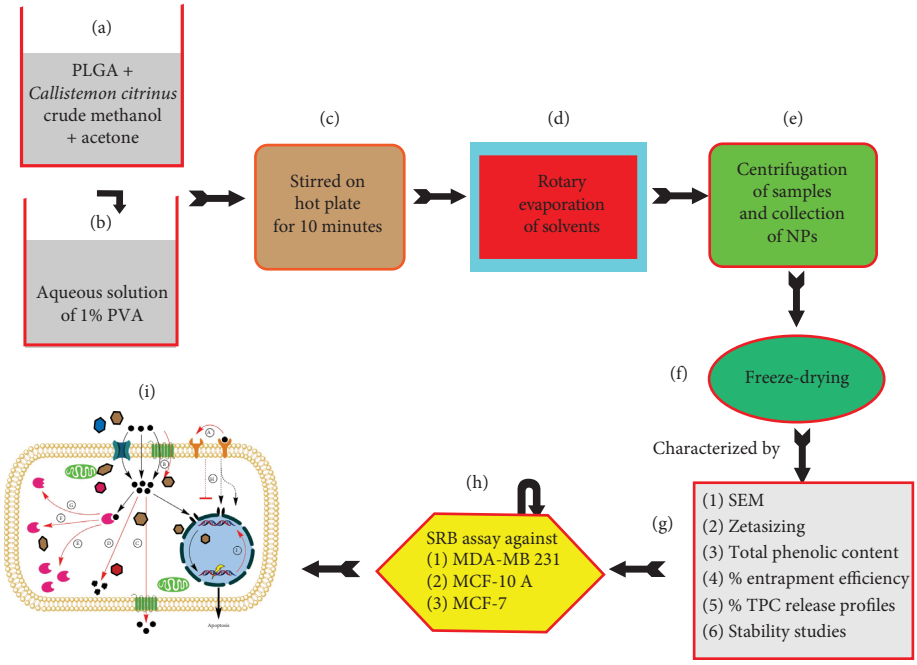

Figure 1: Flow chart for the synthesis and characterization of *C. citrinus* extract-loaded PLGA nanoparticles. (a) Mixing of *C. citrinus* extract solution with PLGA solution. (b) Preparation of 1% PVA solution. (c) PLGA solution containing *C. citrinus* extract was constantly stirred at 150 rpm for 30 minutes, leading to precipitation of PLGA NPs. (d) The solvent was evaporated by rotary evaporation. (e) Collection of PLGA nanoparticles by centrifugation at 14,000 rpm. (f) Collection of PLGA NPs was freeze-dried and stored at −4°C. (g) Characterization of PLGA-loaded nanoparticles by scanning electron microscopy, zetasizing, total content in extracts, loading of total phenolics (TP) into PLGA nanoparticles, and percentage TP release studies and stability profiles. (h) Assessment of growth inhibitory effect of PLGA NPs and their free counterparts against MDA-MB 231, MCF-10A, and MCF-7 breast cancer cells. (i) Mode of action of PLGA NPs on model cells.

Table 1: Estimates of zeta diameter, polydispersity index, and zeta potential of nanoformulations of BBR PLGA, CCE PLGA, and CCE BBR PLGA.

NPs	Incubation temperature (°C)	Zeta diameter (nm)	Aggregation (%)	Polydispersity index	Zeta potential (mV)
BBR PLGA	25	250.6 ± 0.08	0	0.361 ± 0.06	−8.50 ± 0.06
CCE PLGA	25	278.8 ± 0.00	0	0.545 ± 0.07	−20.06 ± 0.10
CCE BBR PLGA	25	274.8 ± 0.02	0	0.256 ± 0.04	−5.80 ± 0.09

Abbreviations: BBR, berberine; CCE, *Callistemon citrinus* extract; PLGA, poly(lactic-*co*-glycolic acid); NPs, nanoparticles. Data points represent mean ± SD ($n = 3$).

and clumping of loaded PLGA nanoparticles. The surface charge of PLGA nanoparticles loaded with berberine and a combination of berberine and *C. citrinus* was −8.50 ± 0.06 and −5.80 ± 0.09, respectively, whereas the surface charge of PLGA nanoparticles loaded with *C. citrinus* alone was 2.4- to 3.5-fold lower than that loaded with berberine and mixture of berberine plus *C. citrinus*. Since nanoparticles' surface charge plays a significant role in inhibition of cancer growth [35], PLGA nanoparticles loaded with berberine plus *C. citrinus* could be more cytotoxic or more effective against cancer cells compared to PLGA nanoparticles loaded with *C. citrinus* alone. The PDI data in Table 1 and size distribution in Figures 2(c) and 2(d) show that the PLGA nanoparticles were of diverse sizes. Formation of diverse-sized PLGA nanoparticles is advantageous due to variation in their diffusion rates with smaller nanoparticles having higher diffusion than larger nanoparticles.

3.4. Total Phenolics and Their Entrapment in LPGA Nanoparticles. Berberine, an alkaloid antioxidant, reacted positively and linearly with the phenolic assay, but at a rate lower than that of *C. citrinus* phenolics. The total phenolic content of berberine, *C. citrinus*, and the mixture of *C. citrinus* and berberine was 1942.46 ± 0.01, 609.35 ± 0.02, and 1979.58 ± 0.006 μg/ml, respectively (Figure 3(a)). The data obtained suggest that phenolic concentration in the *C. citrinus* extract and *C. citrinus* extract plus berberine was 4-fold higher than that in berberine alone. Numerous articles have linked antioxidant properties of total phenolics to human health benefits but have very little information on the health benefits of their entrapment in nanoformulations.

Total phenolic entrapment of about 88% was observed in PLGA nanoparticles loaded with a blend of *C. citrinus* and berberine, followed by 82% entrapment from *C. citrinus* extract alone and only about 23% entrapment from berberine alone (Figure 3(b)). The solubility of the entrapped

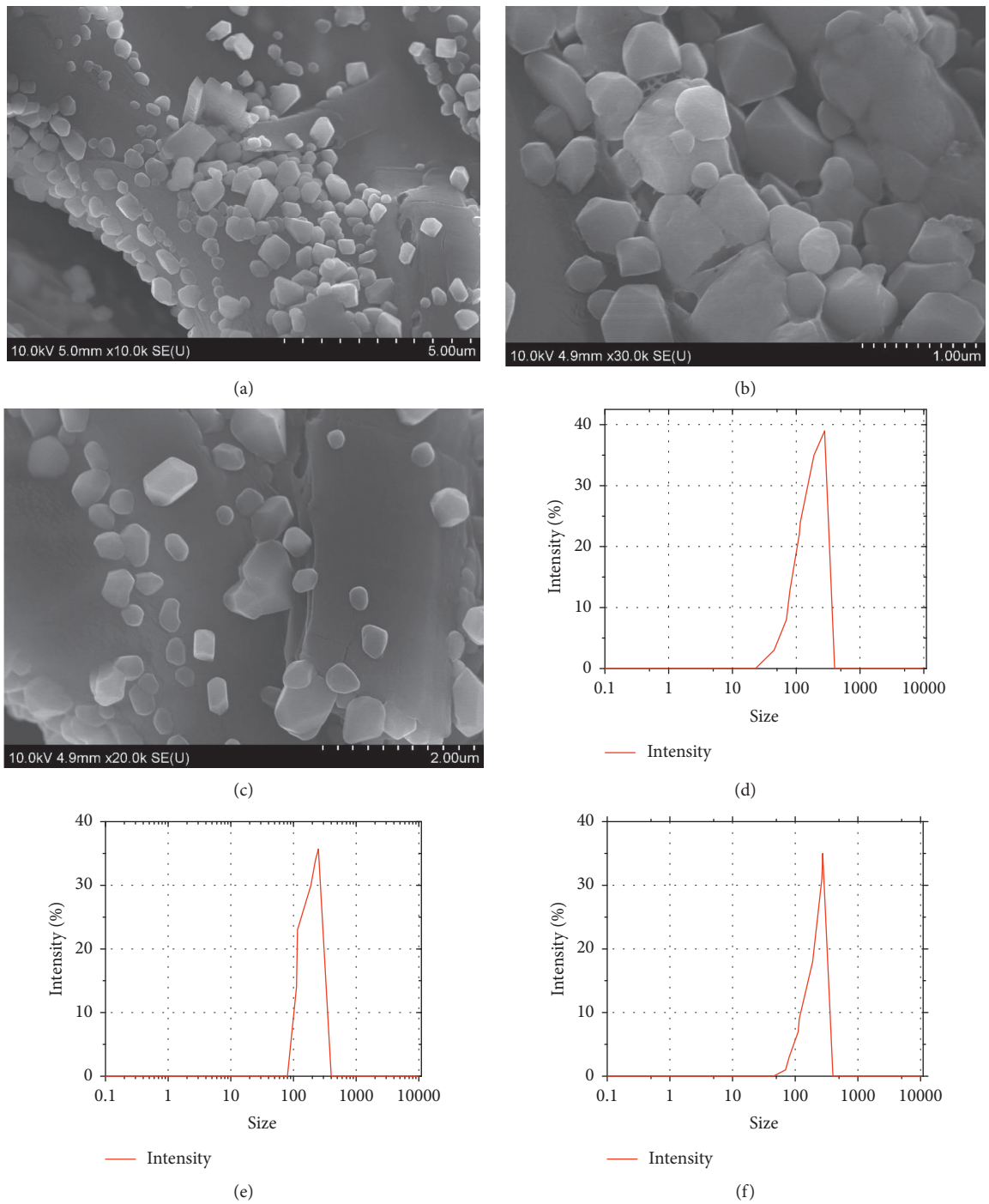

Figure 2: Size distribution images of PLGA NPs obtained through Malvern Zetasizer and Hitachi S-4800 scanning electron microscopy synthesized by a nanoprecipitation technique. SEM images of (a) *C. citrinus*, (b) berberine, and (c) *C. citrinus* + berberine nanoparticles. Histograms of (d) *C. citrinus*, (e) berberine, and (f) *C. citrinus* + berberine nanoparticles.

material in water determines the percentage of entrapment into PLGA nanoparticles as extracts having more solubility will move out into the aqueous phase from the organic phase during synthesis of PLGA nanoparticles, resulting in a decrease in percentage entrapment efficiency as described by Pereira et al. [37]. The entrapment of berberine and *C. citrinus* extracts into PLGA is consistent with the encapsulation of the hydrophobic extract of guabiroba fruit which was reported at between about 84 and 99% encapsulation into PLGA nanoparticles [38].

3.5. Percent Release of Total Phenolics from Loaded Nanoparticles. Release of total phenolics from PLGA nanoparticles was carried out at pH 7.4 and 37°C, resembling conditions of the gastric juices in the human body [39]. Data

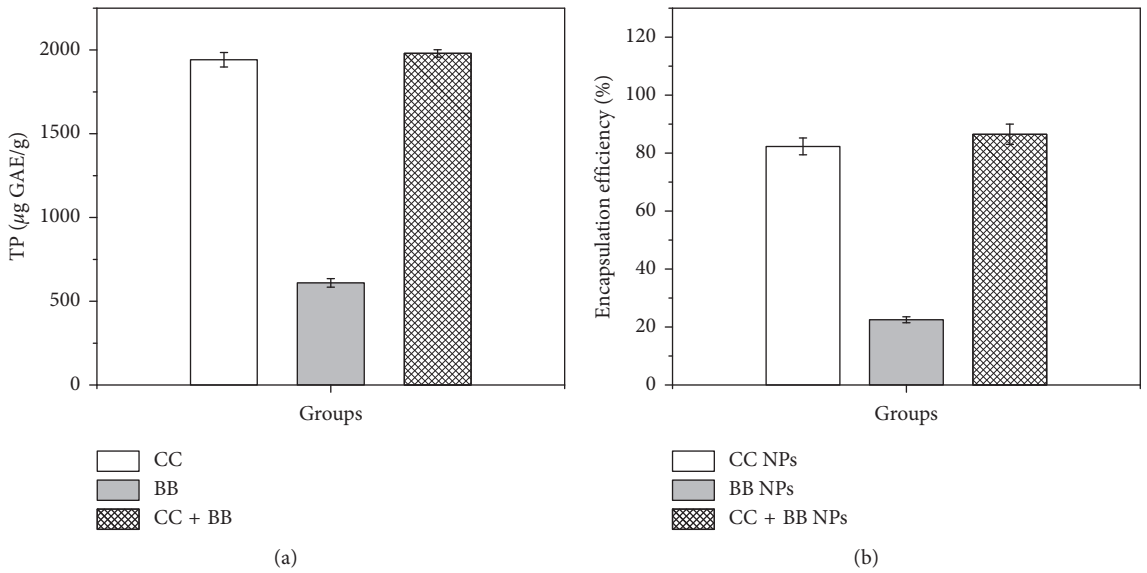

Figure 3: (a) Total phenolic content of *C. citrinus* (CC), berberine (BB), and *C. citrinus* extract + berberine (CC + BB) encapsulated in PLGA nanoparticles. (b) Percent encapsulation efficiency (η%) of PLGA nanoparticles loaded with *C. citrinus*, berberine, and *C. citrinus* + berberine. Data are represented as mean ± SD ($n = 3$).

in Figure 4(a) show linear curve responses with fast releases of berberine, *C. citrinus* extract, and mixture of *C. citrinus* and berberine from PLGA nanoparticles within the first 24 h, reaching their peaks at 7 days and then levelling off to negligible levels thereafter. The immediate fast release of berberine and *C. citrinus* extract could be attributed to rapid desorption and subsequent release from the outer boundary of PLGA nanoparticles as previously described during chlorambucil encapsulation [40]. All three treatments exhibited a similar pattern of release with encapsulated berberine being significantly higher than the other two treatments during the first 7 days, while combination of berberine and *C. citrinus* release was significantly lower than the other two treatments during the remainder of the study period (Figure 4(a)). The slow and sustained release of total phenolics was due to difference in their concentration within PLGA nanoparticles and to the phosphate saline buffer. The change in sink conditions significantly affected the release profile of total phenolics *in vivo* where it was enhanced due to rapid degradation of PLGA nanoparticles [39].

3.6. Stability of PLGA Nanoformulations during Storage. Total phenolic release profiles in Figure 4(b) show that the prepared nanoformulations were very stable. Leakages from prepared PLGA nanoparticles containing berberine, mixture of berberine and *C. citrinus* phenolics, and *C. citrinus* phenolics alone showed less than 2% reduction over a 28-day storage period at 4°C (Figure 4(b)). The stability of these nanoformulations may be due to the presence of a PVA coating barrier, allowing for better formulation retention. The low percent leakage of phenolics over the 28-day storage suggests that the prepared PLGA nanoformulations are a very stable carrier making them suitable for drug delivery applications. The stability of the encapsulated liposomal formulations reported in this study was very similar to that observed using curcumin under similar storage conditions [41], which indicates that storage of PLGA nanoparticles has no effect on their retention of polyphenols, and thus, they can be used as stable carrier systems for berberine and *C. citrinus* extract.

3.7. Cytotoxic Activity of Nanoformulations against Breast Cancer Cell Lines. The nanoformulation technique presented in this study is a useful tool for loading of complex extracts into polymeric nanoparticles to evaluate their anticancer potential for *in vitro* trials. Recent studies have used nanoencapsulation of a single or multiple bioactive compounds to evaluate their anticancer effectiveness against several types of cancers *in vitro* [42, 43]. Sampath et al. [44] examined the bioactivity of *C. citrinus* extracts against skin carcinoma A431 and human keratinocyte HaCaT cell lines. They attributed the apoptotic effect of *C. citrinus* extracts to their content of the monoterpenoid 1,8-cineole, which they reported to enhance the expression of p53, a tumor-suppressing protein. Similarly, essential oils extracted from *C. citrinus* leaves and flowers exhibited highly significant growth inhibition of A549 cells and C-6 cancer cells (61% and 69%, respectively) but had no effect on growth of normal cells.

Data in Figures 5(a)–5(d) of nanoformulated and nonformulated treatments at different concentrations show different growth inhibition efficacies against the tested breast cancer cell lines. Figure 5(b) shows that, at 0.1 mg/ml, a mixture of nanoformulated berberine and *C. citrinus* induced the most potent inhibition (33%) of MDA-MB 231 cells. In contrast, the same concentration in nonformulated form induced only about 12% growth inhibitory, suggesting that encapsulation resulted in nearly a 3-fold increase in effectiveness of the

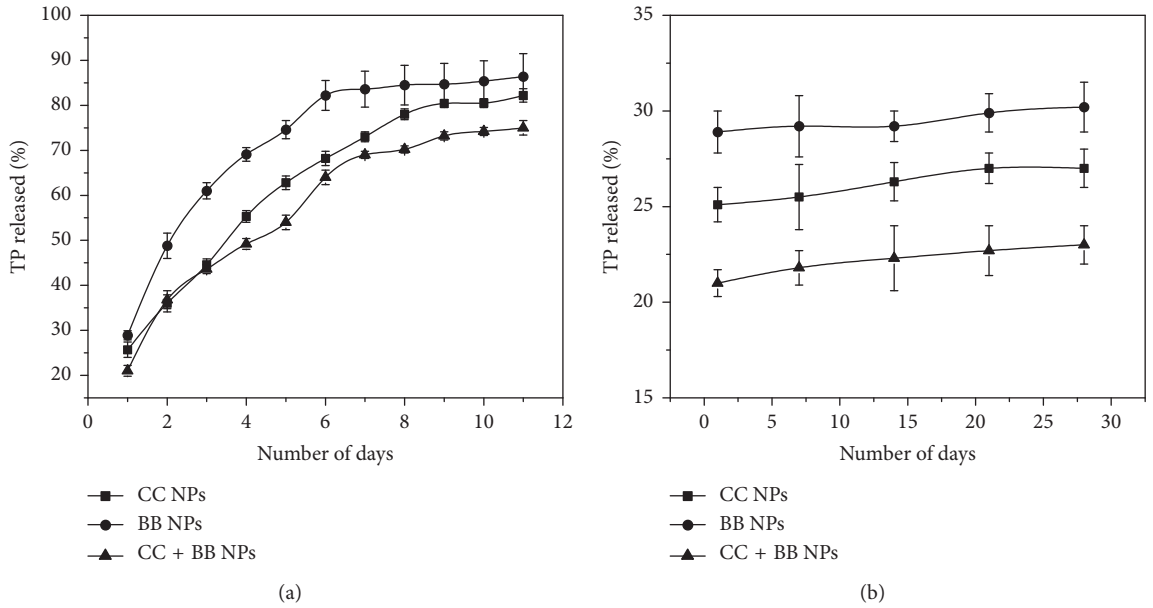

Figure 4: (a) Percentage release of TP of CC-, BB-, and CC + BB-loaded PLGA NPs over a period of 11 days under conditions resembling the human gastrointestinal tracts. (b) Percent release of TP from BB PLGA NPs (closed circle), CC PLGA NPs (closed square), and CC + BB PLGA NPs (closed triangle) over a period of 28 days in cold storage at 4°C. Data represent mean ± SD ($n = 3$).

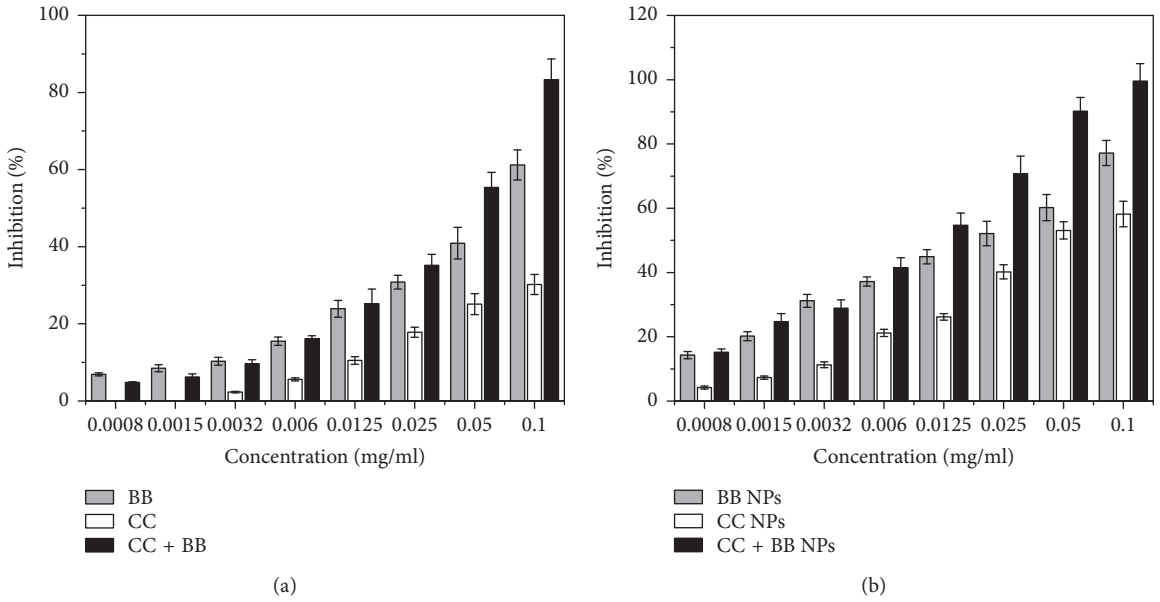

Figure 5: Continued.

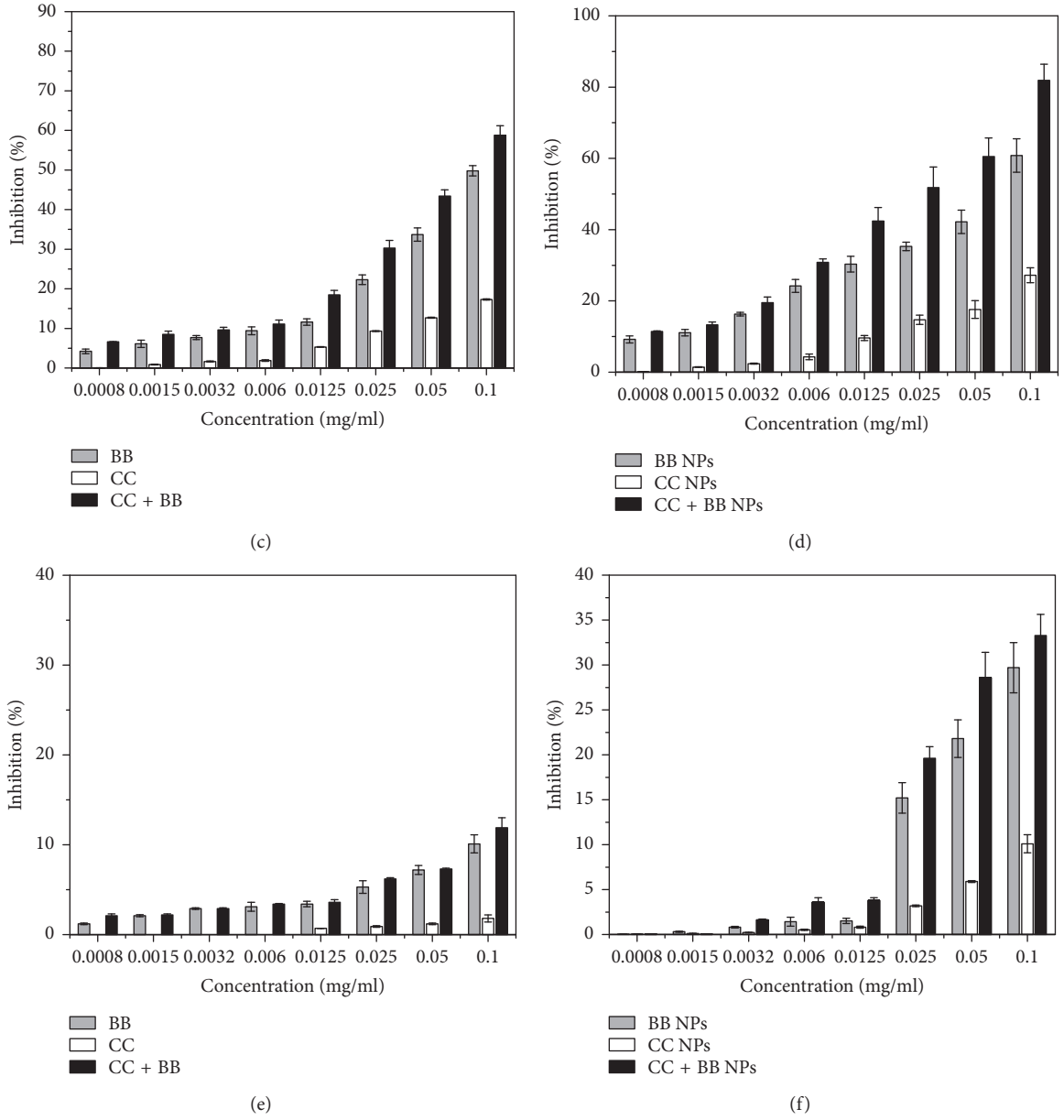

Figure 5: (a) Cytotoxic effects of BB, CC, and CC + BB extracts against MDA-MB 231 breast cancer cell lines. (b) Cytotoxic activity of BB PLGA NPs, CC PLGA NPs, and CC + BB PLGA NPs against MDA-MB 231 breast cancer cell lines. (c) Cytotoxic effects of BB, CC, and CC + BB extracts against MCF-10A breast cancer cells. (d) Extract-loaded PLGA nanoparticles against MCF-10A breast cancer cells. (e) Cytotoxic effects of BB, CC, and CC + BB extracts against MCF-7 cancer cells. (f) Cytotoxic effects of BB-, CC-, and CC + BB loaded PLGA NPs against MCF-7 breast cancer cells (mean ± SD, $n = 3$).

nanoformulated treatments (Figure 5(b)). The cytotoxicity of free and encapsulated *C. citrinus* forms reported in this study is in agreement with that reported in other studies [45], showing a marked difference in their anticancer potential. Contrary to an earlier study [46], we have observed a marked increase in effectiveness of the treatments against MDA-MB 231 breast cancer when their concentrations were increased (Figures 5(a) and 5(b)). The effect of the treatments against MCF-10A, a more invasive cancer cell line, is shown in Figures 5(c) and 5(d). The patterns of MCF-10A response to the treatments' concentrations were similar to those for MDA-MB 231; however, at 0.1 mg/ml nonformulated and nanoformulated doses, the rate of inhibitions was about 2.5- and 5-fold higher, respectively, than those for MD-MB 231. The highest anticancer activities of the treatments were observed against MCF-7 cell lines (Figures 5(e) and 5(f)). More than 83% and nearly 100% cell growth inhibitions were observed in the nonformulated and nanoformulated treatments at 0.1 mg/ml, respectively (Figures 5(d) and 5(f)).

Treatment of cancer cells with *C. citrinus* extracts induced higher growth inhibition compared to berberine in both nonformulated and nanoformulated forms. However, cancer cell growth inhibition was significantly higher when a mixture of both materials was used in either nanoformulated

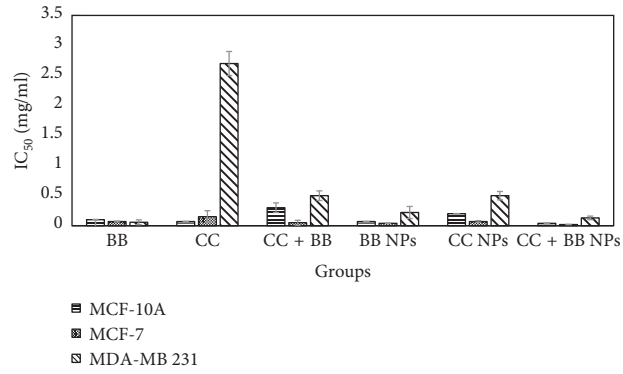

Figure 6: Values of IC_{50} of MDA-MB 231, MCF-7, and MCF-10A breast cancer cell lines treated with nanoformulated and nonformulated BB, CC, and CC + BB extracts (mean ± SD, $n = 3$).

or nonformulated forms. This combination was highly effective against MCF-7, intermediately effective against MCF-10A, and slightly effective against MDA-MB 231 cancer cell lines. Synergistic effects were observed between the tea polyphenols theaflavin-3-3′-digallate, ascorbic acid, and (−)-epigallocatechin-3-gallate [47]. In contrast, PLGA nanoparticles loaded with berberine alone were minimally effective. Results of this study support the previous study using MCF-7 breast cancer cell lines [24]. Thus, PLGA nanoformulations loaded with complex polyphenol matrices could provide effective treatment to reduce and/or inhibit the proliferation of less invasive breast cancer cells. These results highlight two important observations: (a) significant inhibition of cancer cell growth when extracts were encapsulated into PLGA nanoparticles and (b) higher breast cancer cell inhibition when cells were treated with a combination of berberine plus *C. citrinus*.

Another important observation was the correlation between the degree of invasiveness of a cancer cell line and the degree of its growth inhibition by the treatment. For example, *C. citrinus* and berberine PLGA loaded nanoparticle treatment had minimal effects against the growth of the extremely metastatic MDA-MB 231 cancer cell line (Figure 6), while very effective against the other two less invasive lines. Our data confirm earlier observations by Kim et al. [48] using berberine against the same cell lines. In another study [49], treatment of MDA-MB 231 cancer cell lines with magnolol, a natural compound, significantly inhibited cell growth by downregulating the matrix of metalloproteinase-9 (MMP-9), an enzyme required for invasion and tumor metastasis. In our study, nanoformulated *C. citrinus* extract plus berberine showed a more pronounced growth inhibition of MCF-7 cells, which suggests that the degree of invasiveness/proliferation is related to growth inhibition. A similar pattern of growth inhibition was demonstrated against MDA-MB 231 and T47D cells using metformin in nanoencapsulated and free forms [50].

4. Conclusion

Nanoencapsulation of *C. citrinus* into PLGA nanoparticles enhanced its bioactivity against MDA-MB 231, MCF-10A, and MCF-7 breast cancer cell lines by about 2-fold. In addition, the combination of *C. citrinus* extracts with berberine further increased their cytotoxic potential in free and encapsulated forms. Data suggest that nanoencapsulation of anticancer compounds either alone or in combination with other bioactive compounds is a valuable tool for cancer chemotherapy.

Data Availability

Data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest. Additionally, University of Illinois is an Affirmative Action/Equal Opportunity Employer dedicated to building a community of excellence, equity, and diversity.

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Review Article

Functional Properties of Polyphenols in Grains and Effects of Physicochemical Processing on Polyphenols

Shuangqi Tian, Yue Sun, Zhicheng Chen, Yingqi Yang, and Yanbo Wang

College of Food Science and Engineering, Henan University of Technology, Zhengzhou 450001, China

Correspondence should be addressed to Zhicheng Chen; chen_1958@163.com

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Phenolic compounds are important products of secondary metabolism in plants. They cannot be synthesized in the human body and are mainly taken from food. Cereals, especially whole grains, are important sources of dietary polyphenols. Compared with vegetables and fruits, the content and biological activities of polyphenols in cereals have long been underestimated. Polyphenols in whole grains are non-nutritive compounds, which are distributed in all structural areas of cereal substances, mainly phenolic acids, flavonoids, and lignans. In recent years, the health effects of whole grains are closely related to their phenolic compounds and their antioxidant activities. Now, different physicochemical processing treatments and their effects have been summarized in order to provide the basis for promoting the development and utilization of food. The various functions of whole grains are closely related to the antioxidant effect of polyphenols. As the basic research on evaluating the antioxidant effect of active substances, in vitro antioxidant tests are faster and more convenient.

1. Introduction

Natural polyphenols are mostly found in plants which are a kind of compounds with phenolic hydroxyl structure widely existing in nature [1]. Polyphenols mainly include flavonoid, phenolic acid, tannin, and other substances and have strong antioxidation performance, which eliminates free radicals generated by the human body, and the effect of preventing cardiocerebral syndrome and deferring decrepitude. Polyphenols not only have a strong antioxidation characteristic [2] but also have anticancer [3], bacteriostatic [4], liver-protecting [5], anti-infection [6], cholesterol lowering [7], and immunity enhancement [8] properties, and they also prevent various biological activities such as type 2 diabetes [9, 10]. As far as their formation mechanism is concerned, most polyphenols are the secondary metabolites of the phenylpropanoid biosynthesis pathway, which are also known as phenyl propane compounds [11]. Polyphenols and polyphenol-enriched by-products have been widely used in bakery foods because of their nutraceutical properties. While their use in pharmaceutical and cosmetic industries is largely documented, several environmental conditions (e.g., light, temperature, or oxygen) may affect the physicochemical stability of polyphenols. To overcome these limitations, the loading of polyphenols into nanoparticles has been proposed [12].

Although polyphenols cannot provide nutrition for growth and development, they can play a role as defense compounds, such as plant antitoxin as pollinators, prevention of pathogenic bacteria and parasitic bacteria, prevention of ultraviolet rays, and giving color to plants. The existing whole grains mainly include rice, wheat, corn, sorghum, millet, oat, barley, and buck. The process of converting the whole ingredient into one product is referred to as whole grain utilization and the processed food product is referred to as whole grain food [13]. Common whole grain foods are brown rice flour, oatmeal, whole meal flour, whole meal bread, whole meal noodle, cornflakes, and popcorn. The phenolic content of different grains is also different, of which corn has the highest polyphenol content (15.55 μg/g), followed by wheat (7.99 μg/g), oats (6.53 μg/g), and rice (5.56 μg/g) [14]. As shown in Figure 1, whole grains contain more phenols than the processed grains. For example, brown rice contains more ferulic acid than polished rice because

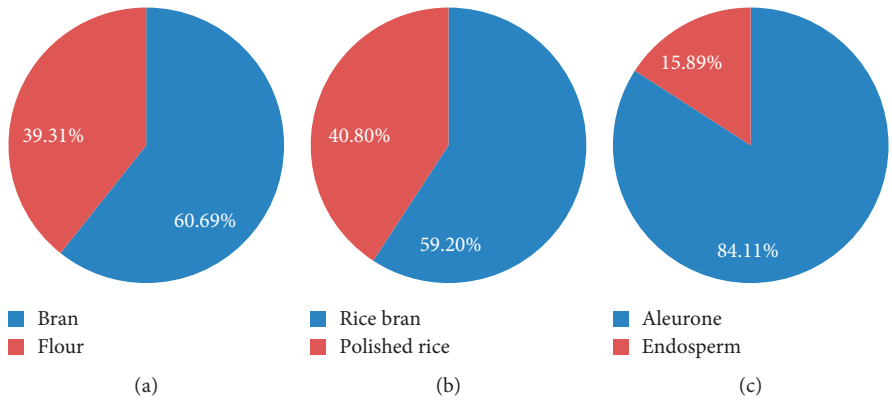

Figure 1: Distribution of phenolic compounds in different tissue parts of whole grains. (a) Wheat. (b) Brown Rice. (c) Corn.

phenols are mainly found on the cortical layer of rice grains. Smaller grains contain more ferulic acid than larger grains, such as rye, oats, and millet than rice, because ferulic acid is associated with total fiber and insoluble dietary fiber content, and the higher the fiber content, the higher the ferulic acid content [15]. After germination, the content of polyphenols is much higher than that before germination [16]. The content of polyphenols in grains is equal to that of fruits and vegetables, and even some highly active phenolic compounds only exist in whole grains. Whole grain polyphenols are a general term for phenolic substances distributed in the structure of grains.

2. Main Constituents of Grains

Polyphenols in whole grains are non-nutritive compounds, which are distributed in all structural areas of cereal substances, mainly phenolic acids, flavonoids, and lignans. Phenolic acid is mainly found on the cortical layer of grains, in which ferulic acid is higher, followed by oxalic acid, p-coumaric acid, and caffeic acid. It has a strong antioxidant effect and can prevent the tissues inside the cortex from oxidation [17, 18]. It also has antimutagenic effects on toxic substances such as nitrosamine and mycotoxins in the environment. Flavonoids are widely distributed in plants, mainly in the cortex and green leaves of plants. Lignans are the primitive substances that make up the cell wall component of lignins in cereal cells [19]. Grain food is the most important source of lignins in human food. The grain content is 2–7 mg/kg, lower than that in flaxseeds, but much higher than that in vegetables. It not only affects the metabolism and biological activity of endogenous hormones, but also affects the synthesis of enzymes and proteins in cells as well as cell proliferation and differentiation. As shown in Table 1 and Figure 2, polyphenols in cereals exist in free form, soluble binding form, or insoluble binding form. The vast majority of them exist in the binding form. Free-form polyphenols mainly include ferulic acid, parabiosanoic acid, protocatechuic acid, gallic acid, coffee acid, and erucic acid can also be detected. The polyphenols in the binding form are made up of ferulic acid, vanillic acid, coffee acid, and syringic acid [36–38].

3. Whole Grain Phenolics Antioxidant Activity

Modern epidemiological studies show that whole grain foods can prevent chronic diseases such as type 2 diabetes, coronary heart disease, and bowel cancer [39–44]. Although the mechanisms underlying these effects are not fully understood, they are likely to be closely related to the antioxidant activity of whole grains [45, 46]. The bioactivity of whole grains is closely related to the natural antioxidants, and polyphenol is the important one.

Polyphenols are very important secondary metabolites in plants. They are synthesized in plants mainly through shikimic acid and malonic acid [47]. In humans, polyphenols in the diet can also enhance the immune defense ability of the body, reduce the incidence of chronic diseases, and have significant effects such as antiallergy, antiarterial atherosclerosis, anti-inflammation, antioxidation, antibacterial, antithrombotic, and protecting heart and blood vessels [48]. The health benefits of polyphenols on the human body are mainly due to their oxidation resistance. Polyphenols in grains have a stronger antioxidant effect in the body through the synergistic effect of multiple bioactive compounds than the single active ingredient and can eliminate too many oxidation free radicals in the body as antioxidants or after the intestinal digestion. The free-form polyphenols are absorbed into the mouth and the protein in the mouth which is rich in protic acid, which is absorbed by the body in the stomach or in the small intestine. Free polyphenols are more easily digested in the upper digestive tract than in the combined state [49]. Due to the combination of the combined polyphenols in the more difficult digested cell walls, the digestion and absorption process mainly occurs in the large intestine. Binding polyphenols in the large intestine are released from the cell wall in the form of glycosidic ligand through the action of microorganisms or related enzymes and then reformed into glucoside, which is used by the human body through the glucose transporter in the cell. Furthermore, the intestinal microbial environment was effectively improved by the interaction of binding polyphenols with microorganisms in the large intestine, and the risk of colon cancer was significantly reduced [14].

Table 1: Composition and existing forms of phenolic acids in whole grains.

Cereal species	Free phenolic acid	Combined with phenolic acid	References
Wheat	Gallic acid, protocatechuic acid, chlorogenic acid, coffee acid, syringic acid, ferulic acid, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid	Gallic acid, syringic acid, ferulic acid, vanillic acid, p-coumaric acid, isoferulic acid	[20–23]
Brown rice	Ferulic acid, coffee acid, syringic acid, protocatechuic acid, chlorogenic acid, coumaric acid, salicylic acid, o-coumaric acid, syringol, 2-4-dihydroxybenzaldehyde, sinapic acid, 2-hydroxycinnamic acid	Ferulic acid, chlorogenic acid, coffee acid, syringic acid, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, syringol, protocatechuic acid, o-coumaric acid, 2-4-dihydroxybenzaldehyde, sinapic acid,	[24–28]
Corn	Ferulic acid, coffee acid, gallic acid, p-coumaric acid, o-coumaric acid,	Ferulic acid, coffee acid, p-coumaric acid, p-hydroxybenzoic acid, syringic acid, protocatechuic acid,	[29–32]
Oats	Chlorogenic acid, coffee acid, syringic acid, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, 2-4-dihydroxybenzaldehyde, sinapic acid, chlorogenic, ferulic acid	Coffee acid, syringic acid, vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, 2-4-dihydroxybenzaldehyde, sinapic acid, chlorogenic, ferulic acid	[33–35]

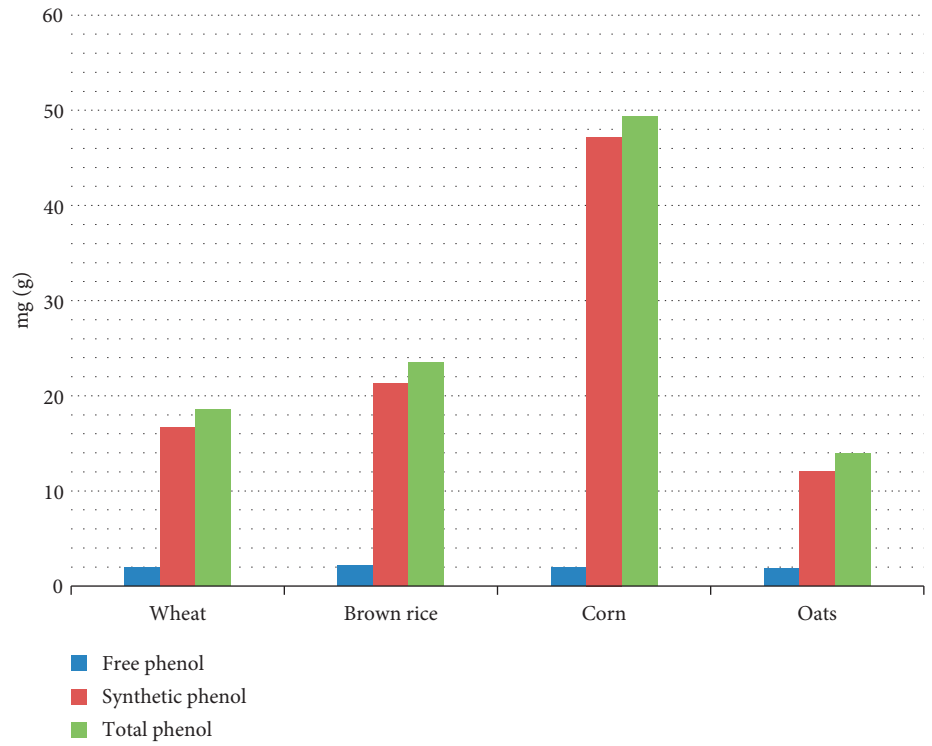

Figure 2: Total phenolic content of whole grains (mg/g) [36, 37].

According to the antioxidant mechanism of polyphenols, free radical-scavenging and chelating transition metal ions were used to determine the antioxidant capacity of grain in vitro [50, 51]. The method of in vitro antioxidant experiment is simple, and as a basic research to evaluate the antioxidant activity of active substances, it is faster and more convenient. Previous studies showed the correlation between the content of combined total phenol and the content of total phenolic acid in wheat, corn, oat, and other grains and DPPH free radical-scavenging capacity and found that the former two have significant correlation with the latter and the antioxidant can be used as the evaluation index of functional food. The antioxidative activity of the whole grain is the same as that of the polyphenol, and it is in close relationship with the variety, tissue, form, and content [52]. It is reported that the antioxidant ABTS values of 24 kinds of whole grains was analysed and found that sorghum had higher antioxidant activity compared with brown rice, wheat, corn, and other whole grains [53]. Choi compared the antioxidant DPPH values of 9 whole grains and reached a similar conclusion [54].

At present, the rapid screening method is the most used to evaluate the antioxidant activity of the phenols in cereals (DPPH, ORAC, HO, and ABTS).The advantage is simple operation and can rapid screening of whole grain varieties that are rich in polyphenols and areas of polyphenol concentration. However, the results of these in vitro experiments cannot reflect the real antioxidant effect of

polyphenols in vivo, so it is necessary to introduce the method of in vivo experiments into the evaluation of the antioxidant activity of phenols in whole grains.

4. Application of Grain Polyphenols

At present, the extraction and product development of polyphenols in grains mainly focus on the research of the outer seed coat of grains, but the research on the extraction of polyphenols in grains and their antioxidant activity is less. With the development of social economy and the progress of grain processing technology, consumers are chasing taste and delicacy. As grain processing becomes more and more refined, it is required to be able to control it more skillfully under various conditions.

4.1. Physical Treatment

4.1.1. Effects of Grinding on Polyphenols in Grain. Milling is the main process of grain physical processing, which is divided into dry grinding and wet grinding [55]. Dry grinding is the separation of outer fibers and germs, while wet grinding is considered the best way to produce cereal endosperm products. Dry grinding consists of two parts: grinding and screening. Screening mainly refers to removing impurities, pest-infested grain, bacterial discolored grain, weeds, and foreign substances [56]. Tylewicz found that overmilling destroys the nutrients in the grains [57]. Compared with dry grinding, wet grinding is the process of soaking grains in water and then separating starch, protein, and fiber components [58]. However, due to the high content of pentosan and poor formation capacity of gluten, it is difficult to realize the industrial wet milling process. The bran layer and protein cortex are difficult to completely separate, and the high content of glucan leads to increased viscosity, which makes it difficult to pass screening and centrifugation [59].

Through milling, the taste and flavor of grains can be greatly improved, and the digestibility can also be improved to a certain extent. However, the nutritional value of grains is greatly reduced. This is because the bran and germ, which are rich in vitamins, minerals, and biologically active ingredients, are removed by milling. As the antioxidant components of whole grains are mainly located in the cortex, aleurone layer, and germ part, the milling process inevitably has adverse effects on the polyphenols and their antioxidant activities. With the improvement of processing accuracy, the total phenol content of both japonica brown rice and indica brown rice decreased significantly, and the free phenol content of both types of brown rice showed a downward trend, and the reduction of combined phenol content was more significant than that of free phenol. The free and combined phenolic acid composition of brown rice with different milling degrees is basically the same, while the phenolic acid content is significantly different. Under the influence of milling treatment, the antioxidant activity of brown rice decreased significantly. After 30 s milling (up to the national first-grade rice quality standard), the total phenol and cellular antioxidant activity (CAA) value of brown rice decreased by 55.50% and 92.85%, respectively [60]. It is mainly caused by the removal of the skin rich in polyphenols and the thermal effect caused by grinding, which leads to the oxidation of polyphenols. The results showed that the polyphenols content of sorghum and millet and their antioxidant effects were similar. In the process of milling, the flavor and nutritive value should be taken into consideration [61, 62].

4.1.2. Effect of Heat Treatment on Polyphenols in Grains. Grains are digested and absorbed by the body before they are assimilated. Grains are usually cured by heat treatment type. Common heat treatment methods include baking, boiling, steaming, etc. The effects of different heat treatment on polyphenols in different grains were studied [63, 64]. Therefore, it is important to clarify the composition, content, and properties of polyphenols after different processing of grains to study the quality of polyphenols assimilated by the human body and its value in the human body.

The effects of the curing process on nutrition and processing quality of oat traditional food were studied. It was found that the physical and chemical indexes of protein, starch, fat, and wech-glucan content of oat were significantly improved after being processed, and the processing characteristics and flavor were greatly affected by them. The effects of evaporation treatment on the properties of oat starch were most significant. However, compared with other treatments, the effect of scalding on oat was minimal [65]. At present, in China, there are few studies on the change in polyphenol characteristics after heat treatment [66].

The effects of various commercial hydrothermal processes (steaming, autoclaving, and drum drying) on levels of selected oat antioxidants were investigated. Steaming and flaking of dehulled oat groats resulted in moderate losses of tocotrienols, caffeic acid, and avenanthramide Bp (N-(4′-hydroxy)-(E)-cinnamoyl-5-hydroxy-anthranilic acid), while ferulic acid and vanillin increased. The tocopherols and the avenanthramides Bc (N-(3′,4′-dihydroxy-(E)-cinnamoyl-5-hydroxy-anthranilic acid) and Bf (N-(4′-hydroxy-3′-methoxy)-(E)-cinnamoyl-5-hydroxy-anthranilic acid) were not affected by steaming [67]. The content of free polyphenols in wheat and sorghum was significantly increased after baking, while the content of total flavonoids was significantly decreased [68]. What is more, the effects of different heat treatments on polyphenols were also different. Compared with baking treatment, free polyphenol content in wheat and sorghum significantly decreased after high-pressure and atmospheric-pressure cooking. In addition, variety is also an important factor affecting the content of polyphenols after heat treatment or high-pressure steam treatment of purple waxy corn [69]. However, Harakotr et al. [70] carried out high-pressure steaming on sweet corn and found that free ferulic acid and total phenol content were significantly increased, and the content showed an upward trend with the extension of heat treatment time and the increase of temperature, but the content of combined ferulic acid and total phenol decreased, the total antioxidant capacity was consistent with the variation trend of total phenol

content. It is found that the research about the influence of traditional heat treatment on the composition and properties of free and combined polyphenols in oat is not comprehensive [71].

4.1.3. Effects of Extrusion on Polyphenols in Grain. Extrusion is a new technology which combines heating, cooking, and extrusion molding. Short-term high temperature and high pressure can change the texture of food, change the composition of food, and even promote the interaction effect between food components. Similarly, extrusion treatment may also affect the polyphenols content and their antioxidant activity of whole grains [18]. The research in China is limited to the taste of processed grains and the determination of the changes of protein, starch, oil, and other components [72]. It was reported that the content of 5 kinds of sorghum total phenol (free phenol) decreased by 33.33%, 56.60%, 69.54%, 70.09%, and 78.37% and 81.82%, 76.92%, 86.63%, 84.90%, and 87.59%, respectively, after the extrusion treatment through a friction-type Maddox single screw extruder [73].

The extrusion treatment destroys the cell wall, promotes the transformation of binding polyphenols to free phenol, and also facilitates the extraction of free phenol. Compression heat inevitably leads to partial degradation of polyphenols and a certain degree of polymerization and changes in molecular structure. In addition, during extrusion, if the water content of the feed is too high (more than 18%), the polymerization of polyphenols will be promoted, and the extraction rate and antioxidant activity will be reduced. If the water content of the feed is too low (less than 15%), the depolymerization of condensed tannin will be accelerated and converted into oligomers with low molecular weight which can be extracted more easily. Extrusion treatment will produce or increase or decrease the content of total phenol and its monomer phenol in different parts of grain and then affect its antioxidant activity.

4.2. Chemical Treatment

4.2.1. Effects of Germination on Polyphenols in Grains. Germination is a complex physiological process. In the process of grain germination, a large number of endogenous enzymes are activated and released, which leads to the decomposition and recombination of the internal material components of grains, which may have a certain impact on the polyphenols of whole grains and their antioxidant activities. During germination, the biological, chemical, nutritional, and sensory properties of grains change significantly. Moreover, the relevant enzymes in the grains were activated, and the nutritive starch in the endosperm was decomposed into reducing sugars, the protein was degraded, and the content of soluble protein was increased. The embryo develops into roots and buds. Studies have shown that the germination process can significantly increase the content of polyphenols in grains such as millet and brown rice [74, 75].

The effects of germination on the nutritional quality of grains include high protein, low unsaturated fatty acids, low carbohydrates, mineral content, and vitamins. After 47 hours of germination treatment, the content of total phenol increased by 38.71% [74]. Similar reports have been reported [76, 77]. After the brown rice was germinated for 47 hours, the content of free phenol increased by 76.67% and binding phenol increased by 44.64%, respectively [78, 79]. The content of free phenol was obviously higher than that of the combined phenol. The composition of phenolic acid in free state and combined state of brown rice with different germination time is basically the same. However, the phenolic acid content is significantly different, which may be caused by the improved extraction rate of phenolic acid in germination or the concentration effect caused by the resynthesis and polymerization of tannin and hydrolysis of pentanine [80, 81]. The reduction of total phenol content in grains may be caused by the dissolution of some polyphenols from grains into the external water environment during the germination process or by the oxidation and decomposition of polyphenols by activated polyphenols oxidase and esterase.

4.2.2. Effects of Fermentation Treatment on Polyphenols in Grains. In the process of grain fermentation, many small molecules with physiological activity are produced while large molecules such as carbohydrates and proteins are consumed. Zhai et al. [82] used seven grains such as wheat, corn, brown rice, millet rice, oat, and sorghum in solid fermentation of agaricus matsutake and found that the content of total phenol (free phenol) of all grains except sorghum was significantly increased after the solid fermentation of agaricus matsutake. This may be related to the strong metabolism of agaricus matsutake to produce phenolic compounds. Except that, the content of total phenol (free phenol) in sorghum was negatively correlated with the fermentation time, the content of total phenol (free phenol) in other grains was positively correlated with the fermentation time, and the extension of fermentation time was beneficial to the improvement of total phenol (free phenol) content. Solid-state fermentation (5 d) can significantly increase the content of total phenol (free phenol) and antioxidant activity (ABTS) of corn, with an increase rate of 20.05% and 36.73% [83], respectively. Liquid fermentation also had significant effects on the content of total phenol (free phenol) and antioxidant activity (ABTS) of 5 kinds of sorghum (slurry and porridge) [71].

5. Conclusions

(1) Polyphenols exist in large quantities in grains and are an indispensable source of nutrition for the human body. When the research and application of polyphenols in grains are not common at the present stage, strengthening the research in this aspect is the most important task.

(2) Although the polyphenol contents in grains are high, the complex composition of polyphenols is mostly

located on the surface and outer shell of grains. It is of great help to the research of different kinds of grains and polyphenols to overcome some diseases.

(3) The various functions of whole grain foods are closely related to the antioxidant effect of polyphenols. As the basic research on evaluating the antioxidant effect of active substances, in vitro antioxidant tests are faster and more convenient.

(4) The effects of physical and chemical treatment on polyphenols in grains during food processing need to be further studied, and more effective treatment methods must be investigated to improve taste and flavor while preserving nutritional value.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Centrifugation, Storage, and Filtration of Olive Oil in an Oil Mill: Effect on the Quality and Content of Minority Compounds

Alfonso M. Vidal, Sonia Alcalá, Antonia de Torres, Manuel Moya, and Francisco Espínola

Centre for Advanced Studies in Energy and Environment (CEAEMA), Agrifood Campus of International Excellence (ceiA3), Dept. Chemical, Environmental and Materials Engineering, University of Jaén, Paraje Las Lagunillas, Edif. B-3, 23071 Jaén, Spain

Correspondence should be addressed to Alfonso M. Vidal; amvidal@ujaen.es

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Centrifugation, storage, and filtration of olive oil were evaluated in an oil mill to determine their effect on the final quality of virgin olive oil. The main functions of these processes are to clarify the olive oil by removing water, solids, and other possible suspended particles. Although some changes were detected in the oil quality parameters after these processes, all the samples were extra virgin olive oil. The phenolic and volatile compound content of the olive oil was influenced by vertical centrifugation processing. Significantly, vertical centrifugation led to a 53% reduction in ethanol content. Oil storage before filtration resulted in a significant increase of around 30% in the peroxide index, while the antioxidant capacity decreased by 78%. Comparison of the results for filtered and unfiltered oil samples revealed that the most significant change was the reduction in the photosynthetic pigment content, with a decrease of around 50% in chlorophyll. Due of all this, the conditions applied in vertical centrifugation and the time of storage of the olive oils should be further controlled, enabling cleaning and decantation but avoiding the reduction of the antioxidant capacity and the content of phenolics compounds.

1. Introduction

Virgin olive oil (VOO) is a fat known worldwide for its beneficial properties for human health. The consumption of olive oil in the Mediterranean diet is associated with low mortality from cardiovascular disease [1]. Several health benefits have been associated with certain antioxidant compounds such as phenols [2]. The health claims on "olive oil polyphenols" by the EEC [3] refer to the impact of bioactive phenolic compounds on the protection of blood lipids against oxidative stress [4]. High nutritional quality arises from large amounts of unsaturated fatty acids in the composition of oil, such as oleic acid and linolenic acid. The production of VOO is solely carried out by physical and mechanical extraction processes. Oil washing is a step of the process, which is performed in a vertical centrifuge (VC). After obtaining the oil, it is filtered to eliminate any solids in the suspension.

Washing represents an important source of oxidative reactions arising from the contact between water and oil [5]. The distribution of phenolic compounds in the water and oil phases depends on their solubility in the phases [6]; phenolic compounds may thus be found in the wastewater and pomace. Vertical centrifugation has a great effectiveness in clarifying the oil, although this process reduces the concentration of minor compounds in the extra virgin olive oil (EVOO) [7]. The maximum oxygenation levels have been detected after VC treatment. The oxidation of olive oil during its shelf-life is negatively affected by the concentration of dissolved oxygen [8].

Inert gases have been used for oil oxygenation prevention and found to significantly extend the oil shelf-life [9]. Other researchers have focused on the effect of the water employed in the VC and on the content of alkyl esters in olive oils [10], where the content of ethyl and methyl esters were found to decrease with the use of water in the VC.

According to Gila et al. [11], minimal water addition to the VC is the optimum option to improve the oil quality.

The content of certain compounds such as hydroxytyrosol, tyrosol, and the dialdehydic form of elenolic acid linked to hydroxytyrosol, underwent the most significant changes [12]. Other authors such as Masella et al. [13] have described slight variations in the concentrations of phenolic compounds while comparing the composition of olive oil before and after the centrifugation process. Generally, a decrease in the content of these compounds is observed [14], that is, by diffusion from the oil phase to the aqueous phase. Moreover, the temperature of the added water was also found to influence the extraction process [15]. Comparative trials have also been performed on oil samples filtered using a conventional filtration method instead of a VC [16].

The turbidity of oil is caused by particles from plant tissue in suspension and water droplets. Such solids, particles, and water can deteriorate the quality by promoting the oxidation and hydrolysis of olive oil [17]. The aim of filtration is eliminate these to increase oil shelf-life. Several changes in the oil composition can occur during filtration, such as changes in the phenol and volatile compound content or the color of the oil [18, 19]. Natural sedimentation is more favorable than filtration in delaying the oxidative deterioration of oil; nevertheless, filtration provides a more stable sensory profile than do sedimentation and decantation [20].

Regarding filtration, a laboratory-scale study has shown that similar amounts of phenolic compounds are present in filtered and unfiltered EVOO [21]. However, another study, this time at pilot plant scale using filtration systems with inert gas flow (argon and nitrogen) and polypropylene filter bags, showed that the content of most phenolic compounds seemed to increase after filtration [22]. Quantitative and qualitative changes, especially on minor components were detected, which affected the EVOO quality [17]. The volatile compound and sensory characteristics of EVOO can be influenced by oil filtration [23, 24].

The objective of this work was to determine the influence of oil centrifugation, storage, and subsequent filtration on the regulated quality parameters and the phenolic and volatile compound contents of olive oil produced in a mill.

2. Materials and Methods

2.1. Raw Material. Olive fruits (*Olea europaea* L.) cv. Picual were harvested from irrigated land during the 2016–2017 crop season in Mancha Real (Jaén, Spain) and processed after the harvest at a local olive oil mill. A lot of approximately 5000 kg of olives was used for the experimental trials. The maturity index (MI), or ripening degree, was obtained following the method described by Espínola et al. [25]. The Soxhlet method is used to analyse the oil content.

2.2. Olive Oil Mill. The oil mill where the centrifugation, storage and filtration trials were carried out is located in the "Cortijo Virgen de loss Milagros," Mancha Real (Spain), and has a plant for the extraction of EVOO. The experiments were performed with the mill working continuously. The VC (Pieralisi, Jesi, Italy) was operated at 6400 rpm. The optimum water addition content was determined by the millworkers to be 5%. Samples of oil, pomace, and paste were collected in triplicate at different times, at approximately 20 min intervals throughout the experiment. The extracted oil was stored in a stainless-steel tank for 25 days. Then, the oil was filtered through a layer of hydrophilic cellulose acetate. The filtration was carried out continuously with an industrial filter and three oil samples were collected, at both the filter inlet and outlet. All oil samples were stored in amber glass bottles, filled with nitrogen, and kept at −18°C until further analysis. The samples for the sensory analysis were sent to an external laboratory.

2.3. Analysis of Olive Oil Quality Parameters. The free acidity, peroxide index, and extinction coefficients K_{232} and K_{270} were determined according to the European Union standard method [26].

2.4. Analysis of Photosynthetic Pigments. The photosynthetic pigments composition was determined according to the method of Mínguez-Mosquera et al. [27]. The spectrophotometer used was a Shimadzu (model UV-1800). The carotenoids and the chlorophylls were measured at a wavelength of 470 nm and 670 nm, respectively. The pigment concentration of the olive oils was expressed as mg of pigment per kg of oil.

2.5. Analysis of Volatile Compounds. The volatile compounds were quantified by following the method previously described by Vidal et al. [28]. They were analyzed by headspace solid-phase microextraction (HS-SPME) and gas chromatography-flame ionization detection (GC-FID). The SPME fiber is formed of Carboxen/DVB/polydimethylsiloxane and had 2 cm length and 50/30 μm of film thickness. It was acquired from Supelco (Bellefonte, PA, USA). The fiber had been previously conditioned following the instructions of the manufacturer.

GC-FID analysis was carried out on a gas chromatograph, model 7890B (*Agilent Technologies*, CA, USA). The capillary column used to the separation was a DB-WAXetr (*Agilent Technologies*, USA), (30 m of length, 0.25 of mm internal diameter, and 0.25 of μm coating) formed by polyethylene glycol. The chromatographic peaks were quantified by the "Internal Standard" method. This method uses internal and external standards. A calibration curve was made with the relationship between the external and internal standard (4-methyl-2-pentanol). The purpose was to improve the quantification. The results are expressed as mg of compound per kg of olive oil.

2.6. Analysis of Phenolic Compounds. The phenolic compounds present in the VOO were determined according to the method of International Olive Council [29]. A liquid chromatograph (*Shimadzu Corp*., Kyoto, Japan) was used. The column C18 BDS Hypersil (*Thermo Scientific*, USA) was

employed in the chromatographic separation and its characteristics were 25 cm length, 5 μm of particle size, and 4.6 mm of internal diameter. The quantification was carried out through the addition of syringic acid and tyrosol, as internal and external standard, respectively. The analytical standards were used to identify the phenol compounds. The results are showed as mg of tyrosol per kg of oil.

2.7. Determination of the Antioxidant Potential. The antioxidant potential was determined according to the method described by Vidal et al. [28]. The free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to determine the antioxidant potential. The absorbance was measured at 515 nm of the sample and the DPPH solution. Methanol was used as solvent and as the control. The absorbance obtained was converted into the DPPH concentration by interpolation of the calibration curve of absorbance versus DPPH concentration. The percentage of inhibition of DPPH radical was calculated according to equation (1):

$$\mathrm{DPPH}_{\mathrm{inhibition}}\ (\%) = \left([\mathrm{DPPH}]_0 - \frac{[\mathrm{DPPH}]_{\mathrm{sample}}}{[\mathrm{DPPH}]_0} \right) \times 100, \quad (1)$$

where $[\mathrm{DPPH}]_0$ and $[\mathrm{DPPH}]_{\mathrm{sample}}$ are the concentration of the control and sample, respectively. The percentage of inhibition DPPH was converted into the Trolox concentration using a calibration curve of the percentage of inhibition versus the Trolox concentration. The antioxidant capacity is expressed as μmol Trolox per kg of olive.

2.8. Sensory Analysis. A panel formed by highly experienced people carried out the quantitative descriptive sensory analysis of the EVOO. The method proposed by the International Olive Council described in the EEC, Annex XII, [26] was used. The determination was carried out in the Agri-food Laboratory of Granada (Granada, Spain). The positive attributes: fruity, bitter, and pungent, and the possible presence of defects were determined.

2.9. Statistical Analysis. The results were processed with the StatGraphics Centurion software, version 17.2.00, (*Statpoint Technologies*, Inc., Warrenton, VA, USA). The mean values of the repeats and the Fisher significant least differences (Fisher's LSD) for each response analyzed was obtained.

3. Results and Discussion

Olives were characterized by a MI of 1.74 and a content of oil of 20.69%. This means that the skin of the olives had a green color with above less than 50% of purple. The percentage of oil content is acceptable to extract considerable oil content, for the early maturation stage at which the sample is found. The effects of vertical centrifugation, storage and filtration were evaluated at industrial scale to obtain realistic results and thus be able to select the best parameters to produce EVOO. Washing the oil in a VC resulted in some changes in the quality parameters and composition. Likewise, some differences were observed between unfiltered and filtered samples.

3.1. Effect of Centrifugation on the EVOO Characteristics. The effect of oil centrifugation or washing was evaluated. For this purpose, the oil exiting the decanter and VC was analyzed. The results are provided in several tables: quality parameters and sensory characteristics (Table 1), volatile compounds (Table 2), and phenolic compounds (Table 3). An analysis of the quality parameters was also conducted. The acidity was reduced by 20.2% after washing and the peroxide index increased by 9.9%. The K_{232} value experienced a slight drop of 4.2% after washing, while K_{270} was reduced by 7.9%. The photosynthetic pigment (chlorophylls and carotenoids) content showed only a slight decrease after vertical centrifugation of the olive oil. These results are similar to those found in the literature [7]. According to the quality parameters, the olive oil category of every sample remained EVOO, as per the limits of the EEC [30]. The olive oil category did not change after the washing process, even though the quality parameters suffered some changes. Few variations in the sensory characteristics were observed, with just a slight decrease in the bitterness and pungency after oil centrifugation.

The results from the volatile compound analysis are presented in Table 2, and are represented in Figure S1(A). After washing, the total content of volatile compounds from the lipoxygenase (LOX) pathway experienced a reduction of 9.0%. These results are consistent with those by Masella et al. [13]. All volatile compounds from the LOX pathway as well as from other analyzed compounds exhibited a reduction in their content after washing. This reduction is due to partition phenomena between the oil and water phases [13]. Of note is the significant decrease of 53.3% in the ethanol content, which is produced by fermentation. According to Alcalá et al. [10], the use of water in the VC reduces the ethyl and methyl ester content, probably because some of the alcohol in the olive oil is extracted into water.

The phenolic compound content results are presented in Table 3, and are represented in Figure S1(B). The total content of phenolic compounds, mostly belonging to the group of secoiridoids, decreased by 22.9% after washing the oil. Furthermore, the antioxidant capacity decreased by 27.0% during the oil washing process. From an individual analysis of phenolic compounds, hydroxytyrosol, cinnamic acid and lignans did not undergo significant variations during centrifugation. In contrast, tyrosol, ferulic acid, *p*-coumaric acid, vanillin, secoiridoids and flavones had a decrease significant in their content during centrifugation. This reduction may be due to the transfer of the hydrophilic phenols of the oil to the water, and also to the increase of oxygen dissolved in the olive oil during centrifugation, which can cause oxidation reactions on the phenolic compounds [6, 13]. Therefore, the observed decrease in the total content of this type of compounds in EVOO is due to the individual reduction in the amount of each compound.

Table 1: Quality parameters and sensory characteristics for the oil samples before and after vertical centrifugation, storage, and filtration*.

	Decanter exit	Centrifuge exit/beginning of storage	Unfiltered/end of storage	Filtered
Acidity (%)	0.186 ± 0.001^{a}	0.148 ± 0.001^{b}	0.122 ± 0.004^{c}	0.111 ± 0.002^{d}
Peroxide I. (mEq·O_2/kg)	3.07 ± 0.07^{d}	3.38 ± 0.13^{c}	4.45 ± 0.03^{b}	5.00 ± 0.04^{a}
K_{232}	1.33 ± 0.08^{a}	1.27 ± 0.19^{a}	1.46 ± 0.03^{a}	1.35 ± 0.04^{a}
K_{270}	0.13 ± 0.01^{a}	$0.12 \pm 0.02^{a,b}$	$0.106 \pm 0.002^{b,c}$	0.096 ± 0.006^{c}
Chlorophylls (mg/kg)	35.18 ± 1.47^{a}	32.51 ± 0.90^{a}	26.41 ± 0.73^{b}	13.78 ± 0.18^{c}
Carotenoids (mg/kg)	14.17 ± 0.62^{a}	$13.08 \pm 1.06^{a,b}$	12.24 ± 0.32^{b}	7.73 ± 0.05^{c}
Total HPLC phenols (mg/kg)	438.37 ± 3.23^{a}	338.14 ± 3.99^{b}	224.75 ± 5.47^{c}	221.40 ± 3.49^{c}
DPPH (μmol/kg)	1359.83 ± 19.54^{a}	992.07 ± 8.52^{b}	221.09 ± 21.56^{c}	213.71 ± 11.84^{c}
Total LOX volatiles (mg/kg)	9.82 ± 0.26^{b}	8.93 ± 0.43^{b}	11.73 ± 0.88^{a}	11.02 ± 0.37^{a}
Fruitiness	6.4 ± 0.6^{a}	$6.0 \pm 0.3^{a,b}$	5.8 ± 0.1^{b}	5.6 ± 0.9^{b}
Bitterness	$3.5 \pm 0.3^{a,b}$	3.6 ± 0.3^{a}	$3.2 \pm 0.3^{b,c}$	2.9 ± 0.2^{c}
Pungency	4.1 ± 0.1^{a}	4.3 ± 0.2^{a}	$3.9 \pm 0.1^{a,b}$	3.6 ± 0.4^{b}

*Values are expressed as mean ± SD; (a, b, c, d) indicate Fisher's least significant differences (LSD), with statistically significant differences at 95% confidence level.

Table 2: Individual content of volatile compounds before and after vertical centrifugation, storage, and filtration processes, expressed in mg/kg*

	Decanter exit	Centrifuge exit/beginning of storage	Unfiltered/end of storage	Filtered
LOX pathway				
Hexanal	0.42 ± 0.01^{b}	0.40 ± 0.01^{b}	0.56 ± 0.02^{a}	0.55 ± 0.02^{a}
Hexan-1-ol	0.38 ± 0.02^{b}	0.36 ± 0.02^{b}	0.64 ± 0.06^{a}	0.61 ± 0.02^{a}
(*E*)-2-hexenal	3.25 ± 0.03^{a}	2.90 ± 0.11^{b}	3.27 ± 0.22^{a}	$3.13 \pm 0.05^{a,b}$
(*E*)-2-hexen-1-ol	0.24 ± 0.01^{b}	0.23 ± 0.02^{b}	0.62 ± 0.01^{a}	0.65 ± 0.02^{a}
(*Z*)-3-hexen-1-ol	1.80 ± 0.04^{b}	1.61 ± 0.11^{b}	2.33 ± 0.21^{a}	2.16 ± 0.06^{a}
(*Z*)-3-hexenyl acetate	2.33 ± 0.16^{b}	2.25 ± 0.10^{b}	3.07 ± 0.35^{a}	2.44 ± 0.08^{b}
1-penten-3-ol	$0.28 \pm 0.00^{a,b}$	$0.23 \pm 0.03^{b,c}$	0.21 ± 0.004^{c}	0.32 ± 0.07^{a}
1-penten-3-one	0.65 ± 0.02^{c}	0.56 ± 0.04^{b}	0.62 ± 0.01^{b}	0.71 ± 0.02^{a}
(*Z*)-2-penten-1-ol	0.45 ± 0.01^{a}	0.39 ± 0.04^{b}	$0.41 \pm 001^{a,b}$	0.46 ± 0.02^{a}
Sugar fermentation				
Ethanol	8.31 ± 0.17^{a}	3.88 ± 0.09^{c}	4.70 ± 0.07^{c}	6.27 ± 0.18^{b}
Acetic acid	0.64 ± 0.09^{b}	0.53 ± 0.07^{c}	0.77 ± 0.04^{a}	$0.55 \pm 0.01^{b,c}$
Other compounds				
(*E*)-2-pentenal	$0.28 \pm 0.01^{b,c}$	0.25 ± 0.03^{c}	0.29 ± 0.004^{b}	0.34 ± 0.02^{a}
Pentan-3-one	0.34 ± 0.01^{b}	0.31 ± 0.02^{c}	0.35 ± 0.01^{b}	0.41 ± 0.01^{a}
Nonanal	1.96 ± 0.05^{c}	1.75 ± 0.12^{d}	2.39 ± 0.05^{b}	2.63 ± 0.12^{a}

*Values are expressed as mean ± SD; (a, b, c, d) indicate Fisher's least significant differences (LSD), with statistically significant differences at 95% confidence level.

3.2. Effect of Storage of EVOO before Filtration. Oil storage was performed in a stainless-steel tank for 25 days at room temperature. A comparison was made between the oil samples obtained on the day of oil elaboration and the samples collected on the day of filtration to determine any changes in the composition, which will in turn have an effect on the quality parameters. The results are those compared between the columns called "centrifuge exit/beginning of storage" and "unfiltered/end of storage" from Tables 1–3. The most significant changes were the increase in the peroxide index by 31.66% and an increase of 14.96% for K_{232}, similar to the results reported by Rodrigues et al. [31]. In contrast, the other parameters decreased after those 25 days. The most significant changes were observed for the antioxidant capacity with a drop of 77.71%, and a decrease of 54.93% for the chlorophyll content and of 48.05% for the carotenoid content. These results are similar to those reported in the literature by Gutiérrez and Fernández [32]. The phenolic compound content decreased by 33.53%, similar to the data reported by Gutiérrez and Fernández [32] and Kotsiou and Tasioula-Margari [33]. A decrease in the content of most phenolic compounds was also observed, which could explain at least in part the loss of antioxidant capacity. This may be due to the loss of hydroxytyrosol, and the decrease of secoiridoid compounds, since they are compounds with a high antioxidant capacity. Making an individual analysis of the phenolic compounds during storage, it is worth highlighting the total disappearance of hydroxytyrosol. Furthermore, the secoiridoid compounds experiment a great decrease except *p*-HPEA-EA. On the contrary, tyrosol, flavones and cinnamic acid have a slight increase in their content.

In the sensory analysis, only a slight decrease was observed, similar to Gutiérrez results [32].

3.3. Effect of Filtration on the EVOO Characteristics. The effect of filtration on the characteristics of the olive oil samples was evaluated. For this purpose, the characteristics of filtered and unfiltered samples were compared. The quality parameters, sensory data, and phenolic and volatile compound content in the filtered and unfiltered oil samples are

Table 3: Individual content of phenolic compounds before and after vertical centrifugation, storage, and filtration, expressed in mg/kg*

	Decanter exit	Centrifuge exit/beginning of storage	Unfiltered/end of storage	Filtered
Phenolic alcohols				
Hydroxytyrosol	6.24 ± 0.10^{a}	6.15 ± 0.10^{a}	–	–
Tyrosol	3.87 ± 0.07^{a}	2.34 ± 0.15^{c}	2.85 ± 0.02^{b}	2.88 ± 0.03^{b}
Phenolic acids				
p-coumaric acid	3.04 ± 0.15^{a}	1.69 ± 0.09^{c}	2.74 ± 0.01^{b}	1.42 ± 0.05^{d}
Ferulic acid	7.36 ± 0.16^{a}	5.32 ± 0.13^{b}	0.77 ± 0.03^{c}	0.87 ± 0.01^{c}
Cinnamic acid	1.70 ± 0.08^{a}	1.64 ± 0.05^{a}	1.04 ± 0.04^{b}	0.88 ± 0.29^{b}
Secoiridoids				
3,4-DHPEA-EDA (oleacein)	141.57 ± 3.68^{a}	94.63 ± 0.32^{b}	28.93 ± 0.37^{c}	29.95 ± 0.92^{c}
3,4-DHPEA-EA	108.14 ± 2.11^{a}	85.27 ± 2.18^{b}	27.99 ± 0.29^{c}	28.98 ± 0.56^{c}
p-HPEA-EDA (oleocanthal)	75.18 ± 2.98^{a}	62.30 ± 2.90^{b}	32.72 ± 0.32^{c}	32.74 ± 0.24^{c}
p-HPEA-EA	21.98 ± 2.38^{a}	17.80 ± 1.49^{b}	18.45 ± 0.17^{b}	18.61 ± 0.37^{b}
Lignans				
Pinoresinol + acetoxypinoresinol	14.56 ± 1.37^{a}	14.05 ± 1.65^{a}	11.60 ± 0.84^{b}	11.45 ± 0.46^{b}
Flavones				
Luteolin	7.67 ± 0.29^{b}	$7.50 \pm 0.48^{b,c}$	9.75 ± 0.56^{a}	6.76 ± 0.50^{c}
Apigenin	5.24 ± 0.48^{b}	4.49 ± 0.31^{c}	7.37 ± 0.13^{a}	6.99 ± 0.23^{a}
Others				
Vainillin	1.85 ± 0.03^{a}	1.67 ± 0.07^{b}	1.52 ± 0.04^{c}	1.56 ± 0.09^{c}

*Values are expressed as mean ± SD; (a, b, c, d) indicate the Fisher's least significant differences (LSD), with statistically significant differences at 95% confidence level.

presented in Tables 1–3. After oil filtration, slight but significant differences were observed. The acidity and K_{232} value decreased slightly and the peroxide index increased by 12.2%. In contrast, the photosynthetic pigment content was reduced during the filtration process. The chlorophyll concentration decreased by 47.8% in relation to the unfiltered oil, and the carotenoid concentration decreased by 36.8%. This means that the cellulose acetate filter collects a very important fraction of photosynthetic pigments. These results are consistent with those reported by Gordillo et al. [34] and Brkic Bubola et al. [24]. According to the quality parameters determined, the olive oil category was still EVOO for all the samples as per the EEC [30]. Although the quality parameters underwent some changes, the category of the olive oil was not changed by the filtration process. The antioxidant capacity was also similar in both cases.

The results of the volatile compounds are presented in Table 2, and are represented in Figure S2(A). The volatile compounds were analyzed separately to detect any differences between the unfiltered and filtered samples. Overall, no major differences were observed between the two samples, except for some compounds. (*E*)-2-Hexenal, (*Z*)-3-hexenol, and (*Z*)-3-hexenyl acetate were found in greater proportion in the unfiltered sample; in contrast, (*Z*)-2-pentenol, 3-pentanone, and (*E*)-2-pentenal were detected in smaller proportion in the unfiltered sample. The amount of six-carbon-atom volatile compounds decreased after filtration; however, the amount of five-carbon-atom volatile compounds increased after the filtration process. Although the observed differences are minor, they still reveal a slight trend. These results are similar to those previously reported in the literature by Bottino et al. [23] and Brkic Bubola et al. [24].

The results obtained from the analysis of phenolic compounds are shown in Table 3, and are represented in Figure S2(B). The total amount of phenolic compounds was similar in both filtered and unfiltered samples. Certain similarities exist in both samples, except for some particular compounds. Larger amounts of luteolin and *p*-coumaric acid were detected in the unfiltered sample, results similar to those obtained by Bakhouche et al. [19], that finds a reduction of phenolic alcohols and flavones. On the other hand, oleacin and 3, 4-DHPEA-EA were found in smaller proportion in the unfiltered sample, although they are not significant differences. According to Gómez-Caravaca et al. [35], the content of phenolic compounds slightly increases after the filtration process, which may be due to the removal of water from the oil, thus increasing the concentration of dissolved substances in the oil.

All other phenolic compounds presented no differences in the filtered and unfiltered samples. It should be noted that there are some investigations changing the filtering conditions, such as that of Lozano-Sánchez et al. [22], and find some differences in the oils. Also, the type of filter used in the filtration process can be affected the content of phenolic compounds, according to results obtained by Bakhouche et al. [19] and Gómez-Caravaca et al. [35].

4. Conclusion

The use of centrifugation, storage (in order to decant) and filtration in an industrial olive mill have the function of to clean and to clarify olive oils. The olive oil category was not changed after the centrifugation, storage and filtration processes with slight changes in the fruitiness, bitterness and pungency. However, centrifugation, storage and filtration produced some significant changes found in the quality parameters and minor composition.

A relevant result was how the content of phenolic compounds was affected by centrifugation. A reduction in the concentration of these compounds was observed after the vertical centrifugation process. This is probably the result

of the transfer of hydrophilic phenols from the oil to the water phase. Centrifugation led to a 22.9% reduction in the total content of phenolic compounds. Similarly, the content of volatile compounds from the LOX pathway exhibited a decrease after washing, although the loss was just of 9%. It should be noted that a significant decrease of 53.3% of the ethanol compound content was observed after vertical centrifugation of the olive oil.

The most relevant results from the oil samples stored for 25 days before filtration were a significant increase in the peroxide index (around 30%) and a 78% decrease in the antioxidant capacity. A small number of differences were detected after oil filtration, with no differences in the sensory characteristics. The total amount of phenolic compounds and volatile compounds from the LOX pathway was similar in both filtered and unfiltered samples; furthermore, the antioxidant capacity exhibited a similar trend to the phenolic compound content. On the contrary, the photosynthetic pigment content decreased after the filtration process.

From these results, it is concluded that the water addition in the vertical centrifugation and the time of storage of olive oils should be reduced in order to avoid the decrease of the antioxidant capacity and phenolics compounds.

Abbreviations

3, 4-DHPEA-EA:	aldehyde and hydroxylic forms of oleuropein aglycone
3, 4-DHPEA-EDA (oleacein):	dialdehyde form of decarboxymethyl oleuropein aglycone
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
EVOO:	extra virgin olive oil
LSD:	least significant difference
MI:	maturity index
MUFA:	monounsaturated fatty acid
p-HPEA-EA:	aldehyde and hydroxylic forms of ligstroside aglycone
p-HPEA-EDA (oleocanthal):	dialdehyde form of decarboxymethyl ligstroside aglycone
VC:	vertical centrifuge
VOO:	virgin olive oil.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1. Comparison of the volatile (A) and phenolic (B) compound content in oil before and after centrifugation. Data at the decanter exit and centrifuge exit. The error bars show the standard deviation. Figure S2. Comparison of the volatile (A) and phenolic (B) compound content in unfiltered and filtered oil. Data at the unfiltered and filtered oils. The error bars show the standard deviation. (*Supplementary Materials*)

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Review Article

Reducing Phenolics Related to Bitterness in Table Olives

Rebecca L. Johnson and Alyson E. Mitchell

Department of Food Science and Technology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

Correspondence should be addressed to Alyson E. Mitchell; aemitchell@ucdavis.edu

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Olives are one of the oldest food products in human civilization. Over the centuries, numerous methods have been developed to transform olives from a bitter drupe into an edible fruit. Methods of processing table olives rely on the acid, base, and/or enzymatic hydrolysis of bitter phenolic compounds naturally present in the fruit into nonbitter hydrolysis products. Today, there are three primary methods of commercial table olive processing: the Greek, Spanish, and Californian methods, in addition to several Artisanal methods. This review focuses on the technological, microbiological, chemical, and sensory aspects of table olive processing and the inherent benefits and drawbacks of each method. The table olive industry is facing challenges of environmental sustainability and increased consumer demand for healthier products. Herein, we examine current research on novel technologies that aim to address these issues.

1. Introduction

The olive tree (*Olea europaea* L.) was first cultivated approximately 5000–6000 years ago in the early bronze age and is one of the oldest known cultivated plants [1]. Ripe olives contain high levels of bitter phenolic compounds including oleuropein and ligstroside that make the fruit inedible [2, 3]. In order for olives to be considered suitable for human consumption, the fruit must undergo some form of processing, fermentation, or curing to reduce the concentration of these bitter phenolic compounds. Various methods are used worldwide to debitter olives. Many of these methods have roots in ancient antiquity (e.g., salt curing), while others employ recent technological developments (e.g., California black ripe processing).

Today, there are three main commercial approaches used for debittering olives which include Greek, Spanish, and California processing methods (Table 1; Figure 1). In addition, there are several artisanal methods used to produce table olives with limited industrial scalability (e.g., salt curing or air-dried olives). Each method of debittering produces a different style of table olives with a unique texture and chemical, microbial, and sensorial profiles.

The consumption of table olives increased globally by 182% [4], and olive oil consumption increased by 76% between 1990 and 2016 [5]. This increase is attributed to the popularity of the Mediterranean diet, which is linked to reducing cardiovascular disease [6], Alzheimer's disease [7, 8], and other age-related conditions [9]. Consumption of olive oil is an essential component of the Mediterranean diet due to presence of mono-unsaturated fatty acids and phenolic compounds that are unique to *Olea europaea* and exhibit antioxidant [10], anti-inflammatory [11], anticancer [12], antimicrobial, and antiviral properties [13, 14]. This phenolic fraction is also present in table olives. Current commercial table olive processing methods remove many of these bitter phenolic compounds and as a result, can alter the health-promoting potential of various table olive products [15, 16]. Additionally, current commercial table olive processing methods are some of the most water intensive methods used in commercial food processing and can require more than 7,571 liters of water per ton of olives (e.g., California and Spanish methods) and generate highly toxic wastewater. Increased consumer demand for healthier food products that are produced in an environmentally sustainable manner, as well as industrial interest in

Table 1: Comparison of Greek natural, Spanish green, and California style black ripe table olives processing parameters.

Method	Greek natural	Spanish green	Californian style black ripe
Raw fruit	Purple maturation	Green maturation	Green maturation
Debittering mechanism	Diffusion	Base hydrolysis + diffusion	Base hydrolysis
Debittering time	6–12 months	1–7 months	1 week
Final pH	~4	~4	5.8–7.9
Final color	Purple or dark brown	Green or pale yellow	Black (artificial color)
Flavor	Salty, acidic, and fermented	Salty, acidic, and fermented	Soapy, earthy, and buttery
Wastewater per ton olive	0.9–1.9 m^3/t	3.9–7.5 m^3/t	8.0 m^3/t
Sterilization required	No	No	Yes
Drawbacks	Long processing time	—	Carcinogenic acrylamide

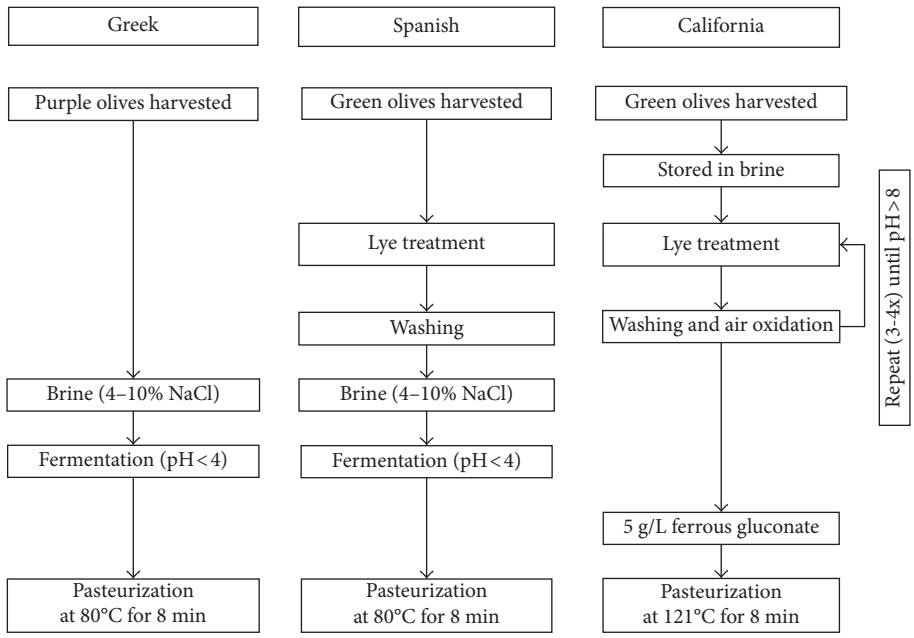

Figure 1: Diagram of Greek natural, Spanish green, and California style black ripe table olives processing methods.

decreasing processing time, water usage, and cost, demonstrates the need for innovation in olive processing technologies. This review focuses on the technological, microbiological, chemical, and sensory aspects of table olive processing, including the benefits and drawbacks of each processing method, and examines proposed novel technologies to improve table olive quality and industry sustainability.

2. Olive Fruit Maturation

Olive fruits are spherical or oval drupes, classified as small (less than 3 grams), medium (3–5 grams), or large (over 5 grams) [17]. During growth, olive drupes are green, and they accumulate bitter phenolics including oleuropein and ligstroside within the flesh and skin. Oleuropein and ligstroside are secoiridoids (i.e., a subclass of monoterpenoid iridoid compounds) that accumulate in the flesh and skin of olives as a protective mechanism against insect, pathogen, and herbivore attack (Figure 2) [18].

Green olives undergo three maturation stages on the tree which include (1) the green stage, (2) the turning color stage, and (3) the purple stage [17]. Color change occurs as the compounds that contribute to the green color in olives decrease (i.e., chlorophylls and carotenoids) and the compounds that contribute to red and purple colors increase (i.e., anthocyanins) [19]. As olives transition from green to purple, the cell wall of the fruit begins to rupture, softening the texture, and releasing enzymes, including the endogenous β-glucosidases and esterases [20, 21]. Endogenous enzymes within the olive fruit hydrolyze oleuropein and ligstroside into derivative compounds (i.e., oleuropein aglycone, ligstroside aglycone, oleocanthal, oleacein, hydroxytyrosol, tyrosol, oleoside methyl ester, and elenolic acid) that can then themselves be further hydrolyzed [22]. (Figure 2) As a result, the phenolic profile of green stage olives is different than purple stage olives, with the former containing a higher concentration of bitter phenolics [23, 24]. Although the purple olive fruit is less bitter than the green, both green and purple fruit of most varieties are far too bitter to be consumed raw without some form of processing or curing to reduce levels of these bitter phenolic compounds.

The olive fruit intended for table olive processing can be picked at any time during the maturation cycle, and olives intended for Greek fermentation, salt curing, or air drying

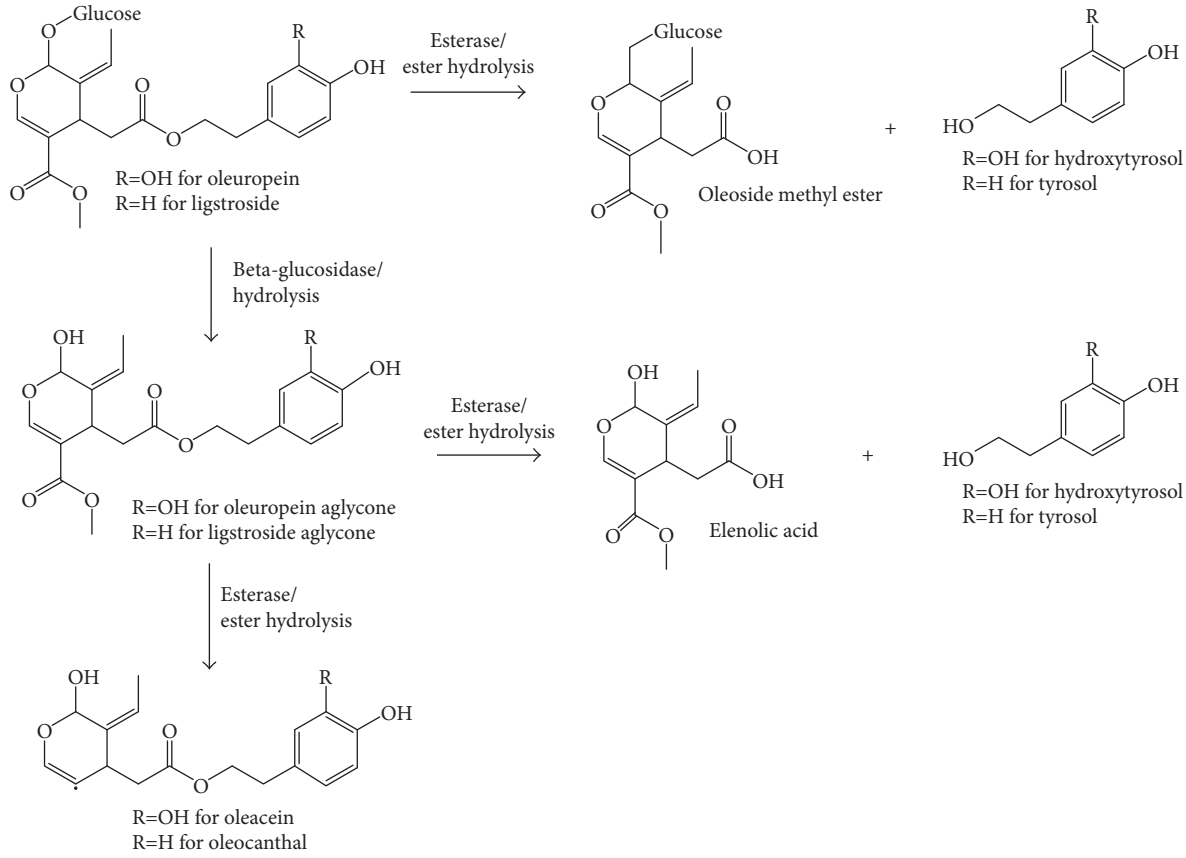

Figure 2: Olive secoiridoids and their hydrolysis products.

are generally harvested when purple, whereas olives intending for Spanish and California processing methods are harvested in the green stage [17]. Choice of the harvest stage will have an impact on the textural, sensorial, and chemical aspects of the final product.

3. Phenolic Compounds in Olives

Bitterness in raw olives is usually attributed to the presence of oleuropein, which is the most prevalent phenolics present at harvest [24]. However, oleuropein is not the only phenolic compound found in olives. Olive phenolics can be grouped into four broad categories: phenolic acids, phenolic alcohols, flavonoids, and secoiridoids [25]. While not all olive phenolics will contribute to olive bitterness, these compounds play an important role in the flavor profile, health-promoting abilities, color, and shelf stability of table olives.

Phenolic acids (C_6C_1) detected in olives include caffeic acid, *p*-coumaric acid, *o*-coumaric acid, ferulic acid, sinapic acid, syringic acid, hydroxybenzoic acid, dihydrocaffeic acid, vanillic acid, 3,4-dihydroxybenzoic acid, and gallic acid. Phenolic alcohols consist of a phenyl group ($-C_6H_5$) bonded to a hydroxyl group (–OH). Phenolic alcohols frequently detected in olive products include homovanillyl alcohol, hydroxytyrosol, and tyrosol. Flavonoids ($C_6C_3C_6$) frequently found in olives include luteolin-7-glucoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, rutin, apigenin-7-glucoside, quercetin-3-rhamnoside, and luteolin. Secoiridoids are phenolic compounds found in very few edible plants apart from olives and are among the more important compounds in regard to sensory perception of bitterness [2, 26]. Secoiridoids are characterized by an exocyclic 8,9-olefinic functionality, comprised of an elenolic acid and a glucosidic residue, also known as an oleosidic skeleton. Notable secoiridoids in olives include oleuropein, ligstroside, and dimethyl oleuropein, as well as their phenolic derivatives and hydrolysis products, which include oleuropein aglycone, ligstroside aglycone, oleacein, and oleocanthal [25], compounds that are either known to be bitter or are considered likely bitter [27].

4. Mechanisms for Debittering Olives

Oleuropein, ligstroside, and related bitter phenolics can be reduced in table olives through several different mechanisms. Strong acids or bases can penetrate the olive flesh directly, where free H^+ and OH^- ions catalyze the hydrolysis of oleuropein/ligstroside at the ester group that connects the hydroxytyrosol/tyrosol to the elenolic acid moiety. This hydrolysis reaction produces the nonbitter hydrolysis products oleoside methyl ester and hydroxytyrosol/tyrosol [2]. Olives flesh contains endogenous enzymes including *β*-glucosidase and esterase that can hydrolyze oleuropein at the ester bond between glucose and the elenolic acid moiety or between elenolic acid and hydroxytyrosol/tyrosol moiety [28]. Endogenous enzymes hydrolyze phenolic compounds within the fruit when the olive is still on the tree in response

to maturation and damage, and hydrolysis continues during fermentation/processing unless inactivated by heat (Figure 2) [29].

During brine-based processing, bitter phenolics are removed from the olive by diffusing from the fruit into surrounding brine. Once these compounds have diffused into the brine, the acid and/or the enzymatic action of exogenous enzymes, β-glucosidase, and esterase from the microbiota present in the brine or surface of the olive can hydrolyze the phenolic compounds [30]. While phenolic compounds diffuse out of the olive, salt and acid from the brine diffuse in, changing the chemical and sensory profile of the product [31]. If the cellular structure of the olive is compromised, either by chemical (e.g., lye), physical (e.g., cracking, slitting, or destoning), or the natural biochemical softening, diffusion can occur more rapidly.

5. Sensory Studies in Bitterness

Sensory studies that examine bitterness in olive products have been conducted using either taste dilution analysis (TDA) or correlating bitterness of olive oil with semiquantitative concentration of measured phenolics [26, 32, 34]. TDA of oleuropein and oleuropein aglycone indicated that these compounds are bitter at a concentration of 50 μg per 1 cm paper square [32]. TDA also confirmed that tyrosol was nonbitter, whereas oleacein, oleuropein aglycone, oleocanthal, ligstroside aglycone, and related isomers were described as bitter, astringent, or burning [33]. Although TDA is the preferred method for characterizing bitterness, it is not always possible due to the lack of commercial standards and the difficulty of isolating pure fractions of these phenolic compounds.

Sensory studies addressed these limitations by evaluating the bitterness of a wide range of olive products and correlating the individual phenolic concentrations obtained though semiquantitative methods to the perceived bitterness. While not ideal, these studies provide valuable information into which compounds are responsible for perceived bitterness in samples. From correlation studies, it can be inferred that oleuropein aglycone, ligstroside aglycone, oleacein, oleocanthal, elenolic acid, and elenolic acid methyl ester correlate well with bitterness perception [26]. Oleuropein and ligstroside derivatives, with the exception of tyrosol and hydroxytyrosol, are also reported as relative predictors of both static and dynamic analysis for bitterness and pungency, while ligstroside aglycone is effective only in predicting pungency [34].

6. Main Commercial Methods

6.1. Greek Fermentation Methods. Greek methods, typical to Mediterranean countries, use olives that are harvested when the fruit is close to full ripeness at the purple maturation stage (i.e., black ripe) but before the olives are overripened. Olives are washed and placed in an 8–10% sodium chloride brine solution (wt/vol) [35, 36]. Fermentation is induced by indigenous microbiota present on the olives and in the environment. This method of debittering takes 6–12 months as it relies on diffusion into the brine to remove the bitter phenolics from the olive flesh [35, 36].

Once olives have achieved the bitterness and flavor profile desired by the producer, olives may be briefly exposed to oxygen to darken the skin via oxidation [36], and the color is stabilized with 0.1% ferrous gluconate [17]. This is done to correct for color losses due to the diffusion of anthocyanins into the brine during fermentation.

Greek olives are packed in cans or jars in a fresh solution of brine, acid, vinegar, or olive oil. Often these packing solutions are flavored by the addition of fennel, garlic, oregano, and other spices or flavoring agents [17]. The final product can vary in the bitterness level, flavor, texture, and taste depending on the length of fermentation, sodium chloride concentration of brine, and the microbiota present. Olive flavor is strongly influenced by the fermentation products including acids (e.g., lactic, acetic, and propionic acid) and alcohols (2-butanol and ethanol) [37]. Popular olives produced with this method include Kalamata (Kalamata variety) [38, 39], Nicoise (Cailletier variety) [40], Gaeta [41], Amfissa (Conservolea variety), Liguria (Taggiasca variety), Bitetto, and Nafplion green olives [40].

Olives produced using Greek methods are enjoyed and consumed all over the world. While bitterness in Greek olives can vary, studies indicate that these olives have higher levels of phenolic compounds as compared to those produced using Spanish processing methods [21]. The final pH of the brined olive is often low enough (~4) that olives do not need to be sterilized, although a pasteurization step may occur. Greek table olives may contain live cultures of lactic acid bacteria with probiotic potential for human health [42]. Although Greek processing methods are time-consuming, they use less water (0.9–1.9 m^3/ton olive) than the Spanish or California processing methods and do not produce phenolic- and lye-enriched wastewater that requires specialized disposal [43].

6.2. Spanish Processing Methods. Spanish processing methods (a.k.a. Sevillian type) use olives picked at the green maturation stage and account for about 60% of the table olives produced worldwide [44]. These olives are immersed in lye for 8–10 hours to hydrolyze oleuropein. Lye treatment (2.0–5.0% sodium hydroxide NaOH for 18–22 hours) penetrates around two-thirds to three-quarters of the flesh, leaving a small area around the stone unaffected [36, 45]. Olives are then rinsed with water (pH 7.0) to remove excess lye and fermented in a brine that can range in sodium chloride concentration around 9–10% NaCl but can drop do 4–5% due to olives high content of interchangeable water [45, 46].

During the initial lye treatment, oleuropein concentration decreases rapidly [47]. The lye solution penetrates the olive flesh where it hydrolyzes the oleuropein and ligstroside, producing nonbitter hydrolysis products such as hydroxytyrosol and tyrosol (Figure 1). In addition, the lye changes the composition of the polysaccharides in the cell wall structure decreasing firmness [48]. The higher the concentration of the lye and the longer the lye treatment, the greater the loss of firmness. The chemical damage to the olive skin and cell structure allows for a faster diffusion of the

remaining olive phenolics and sugars into the brine during the subsequent rinsing and fermentation stages [31]. Lye changes the bacterial populations present both on the surface of the olive as well as in the brine, and a high pH can be an obstacle for desirable bacterial growth [49].

The water-rinsing step between the lye treatment and the fermentation is required to remove sodium hydroxide from the olive and reduce the pH. Free phenolics and sugars will also be rinsed away during this step [43]. This is necessary as oleuropein and related olive phenolics act as inhibitors to desirable lactic acid bacteria growth, and rinsing helps remove phenolic compounds that may prevent the growth of this bacterium [48, 50]. Sugars also decrease during fermentation after a slight increase during the rinsing step [43].

Fermentation occurs through the action of naturally induced microbiota or through the addition of starter cultures. Olive phenolic loss continues during fermentation as phenolic compounds diffuse into the brine. Fermentation can take anywhere from 1-7 months depending on the variety [45].

The flavor of Spanish style green olives is greatly influenced by the presence of organic acids (e.g., lactic, acetic, and propionic) and alcohols formed during fermentation [37, 47]. The main phenolic compounds in brine during Spanish method fermentation are hydroxytyrosol, elenolic acid glucoside, and tyrosol [47]. The final product is canned in a brine or acid solution which may contain additional flavoring agents. Olives processed using this method are often destoned, and the interior is replaced with an almond, pimento, or garlic slice. Olives are placed in cans or glass jars with fresh brine (5–7% NaCl) at a low pH (<3.5). Common olive varieties processed into Spanish method olives include Manzanilla [51], Hojiblanca [51], Gordal [51], Picholine [52], Cerignola [53], and Belice olives [45].

Spanish processing methods are significantly faster than Greek fermentation methods (1–7 months versus 6–12 months), and the final product is firm in texture and green in color. Like Greek olives, Spanish olives can contain live strains of probiotics beneficial to human health [54]. The final pH is ~4, and therefore, these olives do not need to be sterilized but may be pasteurized. In contrast to the California and Greek style olives, the Spanish olives are not oxidized, and therefore, in-field bruising is more of a yield concern. The wastewater fraction is significant (3.9–7.5 m^3/ton olive) and includes a lye fraction that must be treated for disposal [43].

6.3. Microbiota in Fermentation Brines for Greek and Spanish Methods. There are similarities in the fermentation of Greek and Spanish olives. The microbiota present in the olive brine and on the olive epidermis has been extensively studied and can vary between olive producers due to differences in olive variety, sodium chloride concentration in brine, and oxygen exposure [55–57]. In general, there are three primary classes of microorganisms present in olive fermentation brines: *Enterobacteriaceae*, yeasts, and lactic acid bacteria [17]. *Enterobacteriaceae*, which grows naturally on the surface of olives through maturation, can only be found in the brine for the first 7–15 days of fermentation, after which they are no longer detectable, as the acidic conditions of the brine are not conducive to their growth [54]. Yeasts and lactic acid bacteria are the main microorganisms present in olive brines, but the ratio present can vary greatly depending on the sodium chloride concentration and oxygen exposure. Brines with higher sodium chloride concentrations (>8% NaCl) will have a lower population of lactic acid bacteria, when compared to lower sodium chloride concentrations (4–6% NaCl) [36, 58]. Both yeasts and lactic acid bacteria can contain exogenous β-glucosidases and esterases that can hydrolyze oleuropein, ligstroside, and other phenolic compounds that diffuse out of the olive and into the brine (Figure 3) [21].

If brine conditions are conducive to lactic acid bacteria growth, the pH of the brine will decrease during fermentation due to increases in lactic, acetic, and propionic acids [35]. The presence of propionic acid in the brines in considerable amounts indicates that the fermentation process was not controlled successfully and there is a high competition for limited nutrients by different microbial populations [59]. The decrease in pH helps to prevent the growth of spoilage and pathogenic microorganisms. The lactic acid bacteria fraction is made up of a diverse array of bacteria species that contribute to the acidity, flavor, and texture of the final product [57]. Sodium chloride and lactic, acetic, and propionic acids from the brine diffuse into the olive until equilibrium is reached [35]. Olives contain phenolic compounds that can inhibit lactic acid bacteria growth including oleuropein [60], oleuropein aglycone, elenolic acid, hydroxytyrosol [59], oleacein, and oleoside methyl ester [61]. Olive cultivars with high concentrations of oleuropein and related phenolics will have lower levels of lactic acid bacteria in fermentation brines, and yeast growth will dominate. The microbiota of yeasts present in fermentation brines is diverse, and numerous species have been identified in diverse green and black olive preparations [62]. Yeasts are an important component of fermentation as they may influence organoleptic characteristics of table olives including ethanol and other alcohols [47, 63].

If olives are brined in an anaerobic environment, a spoilage problem termed "floaters" can occur. Carbon dioxide builds up under the skin of the olive in response to olive respiration and growth of Gram-negative bacteria. This results in olives that float to the surface of the tank until the carbon dioxide is released, at which point the olive skin wrinkles with the appearance of blisters [64]. Texture defects in brined olives can occur with the growth of lipase-producing yeasts [65, 66]. Clostridia growth can cause butyric and putrid spoilage during the first few days of fermentation. A spoilage problem known as *Zapateria* can occur in the rising temperatures of spring and early summer. *Zapateria* results in an unpleasant taste and odor due to the production of cyclohexanecarboxylic acid and other volatile acids. A salt concentration above 8% and a pH below 4.2 helps prevent this problem [36].

6.4. California Black Ripe Processing Methods. The California black ripe processing method was developed in the late

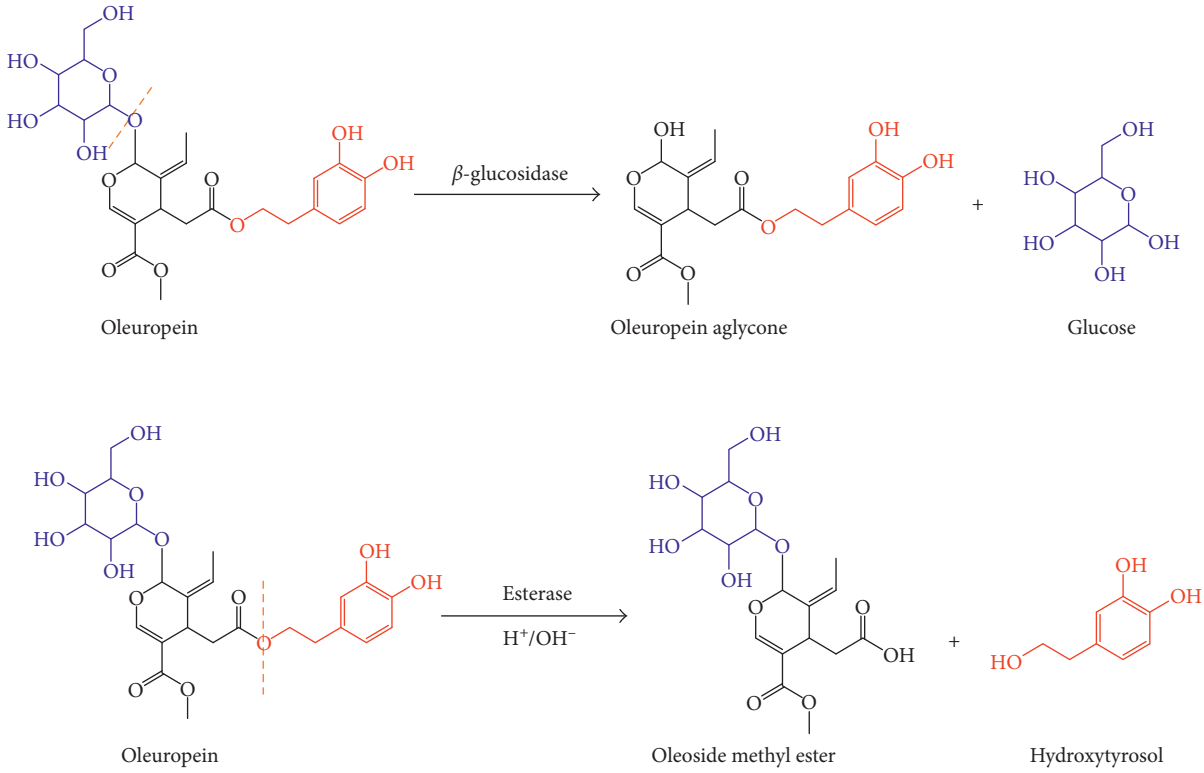

Figure 3: Mechanism of oleuropein hydrolysis.

1800s by a Northern California homemaker, Freda Ehmann. Olives are picked at the green ripe or semiripe maturation stage and stored in salt or acidified brine for 3–12 months prior to processing. During brine storage, fermentation can occur through the growth of yeasts and lactic acid bacteria, and the phenolic content of the olives can be reduced through passive diffusion. Fermentation is not necessary to create these olives as the main action of debittering occurs through the use of lye (sodium hydroxide) [67]. Instead, olives are processed using a series (3–5) of lye treatments (~0.5 M sodium hydroxide) for several hours per treatment [65]. During the lye treatment, sodium hydroxide penetrates the olive flesh, hydrolyzing the olive phenolics and debittering the olives [68]. Anthocyanin concentration decreases during California lye processing [39]. Olives stored in a storage brine for longer periods of time do not need to be treated for as many lye cycles as freshly harvested olives do, and the strength and duration of lye cycles can vary between producers, variety, and season. During the lye treatment, texture changes can occur softening the olive cell walls and texture [69, 70].

In between lye treatments, olives are immersed in rinse water and oxidized with injected compressed air (i.e., oxygen is bubbled through the water) [17]. During oxidation, olives darken and turn brown as the phenolic compounds in the olive skin (predominantly hydroxytyrosol and caffeic acid) polymerize [71, 72]. Black ripe olives are considered debittered when the flesh next to the stone has a pH greater than 8, as indicated by phenethylamine dye. Brown oxidized olives are then colored with the addition of ferric gluconate, which complexes with the compound hydroxytyrosol [68] and turns the fruit black [73].

Olives produced using the California method are packed in cans or glass jars with a salt brine or acidic solution. The final pH of the fruit can vary between 5.8 and 7.9. By FDA regulations, California black ripe olives must be legally sterilized at 115.6°C for 60 min or at 121.1°C for 50 min to prevent the growth of pathogenic bacteria [67]. The high temperature of sterilization can lead to the accumulation of the carcinogen, acrylamide, in olives [74, 75].

California black ripe table olives are made all over the world including Egypt, Morocco, Portugal, and Spain [76]. In the USA, they are consumed as popular toppings for pizza and tacos. The final canned product has a texture and profile very different from olives produced using Greek and Spanish methods. Sensory characteristics to describe California black ripe table olives include alcohol, oak barrel, artificial fruity/floral, buttery, salty, earthy, sour, and ocean-like aroma/flavor while defective olives can exhibit rancidity, metallic, gassy, and soapy/medicinal flavors [76]. American consumers showed a preference for domestic California olives as opposed to imported California black ripe olives [77]. Common olives used to produce California black ripe olives include Manzanillo [67], Hojiblanca [69], Mission [16], Intosso [68], and Sevillano varieties.

The California method produces table olives with the lowest levels of phenolic compounds, the mildest flavor, and the lowest consumer perception of bitterness when compared to other styles of olive [16]. In addition, the final pH of the Californian olives is higher than the Greek and Spanish

olives and therefore more susceptible to pathogenic growth unless sterilized. The sterilization step can result in the formation of acrylamide [67]. The Californian method is highly water intensive and requires 8.0 m^3/ton of olive, of which 2.0 m^3/ton becomes the lye wastewater fraction [78].

6.5. California Green Ripe. California green ripe olives are a variant of the California black ripe olive that do not undergo oxidation (i.e., no compressed air during washing) and color fixation (e.g., ferric gluconate) [25]. This type of olive is processed from freshly harvested olives that have not been stored in a storage brine. The final product is similar in flavor and texture to California black ripe olives, but olives are in green color rather than black.

6.6. Italian Green Olives. Castelvetrano olives (i.e., Italian green olives) undergo a treatment that is cross between Spanish and California green ripe methods. Castelvetrano olives are made from the Nocellara del Belice variety and are large green olives that grow in central and southern Italy [45]. Only large fruits of more than 19 mm in diameter are processed into these table olives. Olives are placed in plastic vessels that contain 1.8–2.5% sodium hydroxide (NaOH). An hour after the lye treatment begins, salt is added to the alkaline solution. Olives are held in the NaOH/NaCl solution for 10–15 days [17]. After treatment, olives are washed to remove the lye, although a portion of the lye flavor remains and is enjoyed by consumers. As recently as 2012, it was discovered that many Italian olives cured in the Castelvetrano style were artificially colored green with E-141ii, copper chlorophyllin complexes, that is not a legally allowed additive for table olives or olive oil by the US Food and Drug Administration (FDA) and the European Union [79], and this continues to be a problem with food fraud today [80].

7. Artesian Methods

Several artesian methods exist which are less common in industrial settings but can be found associated with specific olive cultivars or regions. Because of space, size, and cost limitations, olives produced using water, salt/oil, and air/sun to aid in bitterness reduction are difficult to scale up in industrial settings.

7.1. Water Processing. Water processing methods share similarity with brining olives; however, the soaking water is salt-free, and no fermentation occurs. Olives intended for water processing are picked at any stage in maturation and placed in a large container of water which is then sealed. Bacterial populations are not controlled through acidity or salinity, and water is changed daily [81]. As a result, water processing is highly water intensive and easily susceptible to growth of spoilage and pathogenic bacteria. Water processed olives are usually only found in south of France or in-home kitchens. Destoning, slitting, or cracking open the olive will increase the diffusion rate and decrease the time required for debittering [82]. A low salt solution can be added for flavor, but no fermentation will occur.

Water processing is difficult to adapt to an industrial setting as the great volume of water used in the manufacturing process is cost prohibitive. Water-cured olives are often served in farmer's markets, local stores, and restaurants in salt brine solutions with flavoring agents such as lemon, oregano, vinegar, garlic, chilies, and olive oil. They are enjoyed for the fresh flavor, low acidity, and low salinity.

7.2. Salt/Oil Processing. Debittering olives using salt and oil is an old practice that uses no water. Olives are typically picked at the end of the purple maturation stage when the fruit is ready to fall off the tree. Olives are packed in drums in layers of dry salt for several weeks to several months. The salt removes moisture from the olive and allows a microbiota of yeasts, molds, and *Enterobacteriaceae* to form on the surface of the olive [83], which acts to prevent growth of pathogenic and spoilage bacteria [84].

Oleuropein, ligstroside, and related phenolic compounds are reduced within the olive though the action of endogenous enzymes, (e.g., β-glucosidase and esterase) that hydrolyze bitter phenolic compounds [29]. Olives are then removed from the dry salt layers, washed, and bathed in olive oil. Throuba olives from Crete (Thassos variety) are a popular table olive cured in this manner. Another variant on the salt processing method is the Beldi olive which is briefly treated with lye before being placed in barrels with layers of salt [83]. This processing method results in a wrinkly prune-like appearance, with a mushy texture and an intense concentrated flavor that can be highly bitter.

7.3. Air/Sun Processing. This is a natural method of reducing bitterness by leaving olives on the tree past maturation. Some varieties of olives, known as sweet olives, naturally reduce in the phenolic content while on the tree, and once they reach a low enough bitterness, the fruit can be eaten raw [85]. Olives are then sun-dried, and the final texture is wrinkly and the final flavor strong and intense. Olives that are typically left on the tree to reduce bitterness include Botija Peruvian black olives and Hurma Turkish sweet olives.

8. Novel Technologies

With the growing competitiveness in the international table olive market and changing consumer preferences for healthier products, alternative novel technologies are being explored for growing, harvesting, storage, and processing of table olives with the aim of decreasing processing time, decreasing water usage, improving sustainability, increasing the health properties of processed fruit, and decreasing production cost.

8.1. U.S. Table Olive Market. Table olive consumption in the US market has been in decline at an approximate rate of −2.5% per capita consumption every year, while demand for

olive oil is increasing at an approximate rate of 1.9% per capita consumption [86]. This is in part the perception of the American consumers that olive oil is healthy, while table olives are unhealthy [87]. Both olive oil and table olives contain healthy unsaturated fats and high levels of phenolics that have beneficial health properties. For that reason, olive oil is considered an important part of the Mediterranean diet, a diet linked to the reduction of heart disease and other age-related morbid conditions [6–9]. Additionally, the profit margin for producing table olives has decreased in the US due to rising water costs, labor costs, and lower price points as cheaper international products are available in the market. As a result, the California olive industry has shifted away from table olives to producing olive oil. Between 2000 and 2015, olive processed into oil increased from 4% to 60% of all olives grown in California [88]. Another hurdle that table olive producers face is environmental sustainability. Olive processing is one of the most water intensive fruit or vegetable processing methods. Greek fermentation methods produce 0.9–1.9 m^3/tons of olive wastewater, Spanish methods 3.9–7.5 m^3/tons [43], and California methods ~8.0 m^3/tons olive of wastewater, 2.0 m^3/ton olive of which becomes the lye wastewater fraction [78]. The wastewater that is produced is a dark effluent with high organic burden and polyphenol content that exhibits antibacterial properties. Releasing effluent streams directly into local water systems or on land can result in toxicological consequences and environmental contamination. Therefore, effluent streams are generally redirected to evaporation ponds. However, evaporating wastewater can create unpleasant aromas, and effluents carry the risk of polluting ground and surface water [89]. With ongoing environmental problems created by global warming, including sustained drought, water is becoming increasingly limited and costly. Proposed novel debittering technologies that can reduce water and lye usage and/or produce less toxic effluent streams will provide economic benefits and increase the sustainability of the industry.

Several novel debittering technologies are being considered and include the use of enzymes, resins, ultrasound, and vacuum among others. Most novel technologies focus on methods that remove phenolic compounds more quickly or efficiently and thereby reduce lye washes and the use of large amounts of water. Challenges remain with adapting new technologies to commercial processing without compromising table olive quality and are discussed below.

8.2. Ultrasound Debittering. The use of ultrasound-accelerated debittering of the olive fruit has recently been examined [90]. When ultrasound is used in addition to lye treatments during California method debittering, the rate of phenolic reduction increased by 48%. Ultrasound as a technique showed valorization in reducing the time and number of lye washes needed for debittering olives [90].

8.3. Vacuum Impregnation. Researchers at Uludag University (Bursa, Turkey) investigated the viability of using vacuum impregnation to speed up the rate of olive debittering. Olives treated under a vacuum of 68 kPa demonstrated a shorter required processing time when compared to olives under atmospheric conditions. Green olives under vacuum impregnation and suspended in a lye treatment of 1.5% NaOH took 8 hours to reduce the levels of oleuropein from starting conditions to a third of the initial concentrations, as compared to 48 hours under atmospheric conditions. In brined solutions of 3% NaCl, processing time decreased from 45 days under atmospheric pressure to 11 hours under vacuum impregnation [91].

8.4. Carbon Dioxide Overpressure. The influence of storing olives under a carbon dioxide overpressure on the phenolic content of raw table olives has also been investigated [92]. Green unripe olives were placed under a carbon dioxide overpressure for a period of twelve days and evaluated for bitterness by a trained sensory panel that compared treated olives to control olives exposed to atmospheric conditions. Olives under carbon dioxide overpressure turned red in color and decreased in bitterness without becoming dehydrated or shriveled. It was postulated that the decrease in bitterness was due carbon dioxide promoting the biosynthetic pathways that naturally occur with olives that remain on the branch, including the hydrolysis of oleuropein [92].

8.5. Oxygen Overpressure. Researchers at the University Pablo de Olavide (Sevilla, Spain) investigated a potential method of debittering olives under an overpressure of oxygen. Manzanilla olives stored in brine (9% w/v NaCl and 0.3% w/v acetic acid) for one month after harvest were exposed to an overpressure of 0.3 bars oxygen for 3 days. Oxidized fruit was then placed back in preservation brine for 6 months. Laboratory tests were variable (28–98%) in the amount of oleuropein decreased. Olives exposed to an overpressure of oxygen became darker in color [93].

8.6. Enzymes. The use of exogenous β-glucosidase has also been explored as a method to reduce oleuropein [91]. Manzanilla olives were fermentation with inoculated *L. plantarum*, fermentation with inoculated *L. plantarum* and an extract of β-glucosidase, and cured using the traditional Spanish method of debittering. The phenolic content of the three treatments were measured, and trained sensory panelists scored the olives for texture, appearance, odor, aroma, bitterness and saltiness, and overall appreciation. The Spanish olives were observed to have the lowest bitterness and oleuropein concentration as compared to the inoculated and inoculated plus β-glucosidase treatments. Oleuropein concentration and bitterness perception were not significantly different between inoculated and inoculated β-glucosidase treatments. While β-glucosidase may hydrolyze oleuropein present in brines, under the conditions studied it was not enough to produce significant decreases in oleuropein or bitterness in the olive fruit [94].

8.7. Alternative Salt Solutions. The use of sodium, potassium, and calcium chloride salts to stabilize cell membranes during

the fermentation step in Spanish olive debittering methods was also investigated [95]. Calcium salts can retard the diffusion of sugar from the olive into the brine during fermentation. This results in a decrease in lactic acid bacteria growth and lactic acid production. Lactic acid production is essential for the preservation and flavor of Spanish olives. The effect of alternative salt solutions on oleuropein concentration was not quantified, and therefore, the effect on debittering is unknown. The addition of calcium chloride to brine resulted in a firmer texture olive product as compared with the use of potassium and sodium salts [95].

8.8. ReTain Inhibition. Researchers at the Agricultural University of Athens (Athens, Greece) exposed olives on the tree to aminoethoxyvinylglycine (AVG) also known as ReTain which is an inhibitor of the enzyme 1-aminocyclopronane-1-carboxylic acid synthase, a precursor to the production of ethylene, the plant hormone that stimulates ripening. Olives treated with ReTain demonstrated delayed ripening which extended the harvest, preventing color development and skin softening, while reducing oleuropein content [96].

8.9. Temperature Control. The use of ethylene oxide to delay ripening and promote the endogenous hydrolysis of oleuropein was also investigated [97]. Olives stored in closed container with 30 ppm of ethylene at 40°C showed a significant reduction ($p \leq 0.05$) of bitterness as well as pigment lost as compared to olives stored at 20°C with 30 ppm ethylene. An unpleasant flavor was then observed in ethylene oxide-treated olives indicating that this is not a suitable method of table olive debittering [97].

8.10. Resin Debittering. Our group has investigated using polymeric resins to remove phenolic compounds from untreated olives during brine storage. Adsorptive resins (e.g., Amberlite® resins XAD4, XAD16N, XAD7HP, and FPX66) are nonreactive polymers that can adsorb phenolic compounds in a reversible manner so that adsorbed phenolics can be recovered as value-added products. Preliminary results demonstrate that all resins could remove oleuropein during brine treatments thereby significantly reducing olive bitterness without the need for additional processing.

8.11. Enrichment of Table Olive with Phenolics. With the aim of improving the nutritional value of table olives and creating a novel product (i.e., table olives enriched in olive phenolics), a group in Greece treated table olives with phenolic extracts from olive leaf [98]. Treatment increased the phenolic content of the table olives and also increased the consumer perception of bitterness [98].

9. Conclusions

Table olives are a popular food product consumed worldwide. While traditional and region-specific olive processing methods should be celebrated, there is the opportunity to develop new technologies for debittering olives, that will enable creation of novel products with new textures, flavors, and health-promoting properties that will appeal to contemporary consumers and expand markets. Novel technologies can also reduce water and labor costs and increase environmental sustainability.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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