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Blueberry leaf extracts incorporated chitosan coatings for preserving postharvest quality of fresh blueberries

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ABSTRACT

The phenolic compounds in blueberry (*Vaccinium* spp.) fruit and leaf extracts (BLE) were determined based on HPLC analysis. Antimicrobial assays against *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli*, as well as fungi isolated from the rotting blueberry fruit were conducted. The effects of chitosan coating incorporating different concentrations of BLE on the quality of fresh fruit during postharvest storage at 2±1 ^{o}C and 95±2% relative humidity (RH) for 35 d and then at room conditions for 3 d were also investigated. Five different coating treatments were applied including 2% (w/v) chitosan coating (T1), 2% (w/v) chitosan coating containing 4% (w/v, T2), 8% (w/v, T3), or 12% (w/v, T4) BLE, and 2% (w/v) chitosan coating containing 12% BLE plus modified atmosphere packaging (MAP at 3 kPa O_2+12 kPa CO_2) (T5). A sample of blueberries dipped into distilled water was used as control (T0). BLE had a greater variety of phenolic compounds than fruit extracts with syringic acid the highest concentration (0.259±0.003 g kg^{-1}), but the total phenolic content in BLE was lower ($P < 0.05$) than in fruit extracts. BLE showed good antimicrobial activity against all tested microorganisms, with a minimum inhibition concentration from 25-50 g L^{-1}. The 2% chitosan coating that incorporated 8% or 12% BLE showed some degree of decreasing decay rate of fruit compared with the control, and the coating with BLE plus MAP had more effective control of fruit decay. All treated samples maintained higher total phenolic content and radical scavenging activity than the control. This study suggested that chitosan coating incorporating BLE can be employed to extend shelf-life and maintain high nutritional value of fresh blueberries during postharvest storage.

Key words: Fresh blueberries, Blueberry leaf extracts, Chitosan coating, Postharvest storage, Phytochemicals, Physicochemical quality

1. Introduction

Blueberries (*Vaccinium* spp.) are among the most popular berries in retail markets and are sold in fresh, frozen, and processed forms for various food applications. Blueberries are known for their rich bioactive compounds, including flavonoids, phenolic acids, tannins, and anthocyanins, which individually or synergistically help protect against cardiovascular disease, cancer, inflammation, obesity, diabetes, and other chronic diseases (Wu et al., 2010; Cantín et al., 2011). However, fresh berry fruit deteriorates rapidly due to water loss, juice leakage (stem scar injury), gray mold, and/or ripe rot (Perkins-Veazie et al., 2008). Fruit decay in blueberries is usually caused by fungi, with Anthracnose (*Colletotrichum acutatum*) being the most common fungal disease, followed by Alternaria rot (*Alternaria* spp.) and gray mold (*Botrytis cinerea*) (Wang et al., 2010). At present, cold storage, edible coatings, UV irradiation, modified atmosphere packaging, ozonation, and sulfur dioxide fumigation are among the postharvest preservation technologies applied to reduce postharvest deterioration, prolong shelf-life, and retain the nutritional quality of fresh blueberries (Chiabrando et al., 2006; Ribeiro et al., 2007; Zheng et al., 2008; Cantín et al., 2011).

Many plants present a rich source of antimicrobial agents and natural antioxidants (Mahesh and Satish, 2008). Recent studies have demonstrated that the extracts from the leaves of berry plants (strawberry, cranberry and blueberry) have antileukaemic activity against sensitive HL60 cells *in vitro* (Skupień et al., 2006). The aqueous extract of wild strawberry leaves is a direct, endothelium-dependent vasodilator, and its action is mediated by nitrogen oxide and cyclooxygenase products with the potency similar to that of the hawthorn aqueous extracts (Mudnic et al., 2009). The leaves of wild strawberry have received substantial attention from researchers as a potential source of natural antimicrobial and antioxidant agent over the past few years.

Chitosan (1, 4-linked 2-amino-2-deoxy-β-D-glucan), as a bioactive, biocompatible and

biodegradable polysaccharide has excellent film-forming properties and antimicrobial function, and has been used to form edible coatings for controlling the internal gas atmosphere of the fruit and serving as a barrier to water vapor for reducing moisture loss and delaying fruit dehydration (Ribeiro et al., 2007; Duan et al., 2011). Previous studies have demonstrated that chitosan coatings can effectively decrease mold growth, thus extending shelf-life of strawberries and blueberries (Park et al., 2005; Duan et al., 2011). Meanwhile, modified atmosphere packaging (MAP), when combined with an adequate temperature control, can extend the shelf-life of fresh produce by maintaining the nutritional and sensory qualities of the product (Hancock et al., 2008; Cantín et al., 2011; Cantín et al., 2012), while fungi can be significantly retarded by limited O_2 and elevated CO_2 levels in the package (Schotsmans et al., 2007; Alsmairat et al., 2011). Therefore, combining a coating treatment with MAP may further extend shelf-life of fresh produce.

Chitosan is an excellent carrier of other functional substances. By incorporating blueberry leaf extracts (BLE) into a chitosan coating, it may enhance the antimicrobial function of the coatings. The objectives of this study were first to quantify the phenolic compounds in blueberry fruit and leaf extracts, to evaluate the antimicrobial activity of BLE, and then to investigate the effectiveness of a chitosan coating that incorporates BLE for preserving postharvest quality of fresh blueberries. The study evaluated the physicochemical properties, total phenolic compounds, and antioxidant capacity of the fruit during simulated commercial storage conditions.

2. Materials and methods

2.1. Fruit and leaf materials

Blueberry (*Lanfeng* species) leaves and fruit grown at Qingpu Modern Agricultural Park (Shanghai, China) were hand-picked in the middle of May and June, 2012, respectively.

Leaves were immediately freeze-dried, milled and then stored at -80 ^{o}C prior to extraction. Fresh fruit were transferred to Shanghai Jiao Tong University within two hours after harvest. Berries with similar size and color were selected, and those with visible decay were abandoned. Fresh fruit were packed in 250 g clamshell containers with vents and stored in the dark at 2±1 ^{o}C and 95±2% RH refrigerator after being subjected to coating treatments as described below.

2.2. Preparation of blueberry leaf and fruit extracts

For preparing fruit and leaf extracts for the HPLC analysis of phenolic compounds, the method by Fortalezas et al. (2010) was followed. Briefly, for 1 g of lyophilized leaf or fruit powders, 12 mL of hydroethanolic solvent (50% v/v ethanol) was added and retained in a water bath (Model 2875, Thermo Fisher Scientific Co., USA) and shaken for 30 min at room temperature in the dark. The mixture was then centrifuged at 4,000 x g (Z236K, Hermlmle Labortechnic Gmbh, Germany) for 10 min at room temperature. The supernatant was filtered through the filter paper, and then went through 0.45 μm cellulose acetate membrane filter. The filtrates were used as extract solution and stored at –80 ºC freezer for no more than one month before usage.

For preparing fruit extracts for total phenolic content and antioxidant assay, the method by Rodriguez-Saona and Wrolstad (2001) was employed with some modifications. In brief, a 10 g sample of fruit was powdered using liquid nitrogen. Samples were extracted once with acetone containing 0.1 mL L^{-1} (v/v) HCl, followed by extracting twice with 700 mL L^{-1} (v/v) aqueous acetone containing 0.1% mL L^{-1} (v/v) HCl under ultrasonication (S-10H, Zealway instrument Inc., Xiamen, China) for 30 min. The mixtures were centrifuged at 13,520 x g for 5 min, and the supernatant was evaporated (RE-52AA, Yuhua Instruments Co., Henan, China) under vacuum at 40 ^{o}C to remove residual organic solvent. The extract was retained at

131 constant volume in a 50 mL of volumetric flask. Triplicate extractions were performed for
132 each sample.
133
134 *2.3 Antimicrobial assay of leaf extracts*
135 *2.3.1 Bacterial strains*
136 Two Gram-negative bacteria (*Salmonella Typhimurium* ATCC 14028 and *Escherichia*
137 *coli* DH5α), Gram-positive bacteria (*Staphylococcus aureus* and *Listeria monocytogenes*) and
138 fungi isolated from the surface of rotting blueberry fruit were used to test the antimicrobial
139 activity of BLE. All strains were obtained from Food Safety and Microbiology Laboratory,
140 Shanghai Jiao Tong University, China. Before use, the bacterial strains were incubated at 37
141 ^{o}C for 12 h for activation of the microorganism.
142 *2.3.2 Preliminary antimicrobial screening*
143 Preliminary antibacterial screening assays were conducted following the methods from
144 Khurram et al. (2012) with some modifications. Briefly, sterilized blank disks of 6 mm
145 diameter were punched from qualitative filter papers and dipped into the prepared crude leaf
146 extract. The disks were allowed to dry in a laminar flow hood. The negative control and
147 positive control disks were prepared by applying equal volumes (20 μL) of pure water and
148 0.84% (w/v) Ciprofloxacin in 0.1 mol L^{-1} HCl, respectively. The optimized bacterial cultures,
149 equivalent to 0.5 McFarland turbidity standard (BBL™ Prepared Turbidity Standard, Becton
150 Dickinson and Co., Shanghai, China), were aseptically spread on the entire area of Petri
151 dishes containing plate count agar, and were then placed in a constant temperature control
152 incubator (Hera Therm, Thermo Fisher Scientific Co., USA) for 15 min to dry. The disks
153 containing BLE at the concentration of 50 g L^{-1}, negative control (water), and positive control
154 (standard antibiotics) were aseptically placed on the seeded plates. The inhibition zones were
155 measured after 24 h of incubation at 37 ^{o}C, except for fungi, which was incubated for 48 h in

an inverted position. All the tests were run in triplicate.

2.3.3 Minimum inhibitory concentration (MIC) assay

MIC assay was performed using a broth micro-dilution method following the procedures from Khurram et al. (2012) with some modifications. Ninety-six well culture plates were prepared, and serial two-fold dilutions of the extracts were dispensed into the plate wells. The volume of dispensed extract was 0.1 mL per well in a concentration range of 5%-0.3125%. The same volume (0.1 mL) of bacterial culture at a density of 10^6 CFU mL^{-1} was added to the wells, and the bacterial activity in the test wells was detected by adding 20 μL of 1% (w/v) triphenyl tetrazolium chloride (TTC) aqueous solution. The bacteria were incubated at 37 ^oC for 24 h. The lowest concentration of BLE required to inhibit visible growth of the tested microorganism was designated as the MIC.

2.4. Preparation of chitosan coating solutions and application of coatings on fruit

A 2% (w/v) chitosan (degree of deacetylation > 90% and viscosity of 50-800, Sinopharm Chemical Reagent Co. Ltd, Shanghai, China) coating solution was prepared by dissolving 2 g of chitosan in 1% (w/v) aqueous acetic acid with magnetic stirring apparatus (ETS-D5, IKA Co., Ltd, Germany) overnight at room temperature for completely dissolution. Different concentrations of BLE (4~12% w/v of chitosan solution) were added into the prepared chitosan solutions and fully blended using a magnetic force agitator mixer (ETS-D5, IKA Co., Ltd, Germany) for 30 min. Fresh blueberries were completely immersed in the chitosan coating solutions for 15 s to achieve uniform surface coatings, and then drained and dried at room temperature for 30 min, and finally packed in the clamshell containers for storage.

Blueberry fruit were randomly assigned to six treatment groups: 2% (w/v) chitosan coating (T1), 2% (w/v) chitosan coating containing 4% (w/v, T2), 8% (w/v, T3), or 12% (w/v, T4) BLE, and 2% (w/v) chitosan coating containing 12% (w/v) BLE plus modified

atmosphere packaging (MAP) (3 kPa O_2+12 kPa CO_2) (T5). Fruit dipped in deionized (DI) water were used as control. For each treatment, fruit were packed in three individual clamshell containers containing about 250 g of fruit in each container, stored at 2±1 ^{o}C and 95% RH for 35 d.

2.5. Physicochemical properties of fruit during storage

Weight, pH, titratable acidity (TA), total soluble solid (TSS) and decay rate of the fruit were measured before refrigerated storage and at 3, 6, 9, 12, 20, and 35 d during storage. Fruit were smashed with blender (Model 800S, Waring Commercial Co., Ltd, USA) and filtered through four layers of cheesecloth. The juice was collected and used for the determination of TSS using a refractometer (Master Refractometcr, Japan) and pH using a pH meter (Mettler Toledo Co., Ltd, China). For determining TA, 50 g of fruit samples were mixed with an equivalent amount of water (50 g), meshed with blender (Model 800S, Waring Commercial Co., Ltd, USA). The slurry was transferred to a 250 mL volumetric flask and heated in a 75~80 ^{o}C shaker water bath for 30 min. The heated slurry was cooled to room temperature (25 ^{o}C), and water was added to the scale of the volumetric flask. Subsequently, the volumetric flask was shaken again in the same condition. The solution was filtered through gauze (0.18 mm). Fifty milliliters of filtrate was collected for titrating to pH 8.2 with 0.1 mol L^{-1} NaOH. TA was expressed as percentage of malic acid (%).

The compression firmness (CF) of the fruit was determined using a texture analyzer (TA-XT2i, Stable Micro System, God Alming, England) equipped with a load cell of 25 N. Surface color of the fruit was measured using a colorimeter (Labscen XE, Hunterlab, Japan), and the CIE L*, a* and b* color values were recorded.

Fruit decay was visually evaluated immediately after removal from cold storage at each sampling time. Berries with visible mold growth were considered decayed. Decay rate was

expressed as percentage of fruit showing decay symptoms in each container.

2.6. Analysis of phenolic compounds in blueberry leaf and fruit extracts

The blueberry leaf and fruit extracts were analyzed on an Agilent 2489 infinity HPLC using a Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm, Waters, CA, USA). The complete procedure is depicted in Fig. 1 (Krygier, 1982). Briefly, phenolic compounds were separated using a gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/H_2O, 2:30:68, v/v/v). The gradient employed was: solvent B from 10% - 100% within 42 min and allow 33 min post-run for reconditioning at a flow rate of 0.0167 mL s^{-1}. Identification of the phenolic compounds was accomplished by comparing the retention time and spectrum of the peaks in the samples using the standards under the same HPLC conditions. Each phenolic acid was quantified using external standards and the total area under each peak.

2.7. Electrical conductivity of fruit

Electrical conductivity provides information about plant tissue structure and composition that represents the ability to transport electric charges; thus a direct measurement of ionic behavior in electrolyte solutions (Kuang and Nelson, 1998; Pliquett, 2010; Park et al., 2012; Park et al., 2013). Conductivity was expressed as the relative leakage according to the method of Deng et al. (2011) with some modifications. In brief, 10 g of fruit sample was immersed in 33 mL of DI water and incubated at 25 ^{o}C for 12 h. Initial electrolyte leakage from the berries was determined using a digital conductometer (DDB-6200, Shanghai Leici Apparatus, Shanghai, China) with a DJS-1 conductivity immersion electrode. The fruit sample was boiled for 30 min and cooled to 25 ^{o}C to assess total electrolytes.

231 *2.8. Total phenolic content (TPC) and radical scavenging activity (RSA) of fruit*

232 TPC of fruit was determined by the Folin-Ciocalteu (FC) assay (Singleton and Rossi,
233 1965). A 0.5 mL of diluted sample extract or 0.5 mL of 0-200 mg kg^{-1} gallic acid solutions
234 were mixed with 7.5 mL of DI water and 0.5 mL Folin-Ciocalteau reagent in a series of test
235 tubes, and 0.5 mL of DI water was used as control. After sitting at room temperature (25 ^{o}C)
236 for 10 min, the solutions were mixed with 3 mL of 20% (w/v) Na_2CO_3 and placed in a 40 ^{o}C
237 water bath for 20 min. After heating, all samples were immediately cooled to room
238 temperature in ice bath for 3 min. The absorbance of the samples and standards were
239 measured using UV-spectrophotometer (UV-1800, Shimadzu Co., Ltd. Kyoto, Japan) at 765
240 nm. TPC was calculated as gallic acid equivalents (GAE) on a fresh weight (FW) basis, g
241 kg^{-1}.

242 The DPPH (2, 2-diphenyl-1-picryhydrazyl) assay was used to measure RSA
243 (Brand-Williams et al., 1995). A 1.5 mL of diluted sample extract or 0-0.04 g L^{-1} ascorbic
244 acid were mixed with 3 mL of 0.09 g L^{-1} DPPH solvent in a series of 2.5 mL disposable
245 cuvettes. After sitting at room temperature for 5 min in dark, the absorbance of solution was
246 determined by a spectrophotometer (UV-1800, Shimadzu Co., Ltd. Kyoto, Japan) at 517 nm.
247 Ascorbic acid was used as a standard, and RSA of samples was reported as ascorbic acid
248 equivalents (AAE) on a fresh weight basis, g kg^{-1}.

249

250 *2.9. Experimental design and statistical analysis*

251 A completely randomized design with 5 different coating treatments as independent
252 treatment factors was employed. All measurements were performed in triplicate and
253 evaluated by SPSS statistical software 19.0. The results were reported as the mean ± SD
254 (Standard deviation). Differences between the means of data were compared by least
255 significant difference (LSD) using Tukey HSD's multiple rang test. Differences at $P < 0.05$

were considered significant.

3. Results and discussion

3.1. Phenolic compounds in fruit and leaf extracts

The phenolic compounds in fruit and leaf extracts detected by HPLC are reported in Table 1. Gallic, caffeic, syringic, p-coumaric and ferulic acids were detected in both fruit and leaf extracts, while quercetin and kaempferol were only observed in leaf extracts. Compared with the leaf extracts, fruit extracts had significant higher total phenolic content ($P < 0.05$). As shown in Table 1, the contents of caffeic acid, p-coumaric acid and ferulic acid were higher than other phenolic compounds in the fruit. Syringic acid content in the leaf extracts was significantly higher than other phenolic compounds. Syringic, protocatechuic, caffeic, and p-coumaric acids are well known for their antimicrobial activity. It is believed that the -OH group in the phenolics is largely responsible for the antioxidant and antimicrobial actions because it can interrupt bacterial cell membranes and react with several biomolecules to cause deformation of these molecules, and it is highly reactive under aqueous conditions (Geissman, 1963; Kim et al., 2013).

3.2. Antimicrobial activity of BLE

BLE showed antimicrobial effects on all test strains, and was especially effective against *E. coli* and *S. aureus* with inhibition zone >7.5 mm (Table 2). The MIC values of BLE for all Gram-positive and Gram-negative bacteria were 25 g L^{-1}, while it was 50 g L^{-1} for fungi. The main phenolic compounds responsible for the antimicrobial activity of BLE were known as protocatechuic acid, caffeic acid, and p-coumaric acid (Geissman, 1963; Kim et al., 2013). A previous study reported the antimicrobial activity of ethanol extracts of leafy green vegetable against Gram-positive and Gram-negative bacteria (Kim et al., 2013).

281

282 *3.3. Decay rate, weight loss, firmness and electrical conductivity of fruit*

283 Except T1 samples, all other fruit samples had decay rates <5% at the first 12 d of storage,
284 but increased significantly after that. MAP samples showed significantly lower decay rate
285 than others (Fig. 2A), which was consistent with previous findings about the use of MAP to
286 reduce decay of fresh blueberries (Alsmairat et al., 2011; Cantín et al., 2011; Cantín et al.,
287 2012). The fruit coated with chitosan and 4% BLE (T2) showed a higher decay rate at 20 d of
288 storage, probably due to dual effects - the concentration of BLE added into chitosan coating
289 was too low to provide additional antimicrobial function and the possible chemical damage
290 caused when applying the coatings (Fig. 2A). In general, the higher the BLE concentration in
291 the coatings, the lower the decay rate of fruit during extended storage. However, a
292 significantly higher decay rate in sample T4 was observed at 35 d, which was difficult to
293 explain and further study is needed to explore the reasons. Compared with control, the 2%
294 chitosan coating that incorporates 8% or 12% BLE showed some degree of decreased decay
295 rate of fruit, while coating with BLE and MAP had more significant control on the decay of
296 the fruit.

297 Fig. 3 shows the appearance of fruit samples after 35 d of refrigerated storage followed by
298 an additional 3 d of room temperature storage. The fruit coated with chitosan/BLE plus MAP
299 (T5) had the lowest decay whereas T4 samples had the highest decay, and all coated fruit
300 showed lower decay than the control. Again, the fruit coated with chitosan and 8% BLE (T3)
301 showed lower decay than chitosan alone coating (T1) and chitosan coating containing 4%
302 BLE (T2). However, there was no significant difference among coated fruit (T1 to T5). The
303 exact connection between the decay rate and BLE concentration needs to be further studied.

304 Among all treatments, the highest weight loss (9.7%) was observed in the control samples
305 and the lowest weight loss (1.5%) was found in T5 samples (coating plus MAP) throughout

306 the storage period (Fig. 2B). Overall, coating plus MAP (T5) showed significant control of
307 fruit weight loss during storage, while chitosan coating alone or containing BLE (especially
308 T3 treatment) was only able to reduce weight loss in comparison with control.
309 Chitosan-based coatings have demonstrated superior effectiveness for controlling weight loss
310 in previous studies on pears and strawberries by providing a barrier to water transport (Lin et
311 al., 2008; Rojas-Graü et al., 2007).
312 In general, fruit firmness increased at the first 12 d of storage, but decreased during the
313 following storage period. However, significant fluctuations in the firmness values were
314 observed during the whole storage period (Fig. 2C). During the first 20 d of storage, most
315 treated fruit had significantly increased firmness values compared with their initial firmness
316 (12.34 N) ($P < 0.05$). It is commonly accepted that water loss leads to increased firmness
317 during postharvest storage (Chiabrando and Giacalone, 2011), and the variations among
318 individual fruit cause fluctuations in the firmness values, thus leading to unclear trends.
319 Among all treatments, fruit coated with chitosan/BLE plus MAP (T5) were less firm, fruit
320 subjected to T4 treatment (chitosan coating containing 12% BLE) showed higher firmness
321 values throughout the storage, and T1~T5 samples had less fluctuation in firmness values
322 than controls. Previous studies also found that berries stored under high CO_2 atmosphere have
323 significantly soft texture (Schotsmans et al., 2007; Duarte et al., 2009; Alsmairat et al., 2011).
324 Fruit showed significantly decreased firmness at the later stage of storage. It has been well
325 known that the loss of firmness can be related to the enzymatic hydrolysis of cell wall
326 substances. Additionally, softening is often related to water loss, which is responsible for the
327 loss of turgor and crispness of fresh fruit.
328 Membrane integrity is possibly the major factor in the change of fruit conductivity. As
329 shown in Fig. 2D, the electrical conductivity of fruit remained stable for the first 9 d,
330 increased gradually after 12 d, and then decreased during the rest of storage. These results

suggested that the membranes of fruit had been seriously destroyed by 12 d, and the decrease in conductivity might be caused by fruit decay. Compared with the control samples, coated fruit (T1~T5) generally had higher electrical conductivity and the electrical conductivity was less changed during 35 d of storage. These results may be explained as the coating solutions had very high conductivity, which in turn led to the high conductivity in coated fruit. During storage, the chitosan coatings incorporated BLE helped maintain the membrane integrity of the fruit, thus retaining the electrical conductivity of the coated fruit.

3.4. Titratable acid (TA), total soluble solids (TSS) and color of fruit

TA and TSS were not significantly affected by various treatments during storage (P >0.05) (Fig. 2E and 2F). TA values (Fig. 2E) fluctuated from 0 to 12 d, and decreased in the later storage time. The control samples had significantly lower TA value than other samples during storage and T4 sample showed different trends during the first 9 d. Again, coating generally helped retain TA of fruit similarly to that provided by controlled atmosphere that prevents gas exchange. The high acidity in blueberries stored under controlled atmosphere was reported previously (Duarte et al., 2009).

TSS was relatively stable during the storage period (Fig. 2F). Control (T0) had relatively higher TSS at the first 12 d of storage and then declined. T1- T5 samples had more stable TSS values than the control, and TSS of T5 samples was the lowest at 35 d, probably associated with the different effects of MAP on the respiration rate. It was interpreted that during postharvest storage, acid metabolism converted starch and acid to sugar, thus resulting in the decrease of TA and the increase of TSS (Duan et al., 2011). Similar result was reported in the previous studies on highbush blueberries, in which TSS values generally remained invariable throughout storage period (Chiabrando and Giacalone, 2011). Fruit surface color did not change significantly during storage among different treatments, and, thus were not

356 reported here.

357

358 *3.5. Total phenolic content of fruit*

359 The total phenolic content (TPC) of fruit subjected to different treatments are reported in
360 Table 3. Compared with fresh fruit (1.137 ± 0.014 g kg^{-1}), TPC of fruit increased
361 significantly at the beginning of storage, but dropped thereafter. This might be because
362 'Lanfeng' blueberry is an early-maturing variety, and was not fully ripe when harvested for
363 maximizing the fresh market shelf-life of fruit. During storage, blueberry fruit gradually
364 generated phenolic compounds.

365 Blueberries coated with chitosan/BLE or plus MAP (T2~T5) generally had higher TPC
366 than those coated with chitosan (T1) alone and control (T0) except at 3 d and 35 d. This
367 might be because the control fruit ripened faster than those treated at the beginning of the
368 storage. Phenolic compounds might have reacted with other compounds, resulting in the
369 lower total phenolic content in T3~T5 samples.

370 Postharvest storage impacts phenolic content, and enzymes play significant roles in
371 phenolic metabolism. During refrigerated storage, the postharvest ripening process continued
372 in fruit with TPC accumulation (Wu et al., 2010). Previous studies revealed that a chitosan
373 coating could decrease the loss of phenolic compounds and the occurrence of browning of
374 fruit (Campaniello et al., 2008). However, this is only a hypothesis and needs to be confirmed
375 by further investigations. In the present study, the chitosan coating with BLE helped retained
376 the total phenolic content.

377

378 *3.6. Radical scavenging activity of fruit*

379 The radical scavenging activity (RSA) of fruit during storage is reported in Table 4.
380 Generally, RSA of fruit decreased during storage. At 3 d, T2~T5 samples had lower RSA

than control and chitosan coating alone. From 6 d to 20 d, all treated samples had higher RSA than control, and T5 samples (chitosan/BLE coating plus MAP) had higher RSA than other samples (T0~T4). However, the treatment differences disappeared at 35 d of storage. Generally, the fruit coated with chitosan and high concentration of BLE (T3 and T4) retained higher RSA during storage. These results demonstrated that BLE incorporated in a chitosan coating could maintain higher RSA of fresh blueberries during refrigerated storage. A previous study also found that the TPC and total antioxidant capacity increased substantially at storage temperature of 0 ^{o}C in raspberries and strawberries (Kalt et al., 1999).

4. Conclusions

Blueberry leaf extracts contained a greater variety of phenolic compounds compared with blueberry fruit extracts, but the total phenolic content was lower than that of fruit. Syringic acid, well-known for its antimicrobial activity, was the highest phenolic compound in leaf extracts. Leaf extracts had a broad antimicrobial activity against Gram-positive, Gram-negative and fungi. Adding blueberry fruit leaf extracts into chitosan coatings further enhanced the function of the chitosan coating with respect to delaying the decay and weight loss, and maintaining total phenolic content and radical scavenging activity of fruit during storage, especially when working together with MAP. These results suggested that coatings combined with leaf extracts have the potential of prolonging shelf life and retaining nutraceutical benefits of fresh blueberries. Further studies are under the way to obtain more specific information about the impacts of different extraction methods, harvest times of leaves, and leaves from different blueberry varieties on its antimicrobial and antioxidant activities, as well as the optimal concentrations of leaf extracts to be incorporated into different coating materials for extending postharvest shelf life of fruit and vegetables.

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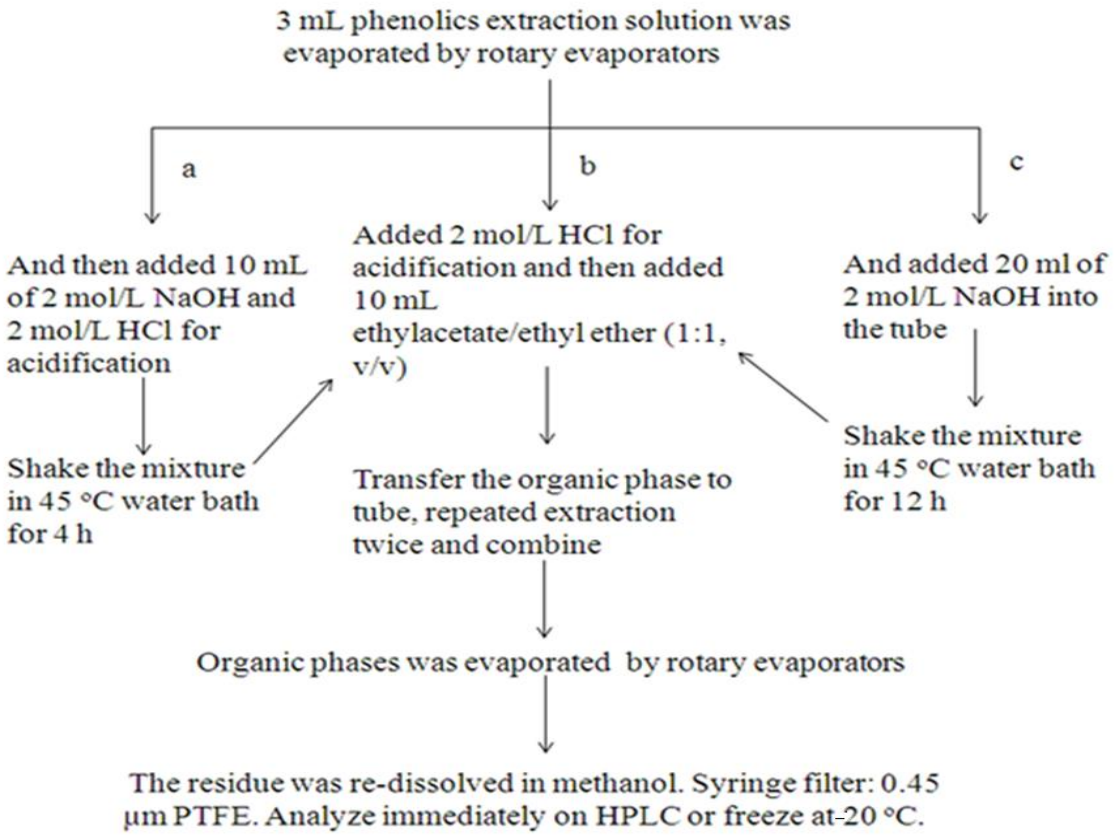

Fig. 1. Procedures for preparing blueberry fruit and leaf extracts for phenolic analysis using HPLC (a: Total soluble phenolics in extracts; b: Free phenolics in extracts; c: Insoluble bound phenolics in extracts. PTFE stands for polytetrafluoroethylene).

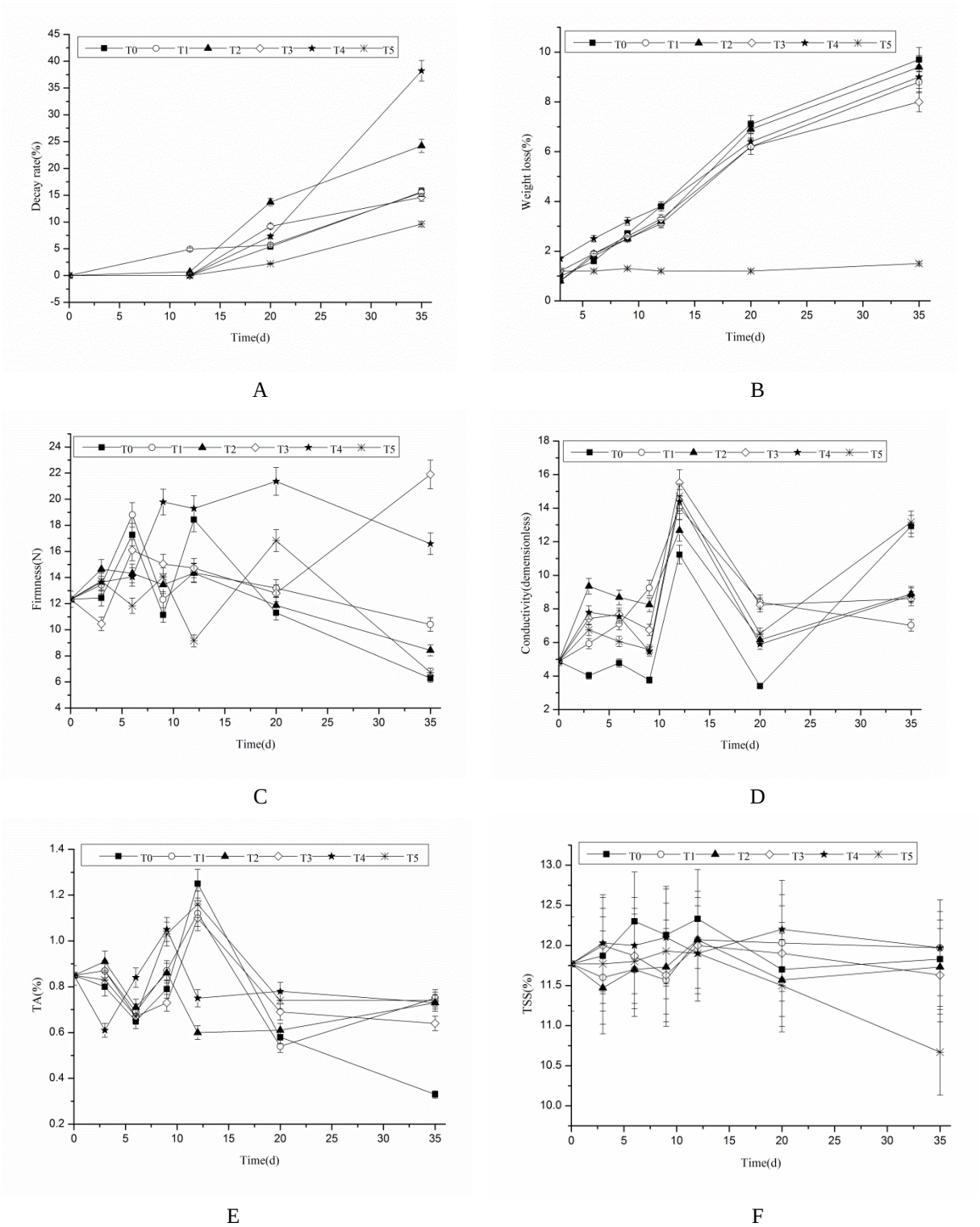

Fig. 2. Physicochemical properties of blueberry fruit subjected to different postharvest treatments during refrigerated storage at 2±1 ^{o}C (Control (T0), 2% chitosan coating (T1), 2% chitosan coating containing 4% (T2), 8% (T3), or 12% (T4) blueberry leaf extracts, and 2% chitosan coating containing 12% leaf extract plus modified atmosphere packaging (3 kPa O_2+12 kPa CO_2) (T5)).

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Fig. 3. Appearance of fresh blueberry fruit after 35 d of storage at 2±1 ^{o}C plus additional 3 d at room conditions (Control (T0), 2% chitosan coating (T1), 2% chitosan coating containing 4% (T2), 8% (T3), or 12% (T4) blueberry leaf extracts, and 2% chitosan coating containing 12% leaf extracts plus modified atmosphere packaging (3 kPa O_2+12 kPa CO_2) (T5)).

Table 1

Phenolic compounds in blueberry fruit and leaf extracts quantified by HPLC

Phenolic compounds	Phenolic content, dry basis (g kg^{-1}) [+]	
	Fruit	Leaf
Gallic acid	0.142±0.002 [Aa ‡]	0.004±0.000 [Ba]
Caffeic acid	1.217±0.007 [Ae]	0.010±0.002 [Ba]
Vanillic acid	0.170±0.002 [Ac]	0.017±0.001 [Bb]
Syringic acid	0.997±0.001 [Ad]	0.259±0.003 [Be]
P-coumaric acid	1.154±0.002 [Ab]	0.083±0.000 [Bd]
Ferulic acid	1.280±0.004 [f]	ND
Quercetin	ND [++]	0.046±0.001 [c]
Kaempferol	ND	0.006±0.003 [a]

[+] Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

[‡] Values within the same column followed by different lowercase letters were significantly different at $P < 0.05$, and values within the same row followed by different capital letters were significantly different at $P < 0.05$.

[++] ND means not detected.

Table 2

Antimicrobial activities of blueberry leaf extracts against tested microorganisms based on inhibition zone and minimal inhibitory concentration (MIC).

Microorganism	*E. coli*	*S. Typhimurium*	*L. monocytogenes*	*S. aureus*	*Fungi*
Inhibition zone [+]	++	+	+	++	+
MIC (mg mL^{-1})	25	25	25	25	50

[+] Inhibition zone 6 - 7.5 mm: antimicrobial activity (+); inhibition zone >7.5 mm: high antimicrobial activity (++).

Table 3

Effect of different postharvest treatments on the total phenolic content of blueberry fruits stored at 2±1 ^{o}C.

Storage time (d)	Total phenolic content, GAE, fresh weight basis (g kg^{-1}) $^{+}$					
	T0 $^{++}$	T1	T2	T3	T4	T5
3	1.74±0.00 ^{Ee}	1.76±0.02 ^{Ee}	1.27±0.01 ^{Bc}	1.49±0.00 ^{Dc}	1.14±0.01 ^{Aa}	1.41±0.05 ^{Cc}
6	1.34±0.01 ^{ABb}	1.22±0.01 ^{Ab}	1.61±0.01 ^{Ff}	1.39±0.01 ^{Cb}	1.47±0.01 ^{Dc}	1.54±0.00 ^{Ed}
9	1.53±0.01 ^{Cc}	1.49±0.01 ^{Bd}	1.57±0.01 ^{De}	1.18±0.01 ^{Aa}	1.61±0.01 ^{Ed}	1.54±0.01 ^{CDd}
12	1.30±0.00 ^{Bb}	1.40±0.01 ^{Cc}	1.17±0.00 ^{Ab}	1.49±0.01 ^{Dc}	1.29±0.01 ^{Bb}	1.51±0.01 ^{Dd}
20	1.47±0.00 ^{Bc}	1.48±0.00 ^{Bd}	1.37±0.01 ^{Ad}	1.65±0.01 ^{Dd}	1.51±0.01 ^{Cc}	1.65±0.02 ^{De}
35	1.67±0.01 ^{Ed}	1.48±0.02 ^{Dd}	1.29±0.01 ^{Bc}	1.38±0.02 ^{Cb}	1.26±0.01 ^{Bb}	1.06±0.01 ^{Aa}

$^{+}$ Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

$^{++}$ Physicochemical properties of blueberry fruit subjected to different postharvest treatments during refrigeration storage at 2±1 ^{o}C (Control (T0), 2% chitosan coating (T1), 2% chitosan coating containing 4% (T2), 8% (T3), or 12% (T4) blueberry leaf extracts, and 2% chitosan coating containing 12% leaf extract plus modified atmosphere packaging (3 kPa O_2+12 kPa CO_2) (T5)).

‡ Values within the same column followed by different lowercase letters were significantly different at $P < 0.05$, and values within the same row followed by different capital letters were significantly different at $P < 0.05$.

Table 4

Effect of different post-harvest treatments on the radical scavenging activity of blueberry fruit stored at 2±1 ^{o}C.

Storage time (d)	Radical scavenging activity, AAE, fresh weight basis (g kg^{-1}) $^{+}$					
	T0 $^{++}$	T1	T2	T3	T4	T5
3	3.00±0.03 ^{Dc}	3.01±0.02 ^{De}	2.48±0.02 ^{Ba}	2.80±0.02 ^{Cc}	2.21±0.02 ^{Aa}	2.77±0.00 ^{Cb}
6	2.72±0.01 ^{Aa}	2.71±0.01 ^{Ab}	3.08±0.00 ^{Dd}	2.84±0.00 ^{Bc}	2.90±0.01 ^{Cd}	3.08±0.01 ^{Dde}
9	2.83±0.05 ^{Bb}	2.80±0.01 ^{Bc}	2.90±0.00 ^{Cc}	2.49±0.01 ^{Aa}	3.05±0.02 ^{De}	3.02±0.02 ^{Dc}
12	2.81±0.02 ^{Bb}	2.91±0.01 ^{Cd}	2.70±0.00 ^{Ab}	3.01±0.01 ^{Dd}	2.83±0.02 ^{Bc}	3.05±0.02 ^{Dcd}
20	2.87±0.01 ^{BCb}	2.82±0.05 ^{ABc}	2.73±0.07 ^{Ab}	3.30±0.02 ^{Ef}	2.94±0.01 ^{Cd}	3.05±0.00 ^{Dcde}
35	2.70±0.02 ^{Da}	2.64±0.03 ^{CDa}	2.51±0.04 ^{Ba}	2.58±0.08 ^{BCDb}	2.56±0.05 ^{BCb}	2.20±0.03 ^{Aa}

$^{+}$ Data were presented as the mean ± standard deviation (SD) of triplicate experiments.

$^{++}$ Physicochemical properties of blueberry fruit subjected to different postharvest treatments during refrigeration storage at 2±1 ^{o}C (Control (T0), 2% chitosan coating (T1), 2% chitosan coating containing 4% (T2), 8% (T3), or 12% (T4) blueberry leaf extracts, and 2% chitosan coating containing 12% leaf extract plus modified atmosphere packaging (3 kPa O_2+12 kPa CO_2) (T5)).

‡ Values within the same column followed by different lowercase letters were significantly different at $P < 0.05$, and values within the same row followed by different capital letters.