To my family and friends
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>5</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>14</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>15</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 BACKGROUND</td>
<td>17</td>
</tr>
<tr>
<td>Introduction</td>
<td>17</td>
</tr>
<tr>
<td>Reactive Carbonyl Compounds</td>
<td>17</td>
</tr>
<tr>
<td>Sources of Reactive Carbonyl Compounds</td>
<td>18</td>
</tr>
<tr>
<td>Reactivity of Reactive Carbonyl Compounds</td>
<td>19</td>
</tr>
<tr>
<td>Advanced Glycation End products (AGEs)</td>
<td>20</td>
</tr>
<tr>
<td>Berries and Muscadine Grape</td>
<td>22</td>
</tr>
<tr>
<td>Health Related Benefits</td>
<td>22</td>
</tr>
<tr>
<td>Phytochemicals in Berries and Muscadine Grapes</td>
<td>22</td>
</tr>
<tr>
<td>Reactions Between Phytochemicals and Reactive Carboynlys</td>
<td>23</td>
</tr>
<tr>
<td>Research Objectives</td>
<td>23</td>
</tr>
<tr>
<td>2 FLUORESCENT AGES PRODUCTION FROM IN VITRO INCUBATION</td>
<td>25</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>25</td>
</tr>
<tr>
<td>Chemicals</td>
<td>25</td>
</tr>
<tr>
<td>Model System for Fluorescent AGEs Formation</td>
<td>25</td>
</tr>
<tr>
<td>Emission and Excitation Spectra of Fluorescent AGEs</td>
<td>26</td>
</tr>
<tr>
<td>Fluorescent Intensity Measurement</td>
<td>26</td>
</tr>
<tr>
<td>Data Expression and Grafting</td>
<td>27</td>
</tr>
<tr>
<td>Results</td>
<td>27</td>
</tr>
<tr>
<td>Fluorescent AGEs Formation</td>
<td>27</td>
</tr>
<tr>
<td>Discussion</td>
<td>29</td>
</tr>
<tr>
<td>3 ANTIGLYCATION EFFECT OF BERRY AND GRAPE PHYTOCHEMICALS</td>
<td>34</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>34</td>
</tr>
<tr>
<td>Chemicals and Materials</td>
<td>34</td>
</tr>
</tbody>
</table>
4 FRACTIONATION OF BLUEBERRY PHYTOCHEMICALS, COMPOUND IDENTIFICATION AND ANTIGLYCATION EFFECTS

Materials and Methods

Chemicals and Materials ......................................................... 54
Fractionation of Sugar-free Blueberry Phytochemicals .................. 54
Phytochemical Identification on HPLC-ESI-MS\textsuperscript{n} ........ 55
Total Phenolic Contents, Antioxidant Capacity, Total Anthocyanin Content and Total Procyanidin Content .............................. 55
Anti-glycation Effect in Selected Models ..................................... 56
Direct Methylglyoxal Trapping Assay .......................................... 56
Data Expression, Grafting and Statistics ....................................... 56
Results ....................................................................................... 57
Phytochemical Fractionation ....................................................... 57
Phytochemical Identification on HPLC-ESI-MS\textsuperscript{n} ........ 57
Total Phenolic Content ............................................................... 59
Antioxidant Capacity ................................................................. 60
Total Anthocyanin Content ........................................................ 60
Total Procyanidin Content ........................................................... 60
Anti-glycation Effect in Selected Models ..................................... 61
Direct Methylglyoxal Trapping ..................................................... 62
Discussion ................................................................................... 62

5 REACTIONS BETWEEN PHYTOCHEMICALS AND REACTIVE CARBONYL SPECIES ........................................................................... 75
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Wavelength of maxima excitation and emission of fluorescent AGEs in various model systems.</td>
<td>31</td>
</tr>
<tr>
<td>3-1</td>
<td>Weight of frozen berries, crude extracts, and sugar-free extracts.</td>
<td>46</td>
</tr>
<tr>
<td>3-2</td>
<td>Sugar concentrations in crude and sugar-free extracts.</td>
<td>46</td>
</tr>
<tr>
<td>3-3</td>
<td>Total phenolic, antioxidant capacity, total anthocyanins and total procyanidins in sugar-free extracts.</td>
<td>46</td>
</tr>
<tr>
<td>4-1</td>
<td>Weights of blueberry extracts and fractions.</td>
<td>64</td>
</tr>
<tr>
<td>4-2</td>
<td>Tentatively identified compounds in blueberry fractions.</td>
<td>64</td>
</tr>
<tr>
<td>4-3</td>
<td>Total phenolic, antioxidant capacity, total anthocyanins and total procyanidins in blueberry extract and fractions.</td>
<td>65</td>
</tr>
<tr>
<td>5-1</td>
<td>Empirical degradation half time of phytochemicals in different incubation mixtures.</td>
<td>90</td>
</tr>
<tr>
<td>5-2</td>
<td>Empirical degradation half time of reactive carbonyl compounds in different incubation mixtures.</td>
<td>90</td>
</tr>
<tr>
<td>5-3</td>
<td>Phytochemical-carbonyl adduct identification.</td>
<td>100</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Structures of reactive carbonyls.</td>
<td>24</td>
</tr>
<tr>
<td>1-2</td>
<td>Structures of selected advanced glycation end products (AGEs).</td>
<td>24</td>
</tr>
<tr>
<td>2-1</td>
<td>Excitation (A) and emission (B) spectra of BSA-fructose model.</td>
<td>31</td>
</tr>
<tr>
<td>2-2</td>
<td>Fluorescent AGE generation in bovine serum albumin (BSA)-monosaccharide incubation models at 37 °C.</td>
<td>32</td>
</tr>
<tr>
<td>2-3</td>
<td>Fluorescent AGE generation in bovine serum albumin (BSA)/ reactive carbonyl incubation models at 37 °C.</td>
<td>32</td>
</tr>
<tr>
<td>2-4</td>
<td>Fluorescent AGE generation in amino acids- monosaccharide incubation models at 37 °C.</td>
<td>33</td>
</tr>
<tr>
<td>2-5</td>
<td>Fluorescent AGE generation in amino acid- reactive carbonyl incubation models at 37 °C.</td>
<td>33</td>
</tr>
<tr>
<td>3-1</td>
<td>Extraction and purification of sugar-free phytochemicals from berries and noble muscadine grapes.</td>
<td>47</td>
</tr>
<tr>
<td>3-2</td>
<td>HPLC chromatogram of sugars in (A) cranberry crude extract and (B) cranberry sugar-free extract.</td>
<td>48</td>
</tr>
<tr>
<td>3-3</td>
<td>Anti-glycation effects of berry extracts in bovine serum albumin-fructose model. Bars represent mean±standard deviation of triplicate tests.</td>
<td>49</td>
</tr>
<tr>
<td>3-4</td>
<td>Anti-glycation effects of berry extracts in bovine serum albumin-methylglyoxal model. Bars represent mean±standard deviation of triplicate tests.</td>
<td>50</td>
</tr>
<tr>
<td>3-5</td>
<td>Anti-glycation effects of berry extracts in arginine-methylglyoxal model. Bars represent mean±standard deviation of triplicate tests.</td>
<td>51</td>
</tr>
<tr>
<td>3-6</td>
<td>Chromatogram of methylglyoxal (A), methylglyoxal after reaction with blueberry sugar-free extract (B) and methylglyoxal after reaction with aminoguanidine (C).</td>
<td>52</td>
</tr>
<tr>
<td>3-7</td>
<td>The percentage of remaining methylglyoxal after reacting with berry and grape extracts for 0.5, 1, 2, 4, 6h.</td>
<td>53</td>
</tr>
<tr>
<td>4-1</td>
<td>Fractionation flow chart of blueberry sugar-free phytochemicals.</td>
<td>66</td>
</tr>
<tr>
<td>4-2</td>
<td>HPLC chromatograms of blueberry fraction I at 280 nm (A) and 520 nm (B).</td>
<td>67</td>
</tr>
</tbody>
</table>
HPLC chromatograms of blueberry fraction II at 280 nm (A) and 520 nm (B). ... 67

HPLC chromatograms of blueberry fraction III at 280 nm (A), 360 nm (B) and 520 nm (C).................................................................................................................. 68

HPLC chromatograms of blueberry fraction IV at 280 nm (A) and 520 nm (B). ... 68

HPLC chromatograms of blueberry fraction V at 280 nm (A) and 520 nm (B). ... 69

4-7 Anti-glycation effects of blueberry extract and fractions in bovine serum albumin-fructose model. ........................................................................................................ 70

4-8 Anti-glycation effects of blueberry extract and fractions in bovine serum albumin-methylglyoxal model. .............................................................................. 71

4-9 Anti-glycation effects of blueberry extract and fractions in arginine-methylglyoxal model. ................................................................................................. 72

4-10 Chromatogram of methylglyoxal (A), methylglyoxal after reaction with blueberry fraction III (B) and methylglyoxal after reaction with aminoguanidine (C). ........................................................................................................ 73

4-11 The percentage of remaining methylglyoxal after been incubated with blueberry extract and fractions for 0, 0.5, 1, 2, 4, 6h. ........................................... 74

5-1 Percentage of remaining catechin (CAT) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR).............................................................. 91

5-2 Percentage of remaining epicatechin (EPI) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR).............................................................. 92

5-3 Percentage of remaining chlorogenic acid (CGA) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR).............................................................. 93

5-4 Percentage of remaining quercetin (QUE) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR).............................................................. 94

5-5 Percentage of remaining resveratrol (RES) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR).............................................................. 95

5-6 Percentage of remaining glyoxal (GO) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA)...................................................... 96
Percentage of remaining methylglyoxal (MGO) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA)................................. 97

Percentage of remaining malondialdehyde (MDA) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA)................................. 98

Percentage of remaining acrolein (ACR) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA)................................. 99

HPLC-FLD (Ex=231nm, Em=320nm) chromatogram of the reaction products in the incubation of catechin with phosphate buffer (A), catechin with glyoxal (B), catechin with methylglyoxal (C), catechin with malondialdehyde (D) and catechin with acrolein (E). ........................................................................................................ 101

MS and MS² spectra of the precursor ions of m/z=619[M-H]⁻ (A), m/z=347[M-H]⁻ (B) and m/z=638[M]⁻ (C) in the incubation of catechin and glyoxal in negative electrospray mode................................................................. 102

The proposed structures of reaction adducts formed by catechin and glyoxal. 103

MS and MS² spectra of the precursor ions of m/z=433[M-H]⁻ (A) and m/z=361[M-H]⁻ (B) in the incubation of catechin and methylglyoxal in negative electrospray mode................................................................. 104

The proposed structures of reaction adducts formed by catechin and methylglyoxal................................................................. 104

MS and MS² spectra of the precursor ions of m/z=433[M-H]⁻ (A) and m/z=361[M-H]⁻ (B) in the incubation of catechin and malondialdehyde in negative electrospray mode................................................................. 105

The proposed structures of reaction adducts formed by catechin and malondialdehyde. ................................................................. 105

MS and MS² spectra of the precursor ion of m/z=345[M-H]⁻ in the incubation of catechin and acrolein in negative electrospray mode…………………………………… 106

The proposed structure of reaction adduct formed by catechin and acrolein. ... 106

HPLC-FLD (Ex=231nm, Em=320nm) chromatogram of the reaction products in the incubation of epicatechin with phosphate buffer (A), epicatechin with glyoxal (B), epicatechin with methylglyoxal (C), epicatechin with malondialdehyde (D) and epicatechin with acrolein (E)..................................................... 107
5-20 MS and MS² spectra of the precursor ions of m/z=347[M-H]⁻ (A), m/z=619[M-H]⁻ (B) and m/z=677[M-H]⁻ (C) in the incubation of epicatechin and glyoxal in negative electrospray mode................................................................. 108

5-21 The proposed structures of reaction adducts formed by epicatechin and glyoxal. ........................................................................................................................................................................................................................................ 109

5-22 MS and MS² spectra of the precursor ions of m/z=361 [M-H]⁻ and m/z 433 [M-H]⁻ in the incubation of epicatechin and methylglyoxal in negative electrospray mode............................................ 110

5-23 The proposed structures of reaction adducts formed by epicatechin and methylglyoxal.................................................................................................................................................................................................................... 110

5-24 MS and MS² spectra of the precursor ion of m/z=433[M-H]⁻ in the incubation of epicatechin and malondialdehyde in negative electrospray mode................................. 111

5-25 The proposed structure of reaction adduct formed by epicatechin and malondialdehyde. .................................................................................................................................................................................................................... 111

5-26 MS and MS² spectra of the precursor ions of m/z=345[M-H]⁻ in the incubation of epicatechin and acrolein in negative electrospray mode. .................................................. 112

5-27 The proposed structures of reaction adducts formed by epicatechin and acrolein.................................................................................................................................................................................................................... 112

5-28 HPLC-DAD (360nm) chromatogram of the reaction products in the incubation of quercetin with phosphate buffer (A), quercetin with glyoxal (B), quercetin with methylglyoxal (C) and quercetin with acrolein (D). ........................................... 113

5-29 MS and MS² spectra of the precursor ions of m/z=341 [M-H]⁻ (A, quercetin and glyoxal), m/z=373 [M-H]⁻ (B, quercetin and methylglyoxal) and m/z=357 [M-H]⁻ (C, quercetin and acrolein) in the incubations of quercetin and reactive carbonyl compounds in negative electrospray mode.......................................... 114

5-30 The proposed structures of reaction adducts formed by quercetin with glyoxal, methylglyoxal, malondialdehyde and acrolein................................................................. 115

5-31 HPLC-FLD (Ex=330nm, Em=374nm) chromatogram of the reaction products in the incubation of resveratrol with phosphate buffer (A), resveratrol with glyoxal (B), resveratrol with methylglyoxal (C), resveratrol with malondialdehyde (D) and resveratrol with acrolein (E). ................................................................. 116

5-32 MS and MS² spectra of the precursor ions of m/z=285 [M-H]⁻ (A, resveratrol and glyoxal), m/z=299 [M-H]⁻ (B, resveratrol and methylglyoxal), m/z=299 [M-H]⁻ (C, resveratrol and malondialdehyde), m/z=283 [M-H]⁻ and m/z=339 [M-H]⁻ (D and E, resveratrol and acrolein) in the incubation of resveratrol and reactive carbonyl compounds in negative electrospray mode. .......................... 117
The proposed structures of reaction adducts formed by resveratrol with glyoxal, methylglyoxal, malondialdehyde and acrolein.
LIST OF ABBREVIATIONS

µg  Microgram
µL  Microliter
µM  Micromolar
AGE Advanced Glycation End products
ANOVA Analysis of Variance
BSA Bovine Serum Albumin
DAD Diode array detector
DMSO Dimethyl sulfoxide
FLD Fluorescent detector
Fluorescent AGEs Fluorescent Advanced Glycation End products.
H  Hour(s)
HPLC High performance liquid chromatogram
M  Mole/Liter
Mg  Milligram
min Minute(s)
mL  Milliliter
mM  Millimolar
m/z Mass to charge ratio
nm  Nanometer
ORAC Oxygen radical absorbance capacity
Trolox 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Oxidation of carbohydrates and proteins in foods and human body generates reactive carbonyls, which include glyoxal, methylglyoxal, malondialdehyde and acrolein. Reactive carbonyls react with proteins to form advanced glycation end-products (AGEs) and cause aging, diabetic complications and other chronic diseases. Previous studies suggested that plant extracts and certain phenolic compounds were able to inhibit AGE formation or directly scavenge reactive carbonyl compounds. Berries and Muscadine grapes are known as good sources of antioxidants and phenolic compounds. Therefore, we hypothesized that they may have the inhibitory effects on AGE formation and direct carbonyl trapping capacities.

Phytochemicals were extracted and purified on adsorption resin to remove sugars. Sugar-free phytochemicals purified from blueberries were fractionated into five fractions using a Sephadex LH-20 column. The resultant extracts and fractions were tested in three AGE generation models that simulated physiological conditions. The model systems included bovine serum albumin (BSA)-fructose, BSA-methylglyoxal and arginine-methylglyoxal models. AGEs were detected using florescence. The capacity of sugar-free phytochemicals to scavenge reactive carbonyl was tested on methylglyoxal.
Catechin, epicatechin, quercetin, chlorogenic acid and resveratrol were used to react with glyoxal, methylglyoxal, malondialdehyde and acrolein. Reaction kinetics were evaluated and phytochemical-carbonyl adducts were tentatively identified using HPLC-ESI-MS$^n$.

Results showed that sugar-free phytochemicals from berries and grapes, and blueberry phytochemical subfractions, inhibited AGE generation in BSA-fructose, BSA-methylglyoxal and arginine-methylglyoxal models by 22-88%. Berry and grape phytochemicals and blueberry subfractions scavenged methylglyoxal by 47-80% within six hours. Pure phytochemicals (catechin, epicatechin, quercetin and resveratrol) reacted with glyoxal, methylglyoxal, malondialdehyde and acrolein and formed various phytochemical-carbonyl adducts.

Results in this study indicated that phytochemicals from berries and grapes are effective AGE inhibitory agents that are useful to alleviate AGE-related chronic diseases. Carbonyl scavenging and adduct formation was one of the mechanisms for berry phytochemicals to inhibit AGE formation.
CHAPTER 1
BACKGROUND

Introduction

Reactive carbonyl compounds, also called reactive carbonyl species, have been studied for decades due to their negative impacts on human health. Methods have been developed to detect their presence in various foods and in the environment. Most of the research has focused on their chemical characteristics, their endogenous generation and reactions with biological macromolecules. The capacity of carbonyls to modify proteins and generate advanced glycation end-products (AGEs) is a major cause of various diseases. A new trend in research is to discover food-source compounds that are able to scavenge reactive carbonyl species and prevent the production of AGEs. Based on literature study, we hypothesized that phytochemicals from edible berries and muscadine grapes are able to scavenge reactive carbonyl species by adduct formation, thus reducing the generation of AGEs.

Reactive Carbonyl Compounds

Reactive carbonyl compounds are a group of compounds that contain one or more carbonyl groups. The most reactive carbonyls are dicarbonyls and α,β-unsaturated aldehydes. Dicarbonyl compounds contain two carbonyl groups in each molecule, such as glyoxal, methylglyoxal and malondialdehyde. α,β-unsaturated aldehydes contain a carbon-carbon double bond in conjugation with an aldehyde group, such as acrolein and 4-hydroxynonenal. Reactive carbonyl compounds react with proteins to generate AGEs, which cause various chronic diseases.
Sources of Reactive Carbonyl Compounds

Reactive carbonyls are absorbed from foods and the environment, or they are generated endogenously. In the environment, small amounts of acetaldehyde, acrolein, glyoxal and methylglyoxal exist (1). Higher amounts of acrolein, methylglyoxal and glyoxal were detected in the air from urban areas than from rural areas (1). Acrolein and methylglyoxal were the major constituents in cooking fumes and cigarette smoke (2). Studies show that reactive carbonyls from the air are absorbed into the blood stream by inhalation (3-4). Reactive carbonyls are also absorbed from diet. These compounds were found in various foods, especially foods which have high lipid content and were processed at a high temperature (5). Acrolein, methylglyoxal and glyoxal have been found in heated foods, such as baked meats. Methylglyoxal was also detected in many sugar-containing foods, including soy sauce and carbonated soft drinks (5-6). The mechanisms of the formation of reactive carbonyls are similar under physiological condition or during food processing. Reactive carbonyls are produced by oxidation in vivo and in vitro. Under physiological condition, they are produced via enzymatic metabolism.

In the first mechanism, carbohydrates, lipids and amino acids, which are abundantly present throughout the body and in foods, are the precursors of reactive carbonyls. Glycoaldehyde, methylglyoxal and glyoxal are generated from carbohydrate or ascorbate metabolism or from their autoxidation (1, 7). Lipids, especially polyunsaturated fatty acids, produce malondialdehyde, acrolein and 4-hydroxynonenal through degradation, oxidation and cleavage reactions (8-10). Reactive carbonyls are also produced as intermediates of amino acid metabolism (10-11).
The oxidation of protein glycation products is another source of reactive carbonyl compounds. Protein glycation, known as the Maillard reaction when it occurs in vitro, is the non-enzymatic reaction between reducing sugars and proteins, eventually generating products with browning, fluorescence and cross-linking characteristics. During the reaction, reducing sugars, for instance glucose, react with amino groups of proteins, producing Schiff bases. Schiff bases undergo Amadori rearrangement to produce relatively stable compounds called Amadori products. At low pH or oxidative conditions, the degradation of Schiff bases or Amadori products generates reactive carbonyls, such as methylglyoxal (1, 12-15). These reactive carbonyls continue to attack macromolecules at a much faster speed than do reducing sugars.

Additionally, reactive carbonyls are generated during enzymatic catalyzed metabolism under physiological conditions. For example, when polyamine oxidase catalyzes the oxidation of spermine to form 3-aminopropanal, acrolein is automatically generated from aminopropanal (16-17). Similarly, acrolein is generated when myeloperoxidase catalyzes oxidation of threonine (18). Methylglyoxal can be formed from ketone bodies, catalyzed by cytochrome P450 enzymes (19).

**Reactivity of Reactive Carbonyl Compounds**

Most carbonyls produced from macronutrient oxidation and Amadori products degradation are highly reactive. The most reactive carbonyls are dicarbonyls (glyoxal, methylglyoxal and malondialdehyde) and α,β-unsaturated aldehydes (acrolein and 4-hydroxynonenal) (Figure 1-1)(8). The carbonyl groups of these compounds are electrophilic, readily attacking the amino groups of amino acids in proteins. The reaction rate between reactive carbonyl compounds and proteins is much faster than that between sugars and proteins. For example, glyoxal and methylglyoxal react 20 times
faster than sugars to form protein adducts (20). α,β-unsaturated aldehydes contain a C2=C3 unsaturated double bond linked to the C1 aldehyde group. Therefore, the C3 carbon is a strong electrophile which can undergo Michael addition to the nucleophilic groups on proteins, DNAs and lipids (11, 13, 21). These reactions toward macromolecules cause structural and functional changes of proteins, DNAs and lipids. Reactive carbonyls cause various adverse biological effects, mainly through the formation of AGEs.

**Advanced Glycation End products (AGEs)**

Advanced glycation end-products are a group of compounds with diverse molecular structures and biological functions. They are primarily formed from reactive carbonyls and proteins (12, 22) and have various characteristics, including browning, fluorescence and cross-linking. When similar reactions occur in food, some of the AGEs are known as Maillard reaction products (23). Generally, AGEs are formed from the reaction between sugars and proteins, followed by the oxidation of Amadori products. Pentosidine and N-(carboxymethyl) lysine are such examples. They can also be generated from the cross-linking between reactive carbonyls and proteins. Reactive carbonyls target the amino, guanidinum and sulphydryl functional groups of intracellular and extracellular proteins. Reactions between reactive carbonyl compounds and ε-amino group on lysine or the guanidinium group on arginine results in AGEs such as N-(carboxymethyl) lysine, pyrraline, methylglyoxal-derived lysine dimer and glyoxal-derived lysine dimer (12). Structures of N-carboxymethyl lysine, pyrraline and pentosidine are shown in figure 1-2.

Since reactive carbonyls are able to modify protein structures, the corresponding protein functions can be changed. Reactive carbonyls are able to react with proteins
with various functions, causing deactivation of membrane transporters, enzymes, signaling components, transcription factors, eventually causing protein degradation or cytotoxic effects (24-26). For example, methylglyoxal inhibits mitochondrial respiration and glycolysis by inactivating membrane ATPase and glyceraldehydes-3-phosphate dehydrogenase (14). Reactive carboxyls reduce the intracellular level of glutathione and lead to increased oxidative stress (27).

The formation of AGEs is accelerated under oxidative conditions. In the presence of transition metal ions, sugars undergo auto-oxidation to form hydrogen peroxide and keto aldehydes which speed up the formation of AGEs (28). Once formed, AGEs are more susceptible to degradation and proteolysis when compared to the original proteins. Accumulated AGEs have negative biological effects, causing various cellular process disruptions. Studies have shown that AGEs are the causing factor of many pathological conditions, including diabetic complications, atherosclerosis, Alzheimer’s disease, cataracts, aging and other chronic diseases (1, 9, 13, 29-30). AGEs contribute to diabetic complications through a series of pathological changes, such as increasing atherogenicity of low density lipoprotein, increasing membrane permeability and decreasing insulin binding to its receptors (11, 31-32). AGEs also act as ligands and bind to the AGE receptors on the cell membrane to induce signal cascades, causing inappropriate cellular activities and gene expressions (12). Advanced or intermediate glycation products induce free radical production in vitro and in vivo (33-34). Therefore, the formation of AGEs can trigger an oxidative environment in which more AGEs are generated.
Berries and Muscadine Grape

Berries, such as blueberries, strawberries, raspberries, blackberries and cranberries are popular fruits throughout the United States. Muscadine grapes are cultivated and consumed in the southwestern United States. They are known to contain a wide variety of antioxidant phytochemicals with diverse health benefits.

Health Related Benefits

Epidemiological studies suggest a phytochemical-rich diet with fruits and vegetables decreases the incidence of chronic diseases. Most edible berries have high antioxidant capacities, which are considered important factors for health. They are effective in stimulating apoptosis and inhibiting the proliferation of cancer cells both in vivo and in vitro (35-37). Blueberries inhibited the development of hemangioendothelioma (38), and reduced DNA damage and lipid peroxidation in vivo (39-40). Phenolic compounds from berry extracts inhibited the growth of food borne bacteria and gastrointestinal pathogens, including Escherichia coli, Salmonella enteric and Staphylococcus aureus (41). Clinical research has also shown that cranberry effectively inhibits urinary tract infections (42).

Phytochemicals in Berries and Muscadine Grapes

Berries and grapes contain phenolic phytochemicals of various structures. Major phenolic compounds in berries are anthocyanins, hydrolysable and condensed tannins, flavonoids, phenolic acids and stilbenoids. Flavonoids in berries include anthocyanins, flavonols and flavan-3-ols. Anthocyanins give the red, blue and purple color to berries. Oligomers and polymers of proanthocyanidins were found in blueberries, strawberries, cranberries, blackberries, and raspberries (43). Ellagictannins were found in strawberries, blackberries, raspberries, and Muscadine grapes (44-48).
Reactions Between Phytochemicals and Reactive Carbonyls

Because reactive carbonyl compounds react with proteins to generate AGEs, causing diseases and aging, drugs have been designed to scavenge reactive carbonyls and reduce the production of AGEs. Several synthetic compounds with carbonyl trapping capacity have been tested to prevent and treat diabetic complications. These compounds include aminoguanidine, OPB-9195 [(±)-2-isopropylidenehydrazono-4-oxothiazolidin-5-ylacetanilide] and penicillamine. However, clinical applications of these medications were not successful due to high dose requirement and hepatotoxicity in diabetic patients (49).

Previous research suggested that phenolic compounds in foods can scavenge reactive carbonyls and inhibit AGE generation. For instance, catechin, epicatechin, epigallocatechin, epigallocatechin 3-gallate from green tea and theaflavin 3,3’-digallate from black tea were found to inhibit different stages of protein glycation (6, 50). Proanthocyanidin monomer and oligomers from cinnamon bark directly trapped methylglyoxal by forming adducts (51). Phloretin and other phenolic compounds effectively quenched α,β-unsaturated aldehydes (52). Carbonyl-phytochemical adducts have been isolated and identified for catechin, epigallocatechin-3-gallate, phloretin and resveratrol (52-56).

Research Objectives

1. To evaluate fluorescent AGE generation in different model systems.

2. To extract sugar-free phytochemicals from berries and muscadine grapes; and to investigate their capacities of inhibiting AGE generation and scavenging reactive carbonyls.

3. To fractionate blueberry phytochemicals and investigate their carbonyl scavenging capacities and antiglycation effects.
4. To investigate reaction kinetics between pure phytochemicals and reactive carbonyls and tentatively identify phytochemical-carbonyl adducts on HPLC-ESI-MS.

Figure 1-1. Structures of reactive carbonyls.

Figure 1-2. Structures of selected advanced glycation end products (AGEs).
Stable model systems are needed to study the inhibitory effects of phytochemicals on AGE production. An ideal model system provides a good simulation of physiological conditions and produces a sufficient amount of AGEs that can be easily detected. Several AGE-generation model systems have been used in previous research. The performances of these model systems have not been evaluated. The objective of this chapter was to evaluate fluorescent AGEs generation in different model systems and identify appropriate models for future studies.

**Materials and Methods**

**Chemicals**

Glyoxal (40% solution in water) was a product from Acros Organics (Morris Plains, NJ). Methylglyoxal (40% aqueous solution) was purchased from MP Biomedicals, LLC (Solon, OH). Bovine serum albumin (BSA), glucose, fructose, ribose, galactose, ascorbic acid, arginine, lysine, sodium azide, monobasic and dibasic sodium phosphate, and 96-well plates with clear bottom wells were purchased from Fisher Scientific Co. (Pittsburg, PA).

**Model System for Fluorescent AGEs Formation**

**BSA-monosaccharide model:** BSA (20 mg/ml, 1 ml) was mixed separately with 1 ml of glucose, fructose, ribose, galactose or ascorbic acid (1.0 M). The mixtures were incubated at 37°C, with sodium azide (0.02%, w/v) serving as an aseptic agent.

**BSA-Carbonyl model:** BSA (20 mg/ml, 1 ml) was mixed individually with 1 ml of glyoxal or methylglyoxal (40 mM). The mixtures were incubated at 37°C with 0.02% (w/v) of sodium azide.
**Amino acid-monosaccharide model:** lysine and arginine (40 mM, 1ml) were mixed separately with 1 ml of glucose, fructose or ribose (1.0 M). The mixtures were incubated at 37°C with 0.02% (w/v) of sodium azide.

**Amino acid-carbonyl model:** methylglyoxal and arginine (40 mM each, 1ml each), glyoxal and lysine (40 mM each, 1ml each) were mixed and incubated. The mixtures were incubated at 37°C with 0.02% (w/v) of sodium azide.

All reagents were dissolved in 50 mM phosphate buffer (pH 7.4). Incubation mixtures were placed in 10-ml screw capped glass tubes and were kept in an incubator at 37 °C (Model 304, Lab-Line Instrument, Melrose Park, IL). Individual reactants in phosphate buffer were incubated in the same conditions as the blank controls. Incubations were conducted in triplicates.

**Emission and Excitation Spectra of Fluorescent AGEs**

After incubation for three days, incubation media (20 µl) was injected into an Agilent 1200 fluorescence detector (Agilent Technologies, Palo Alto, CA) without column separation. Emission spectra were obtained using 280 nm as the excitation wavelength. Excitation spectra were obtained using 420 nm as the emission wavelength. Spectra were recorded using ChemStation software (Version B 01.03, Agilent Technologies, Palo Alto, CA).

**Fluorescent Intensity Measurement**

Every 24 h, a 200-µl aliquot from each incubation media were transferred into a 96-well plate with clear, flat bottom to measure fluorescent intensity on a microplate reader (Spectra XMS Gemini, Molecular Device, Sunnyvale, CA). Fluorescent intensity
was recorded and plotted against incubation time until the fluorescent reading reached a plateau.

**Data Expression and Grafting**

Data are expressed as mean±standard deviation for triplicate tests. Data grafting was done with SigmaPlot (Version 11.0, Systat Software Inc, San Jose, CA).

**Results**

**Fluorescent AGEs Formation**

Excitation and emission spectra of BSA-fructose system are shown in Figure 2-1. Wavelength of maxima excitation and emission for different incubation models are listed in Table 2-1. The majority of the incubation models gave maxima excitation at about 340 nm. Wavelength of maxima emission fell in a range of 380 nm to 420 nm. Wavelength of maxima excitation and emission for BSA solution were 270nm and 400 nm, respectively. After BSA was incubated with carbonyl sources, its wavelength of maxima excitation and emission shifted, suggesting structural modification of BSA and generation of AGEs. This is in agreement with a previous study, which showed the maxima excitation and emission of BSA changed to 325 nm and 382 nm, respectively, after incubating with methylglyoxal at 37 °C for six days. Modification of BSA by reactive carbonyl compounds includes irreversible and reversible modifications, targeting mainly arginine, lysine and cysteine residues. For instance, methylglyoxal modifies BSA irreversibly, mainly with arginine residues, and reversibly binds to BSA with arginine, lysine and cysteine residues in the ratio 110:51:0.6. Complex protein-carbonyl adducts, such as imidazolone, contribute to fluorescence (57).

Fluorescent intensities increased with incubation time in most model systems. Among BSA-monosaccharide models (Figure 2-1), the BSA-ribose mixture showed the
most remarkable increase in fluorescent signal. However, it had higher standard deviations than other BSA-monosaccharide models. Fluorescent intensities in BSA-fructose and BSA-galactose systems increased gradually with incubation time and reached maxima value within a week. Data from these two systems showed lower standard deviations, suggesting these two systems are more stable. The increase of fluorescent intensities from BSA-glucose and BSA-ascorbic acid models were not as prominent as the other three models. The fluorescent intensity generated in the BSA-fructose model was much higher than that in the BSA-glucose model. This is in agreement with the work by Lee et al. (25). The BSA-fructose model was chosen for anti-glycation experiment in future studies.

When BSA was incubated with glyoxal or methylglyoxal (Figure 2-3), the fluorescent intensity increased similarly to that in the BSA- monosaccharide models. The fluorescent intensity was much less in the BSA- methylglyoxal model than in the BSA- glyoxal model after one day of incubation. However, since the readings in the BSA- methylglyoxal model increased more rapidly, there was no significant difference between the two models after six days of incubation. The BSA- methylglyoxal model was chosen to represent protein and reactive carbonyl compound reactions.

Models of amino acids and monosaccharides (fructose/ ribose/ glucose) (Figure 2-4) were designed to produce pentosidine, a specific advanced glycation end-product. However, the fluorescent intensity generated from these models showed a lot of fluctuation. It suggested that fluorescent detection may not be suitable to monitor AGE generation in amino acid- monosaccharide models. Therefore no model was chosen from this category.
In the case of amino acid-reactive carbonyl compound models (Figure 2-5), the fluorescent reading increased dramatically in the arginine-methylglyoxal model, with a small standard deviation during the incubation period. But the fluorescent reading was only slightly decreased in the lysine-glyoxal model. Thus, the arginine-methylglyoxal model was selected to represent reactions between amino acids and reactive carbonyl compounds.

Discussion

Reactive carbonyl compounds originated from reducing sugars react with proteins to generate advanced glycation end-products. More than thirty AGEs have been identified. They were generated from carbonyl compounds and proteins of different structures and modification sites (58). Fluorescent detection is a convenient way to monitor AGE generation (14, 59-60). However, this method is not able to detect AGEs that do not fluoresce, such as N-carboxymethyl lysine (11).

BSA-fructose, BSA-methylglyoxal and arginine-methylglyoxal were selected for further experiments, representing the reactions between proteins and sugar, proteins and carbonyls, and amino acids and carbonyls. Methylglyoxal was reported as a potent agent for AGE generation. It modified proteins reversibly or irreversibly (57) by targeting the side chains of arginine (14) at a much faster rate than reducing sugars.

Phosphate buffer saline (50 mM) was used as the matrix, because the protein modification by carbonyls required a sufficient ionic strength (14, 57). The concentration of phosphate buffer used for this experiment was slightly higher than the physiological condition and was sufficient to maintain protein glycation. However, further increase of the concentration of phosphate buffer to 250 and 500 mM did not shorten the incubation period (14, 57, 61). In the BSA-fructose model, fluorescent reading reached a plateau
after six days. It suggested the modification sites on BSA, such as side chains of arginines, lysines and cysteines, were saturated.

In conclusion, BSA-fructose, BSA-methylglyoxal and arginine-methylglyoxal models were stable systems in generating fluorescent AGEs. These model systems are suitable to test the antiglycation effect of berries in future studies.
Table 2-1. Wavelength of maxima excitation and emission of fluorescent AGEs in various model systems.

<table>
<thead>
<tr>
<th>Incubations</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-glucose</td>
<td>280</td>
<td>410</td>
</tr>
<tr>
<td>BSA-fructose</td>
<td>340</td>
<td>420</td>
</tr>
<tr>
<td>BSA-galactose</td>
<td>340</td>
<td>410</td>
</tr>
<tr>
<td>BSA-ribose</td>
<td>340</td>
<td>420</td>
</tr>
<tr>
<td>BSA-ascorbic acid</td>
<td>280</td>
<td>305</td>
</tr>
<tr>
<td>BSA-glyoxal</td>
<td>360</td>
<td>430</td>
</tr>
<tr>
<td>BSA-methylglyoxal</td>
<td>280</td>
<td>375</td>
</tr>
<tr>
<td>Arginine-lysine-glucose</td>
<td>320</td>
<td>410</td>
</tr>
<tr>
<td>Arginine-lysine-fructose</td>
<td>322</td>
<td>425</td>
</tr>
<tr>
<td>Arginine-lysine-ribose</td>
<td>340</td>
<td>380</td>
</tr>
<tr>
<td>Arginine-methylglyoxal</td>
<td>340</td>
<td>380</td>
</tr>
<tr>
<td>Lysine-glyoxal</td>
<td>350</td>
<td>420</td>
</tr>
</tbody>
</table>

Figure 2-1. Excitation (A) and emission (B) spectra of BSA-fructose model.
Figure 2-2. Fluorescent AGE generation in bovine serum albumin (BSA)-monosaccharide incubation models at 37 °C. Data are mean± standard deviation for three independent tests.

Figure 2-3. Fluorescent AGE generation in bovine serum albumin (BSA)/reactive carbonyl incubation models at 37 °C. Data are mean± standard deviation for three independent tests.
Figure 2-4. Fluorescent AGE generation in amino acids- monosaccharide incubation models at 37 °C. Data are mean± standard deviation for three independent tests.

Figure 2-5. Fluorescent AGE generation in amino acid- reactive carbonyl incubation models at 37 °C. Data are mean± standard deviation for three independent tests.
CHAPTER 3
ANTIGLYCATION EFFECT OF BERRY AND GRAPE PHYTOCHEMICALS

Over production of carbonyls causes AGEs production and other adverse biological effects that are collectively called carbonyl stress. Carbonyl stress can be alleviated by using carbonyl scavengers. Berries and grapes are known as rich sources of antioxidants and phenolic phytochemicals (44, 48, 62-63). Eating berries has been shown to reduce oxidative stress in animals and in humans (38-40, 64-65). However, it is not known if berries and grapes can reduce carbonyl stress. The objective of this chapter was to investigate the carbonyl trapping capacities and inhibitory effects on AGE generation by berry and grape extracts.

Materials and Methods

Chemicals and Materials

Strawberries, cranberries and raspberries were purchased from local supermarkets. Southern high-bush blueberries, blackberries and noble muscadine grapes were obtained from Straughn farm (Waldo, Florida), Coggins Farms (Plan City, Florida) and Sirvent’s Vineyard (Florahome, Florida), respectively. AAPH (2,2’-azotis(2-amidinopropane)) was a product of Wako Chemicals Inc. (Bellwood, RI). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (+)-catechin and (-)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO). Aminoguanidin was a product from Acros Organics (Morris Plains, NJ). Amberlite XAD-7 resin was a product of Rohm and Haas Co. (Philadelphia, PA). HPLC grade methanol and other chemicals were purchased from Fisher Scientific Co. (Pittsburg, PA).
Extraction and Purification of Sugar-free Phytochemicals

The phytochemicals extraction and purification procedure is depicted in a flow chart in Figure 3-1. Frozen berries (blueberries, strawberries, cranberries, raspberries and blackberries) and muscadine grapes (Noble cultivar) were thawed under room temperature. Fruits (200 g) were blended with methanol (200 ml with 1% formic acid) in a kitchen blender. The mixture was sonicated in a water-bath sonicater (FS30, Fisher Scientific) for 10 min, then kept at room temperature for 20 min and filtered through Whatman no.4 filter papers. The extraction was repeated once on the remaining solid and the two methanol extracts were combined. The extracts were transferred to weighted containers and solvent was removed using a SpeedVac concentrator (Thermo scientific ISS110, Waltham, MA) at 25°C. The dried crude extracts were stored at 4 °C.

Amberlite XAD-7 resin was used to remove sugars from the extracts. Resin was suspended in 80% methanol and packed into a glass column. Resin (70g) in the column was cleaned by the following process: washed by 300 ml of methanol, 800 ml of distilled water, 300 ml of 0.2% sodium hydroxide, followed by distilled water, until elute was neutral, then 300 ml of methanol followed by distilled water, until all methanol was removed. After cleaning, the resin was ready for sample loading.

Dried berry crude extract was re-dissolved in 20 ml of distilled water containing 1% formic acid and loaded to an Amberlite XAD-7 resin column. After sample loading, elution was halted for 10 min to facilitate the adsorption of phytochemicals on resin beads. Sugars in the crude extracts were removed by elution with 600 ml of acidified (1% formic acid) water. Phytochemicals absorbed on resin were recovered by 250 ml of
methanol (80%, with 1% formic acid). Elutes from resin column were dried on SpeedVac concentrator, and the weights of the dried phytochemicals were recorded.

**Analysis of Sugars in Phytochemical Extracts**

Sugar analysis was conducted using a Hitachi HPLC system with a refractive index detector and a Shodex SP0810 column (300 mm × 8 mm, Shodex, Colorado springs, CO) with a SP-G guard column (2 mm × 4 mm). An isocratic solvent delivery of water was run at 1.0 mL/min. Crude extracts (from raspberries and cranberries) and purified sugar-free extracts (from blueberries, strawberries, cranberries, raspberries, blackberries and noble grapes) were dissolved in distilled water to a concentration of 15 mg/ml. Sample injection volume was 5 µL. Sucrose, glucose, fructose, lactose and maltodextrin were used as standards. Each sample was analyzed in duplicates.

**Folin-Ciocalteu Assay**

The total phenolic contents of berry and grape sugar-free extracts were determined by Folin-Ciocalteu assay which was modified from a published method (66). Sugar-free phytochemical extracts were dissolved in phosphate buffer (50 mM, pH 7.4) with 5% dimethyl sulfoxide (DMSO) at a concentration of 0.75 mg/ml. One hundred microliter of each was mixed with Folin-Ciocalteu reagent (1 ml, 0.2 N) and sodium carbonate (1 ml, 15%). Gallic acid solutions with concentrations ranging from 100-600 mg/L were used to generate a standard curve. Absorption at 765 nm was measured on a microplate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA) after 30 min of incubation at room temperature. Results of total phenolics were expressed as milligrams gallic acid equivalents per milligram of sugar-free extract (mg GAE/mg).
Oxygen Radical Absorbance Capacity

The oxygen radical absorbance capacity (ORAC) assay used a published method with minor modification (67). All sugar-free extracts were dissolved in phosphate buffer (50 mM, pH 7.4) with 5% dimethyl sulfoxide (DMSO) at a concentration of 15 µg/ml. Fifty microliters were mixed with fluorescein solution (100 µl, 20 nM) in a 96-well microplate with clear, flat bottom. The mixtures were incubated at 37 °C for 10 min before the addition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 50 µl, 0.14 M). Fluorescence was measured on a fluorescent microplate reader (Spectra XMS Gemini, Molecular Device, Sunnyvale, CA) using 485 and 530 nm as the excitation and emission wavelengths. Readings were taken at 1 min intervals for 40 min. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) solutions with concentration of 3.125, 6.25, 12.5 and 25 µM were used to generate a standard curve. The results of the antioxidant capacity were expressed as µmol Trolox equivalents per milligram sugar-free extract (µmol TE/mg).

Total Anthocyanin Content

Total anthocyanin content was measured by using a pH differential assay (68). Blueberry and blackberry extracts (5 mg/ml in methanol) were diluted 80 times in potassium chloride buffer (0.025 M, pH 1) and sodium acetate buffer (0.4 M, pH 4.5), respectively. Other berry and grape extracts (5 mg/ml in methanol) were diluted 20 times in the same buffers. Absorbance at 520 nm and 700 nm were measured on a Life Science UV/Vis spectrophotometer (DU 730, Beckman Coulter, Fullerton, CA) after 30 min of incubation at room temperature. Absorbance (A) was calculated using (A520 – A700)_{pH 1.0} – (A520 – A700)_{pH 4.5}. Total anthocyanin content (µg Cy-G/mg) was calculated using (A × 449.2 × 80 × 1000) / (26900 × 1) for blueberry and blackberry
extracts and \((A \times 449.2 \times 20 \times 1000) / (26900 \times 1)\) for other berry and grape extracts. Results of total anthocyanin content for berry and grape extracts were expressed as milligram cyanidin 3-glucoside equivalent per gram of dried berry and grape extracts (µg Cy-G/mg).

**Total Procyanidin Content**

Total procyanidin in berry and grape extracts were determined using 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method (69). Five milligram of berry and grape extracts were dissolved in 5 ml of acetone: water: acetic acid (70:29.5:0.5, v:v:v) then diluted to a proper concentration with ethanol. An aliquot of 70 µl of diluted berry and grape extracts were mixed with DMAC solution (0.1%, 210 µl) in a 96-well plate. Epicatechin solutions with concentrations ranging from 1-15 µg/ml were used to generate a standard curve. Absorption at 640 nm was measured on a microplate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA) after 30 min of incubation in darkness. Results of total procyanidins were expressed as micrograms epicatechin equivalents per milligram of sugar-free extract (µg epicatechin/mg).

**Antiglycation Effects in BSA-Fructose Model**

Fructose (1.5 M, 1 ml) was mixed with the sugar-free phytochemical extracts, catechin or epicatechin (0.15 mg/ml, 1 ml) in sodium phosphate buffer (50 mM, pH 7.4, with 0.02% sodium azide) in capped test tubes and incubated at 37 °C for 2 h. BSA (30 mg/ml, 1ml) was added in each test tube and the mixtures were incubated in an incubator (Model 304, Lab-Line Instrument, Melrose Park, IL) at 37 °C for six days. BSA and fructose with a phosphate buffer (1 ml) or aminoguanidin (30 mM, 1ml) were used for negative and positive controls. Fluorescent AGEs from each incubation were monitored by taking the fluorescent reading of mixtures (200 µl each) using a microplate
reader (Spectra XMS Gemini, Molecular Device, Sunnyvale, CA) using 340 and 420 nm as the excitation and emission wavelengths.

Samples, blanks and positive controls were prepared in triplicate. Percentage of the AGE inhibition of extracts was calculated by the following equation:

\[
\text{Percentage inhibition} = \left(1 - \frac{\text{Fluorescent intensity with inhibitor}}{\text{Fluorescent intensity without inhibitor}}\right) \times 100\%
\]

**Antiglycation Effects in BSA-Methylglyoxal Model**

Procedure of antiglycation effect in the BSA-methylglyoxal model was similar to that of the BSA-fructose model. Methylglyoxal (60 mM, 1 ml) was incubated individually with phosphate buffer (1 ml, negative control), phytochemical extracts (1.5 mg/ml, 1 ml), catechin (1.5 mg/ml, 1 ml), epicatechin (1.5 mg/ml, 1 ml) or aminoguanidin (30 mM, 1 ml, positive control) in sodium phosphate buffer (50 mM, pH 7.4, with 0.02% sodium azide) at 37 °C for 2 h. BSA (30 mg/ml, 1 ml) was added to each test tube and incubated at 37 °C for six days. The fluorescent intensity was taken for each mixture (200 µl) using 340 and 380 nm as the excitation and emission wavelengths.

Samples, blanks and positive controls were prepared in triplicate. Percentage of the AGE inhibition of extracts was calculated by the following equation:

\[
\text{Percentage inhibition} = \left(1 - \frac{\text{Fluorescent intensity with inhibitor}}{\text{Fluorescent intensity without inhibitor}}\right) \times 100\%
\]

**Antiglycation Effects in Arginine-methylglyoxal Model**

Methylglyoxal (60 mM, 1 ml) was incubated with phosphate buffer (1 ml, negative control), phytochemical extracts (0.75 mg/ml, 1 ml), catechin (0.75 mg/ml, 1 ml), epicatechin (0.75 mg/ml, 1 ml) or aminoguanidin (10 mM, 1 ml, positive control) in 50 mM sodium phosphate buffer (pH 7.4, with 0.02% sodium azide) at 37 °C for 2 h.
Arginine (60 mM, 1ml) was added to each test tube and incubated at 37 °C for six days. Fluorescent signal was taken for each mixture (200 µl) using 340 and 380 nm as the excitation and emission wavelengths.

Samples, blanks and positive controls were prepared in triplicate. Percentage of the AGE inhibition of extracts was calculated by the following equation:

\[
\text{Percentage inhibition} = \left(1 - \frac{\text{Fluorescent intensity with inhibitor}}{\text{Fluorescent intensity without inhibitor}}\right) \times 100\%
\]

**Direct Methylglyoxal Trapping**

Test of the direct methylglyoxal trapping used a published method with minor modifications (51). Methylglyoxal (10 mM) and \(\alpha\)-phenylenediamine (derivatization agent, 50 mM) were freshly prepared in phosphate buffer (50 mM, pH 7.4). Berry and grape phytochemicals were diluted to 2.5 mg/ml. Catechin (5 mM) and aminoguanidine (10 mM) were used as a pure compound control and a positive control, respectively. Methylglyoxal solution (0.125 ml) was mixed with 0.125 ml of phosphate buffer (negative control) or berry phytochemicals and incubated at 37 °C. After incubating for 0.5 h, 1 h, 2 h, 4 h and 6 h, \(\alpha\)-phenylenediamine (0.25 ml) was added into each sample and kept for 30 min for the derivatization reaction to complete. HPLC analysis of incubation media was performed on an Agilent 1200 HPLC system (Agilent technologies, Palo Alto, CA). Compound separation was carried out in a zorbax stablebond analytical SB-C18 column (4.6×250 mm, 5 µm, Agilent Technologies, Palo Alto, CA). Mobile phases were composed of 0.1% formic acid in millipore water (mobile phase A) and pure methanol (mobile phase B). The flow rate was 1 ml/min and the injection volume was 15 µl. The linear gradient for elution was: 0-3 min, 5-50% B; 3-16 min, 50-50% B; 16-17 min, 50-90% B; 17-19 min, 90-90% B; 19-19.5 min, 90-5% B;
followed by 1 min of re-equilibration of the column. Methylglyoxal-o-phenylenediamine adduct, 1-methylquinoxaline, was detected at 315 nm using a diode array detector. Its retention time was 12.9 min. The peak area of 1-methylquinoxaline in each sample was integrated.

The percentage of remaining methylglyoxal was calculated by the following equation:

\[
\text{remaining percentage} = \frac{\text{Peak area at different incubation time}}{\text{Peak area at time zero}} \times 100\%
\]

**Data Expression, Grafting and Statistics**

Samples were analyzed in triplicate and data was expressed as mean ± standard deviation unless otherwise noted. One-way analyses of variance with Tukey-Kramer HSD pair-wise comparison of the means were performed using JMP software (Version 8.0, SAS Institute Inc. Cary, NC). A difference of \( p \leq 0.05 \) was considered significant. Data grafting was done using SigmaPlot (Version 11.0, Systat Software Inc, San Jose, CA).

**Results**

**Phytochemical Extraction and Purification**

Frozen berries and grapes, approximately 200 g, were extracted. After extraction, filtration and solvent evaporation, about 20 g of crude extracts were obtained. The crude extracts were sticky due to the high content of sugars. Sugars were not detected after crude extracts were purified on resins. About one gram of sugar-free extracts was obtained. The weights of frozen fruits, crude extracts and sugar-free extracts are listed on Table 3-1.
Sugar concentration was measured in two crude extracts (raspberries and cranberries) and in all the sugar-free extracts. Sucrose, glucose and fructose were detected in raspberry crude extracts. Glucose and fructose were detected in cranberry crude extracts (Figure 3-2). For both crude extracts, they contained approximately 50% of sugars. In contrast, no sugar was found in any of the sugar-free extracts (Table 3-2), which demonstrated that our sugar-removal process was effective.

**Folin-Ciocalteu Assay**

Sugar-free berry and grape extracts contained more than 0.38 µg gallic acid equivalents per µg extract (Table 3-3). Noble grapes and blueberries had the highest total phenolic content (0.56±0.03 and 0.53±0.01 µg gallic acid equivalents per µg extract). The total phenolic content of strawberries, cranberries and blackberries was significantly lower. Raspberries had the lowest phenolic content, but did not significantly differ from the extracts of strawberries and cranberries.

**Oxygen Radical Absorbance Capacity**

Based on ORAC results, the antioxidant capacities of berry and grape extracts ranged from 4.86-6.91 µmol Trolox per mg extract (Table 3-3). Noble grapes showed the highest antioxidant capacity value (6.91±0.08 µmol Trolox/mg extract). However, unlike the result of the Folin-Ciocalteu assay, blueberries showed the lowest antioxidant capacity value (4.86±0.05 µmol Trolox/mg extract) in the ORAC assay.

**Total Anthocyanin Content**

Total anthocyanin content in berry and grape extracts ranged from 20.9-163.5 µg Cyanidin-3-glucoside per mg extract (Table 3-3). Blackberry extract had the highest total anthocyanin content (163.5±2.19 µg Cy-G/mg), followed by blueberry extract.
(153.5±3.19 µg Cy-G/mg). Noble grape and raspberry extracts showed the lowest total anthocyanin content (23.6 and 20.9 µg Cy-G/mg, respectively) among all the extracts.

**Total Procyanidin Content**

Total procyanidin content in berry and grape extracts ranged from 12.9-118.9 µg epicatechin equivalent per mg extract (Table 3-3). Noble grape extract had the highest total procyanidin content (118.9±5.44 µg epicatechin equivalent/mg). Blueberry and raspberry extracts showed the lowest total procyanidin values (20.9 and 12.9 µg epicatechin/mg, respectively) among all the extracts.

**Anti-glycation Effects in Selected Models**

Berry and grape extracts (0.05 mg/ml) were able to inhibit formation of fluorescent AGEs in the BSA-fructose model by over 60% in six days (Figure 3-3). Among sugar-free berry or grape extracts, strawberry extract showed the highest anti-glycation effect (79.5%), and cranberry showed the lowest (60.1%). Results from the other extracts fell in between. Catechin and epicatechin inhibited fluorescent AGE generation by 81% and 72.5%, respectively. The positive control, aminoguanidine was able to inhibit fluorescent AGE generation by 95%.

In the BSA-methylglyoxal model, antiglycation effects by extracts, catechin and epicatechin (0.5 mg/ml) ranged from 34% to 79% (Figure 3-4). Catechin showed the highest antiglycation effect (79%). Blueberry, cranberry, blackberry, noble grape extracts and epicatechin were not significantly different in terms of AGE inhibition in this assay. They inhibited approximately 60% fluorescent AGE generation. Raspberry had the lowest antiglycation effect in this assay (34%).

Berry and grape extracts (0.25 mg/ml) inhibited fluorescent AGE generation in the arginine-methylglyoxal model over 45% (Figure 3-5). Blueberry blackberry and noble
grape extracts had the highest inhibitory effect. They inhibited 56.8%, 52.2% and 53.3% fluorescent AGE generation, respectively. In this assay, strawberry extract had the lowest anti-glycation effect (45.6%).

**Direct Methylglyoxal Trapping**

The direct methylglyoxal trapping curve was obtained by measuring the amount of methylglyoxal at various incubation periods after they reacted with the tested samples. The chromatograms of methylglyoxal, methylglyoxal with blueberry sugar-free extracts and methylglyoxal with aminoguanidine are shown in Figure 3-6. The direct methylglyoxal trapping curve of berry and grape extracts are presented in Figure 3-7. Results showed that the raspberry extract was able to trap 46.9 % methylglyoxal within 6 h, whereas the other tested berries and grapes had higher capacities, trapping approximately 70 % methylglyoxal within 6 h.

**Discussion**

The results of this chapter showed that sugar-free berry and grape extracts had high total phenolic content, antioxidant capacity and notable anti-glycation effects. The antioxidant properties of many plant extracts have been attributed to their phenolic content. Phenolic compounds play an important role in neutralizing free radicals, quenching singlet and triplet oxygen species and decomposing peroxides. It was known that a good correlation existed between the free radical scavenge activity and the inhibitory effect on AGE generation for pure phenolic compounds and phenolic-rich plant extracts (70-71). Oxidative stress was elevated under hyperglycemic conditions, and free radicals accelerated the formation of AGEs (28). Phenolic compounds inhibit AGE formation in part by functioning as free radical scavengers. In this study, phytochemical extracts with high total phenolic content and antioxidant capacities
showed significant anti-glycation effects. This relation is in agreement with previous studies (72-75).

It was believed that direct trapping of reactive carbonyls was one of the major mechanisms for the inhibition in AGE formation. Results from the direct methylglyoxal trapping assay showed that sugar-free berry and grape extracts drastically decreased the amount of methylglyoxal. Because methylglyoxal is an active intermediate for AGE formation, compounds from berry and grape extracts inhibited AGE generation by directly quenching reactive carbonyl compounds.

A number of plant extracts showed anti-glycation activity in vitro (51, 70, 73, 76-77). Leaves and stems of lowbush blueberries (Vaccinium angustifolium) in different seasons had anti-glycation effects (78). However, anti-glycation effects and carbonyl quenching activities of berries has not yet been studied. Results in this study showed berry and grape extracts effectively prevented the formation of fluorescent AGES and directly quenched methylglyoxal. It is likely that a variety of compounds in berries and grapes act together to inhibit AGE formation.

In conclusion, sugar-free phytochemicals from berries and grapes extracts inhibited AGE generation by 34%-80% in BSA-fructose, BSA-methylglyoxal and arginine-methylglyoxal models. They scavenged 47%-80% of the methylglyoxal within 6 h. Such effects were attributed to their high total phenolic contents and antioxidant capacities.
### Table 3-1. Weight of frozen berries, crude extracts, and sugar-free extracts.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Frozen Berries (g)</th>
<th>Crude Extracts (g)</th>
<th>Sugar-free Extracts (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blueberry</td>
<td>200.8</td>
<td>26.0</td>
<td>839.6</td>
</tr>
<tr>
<td>Strawberry</td>
<td>200.8</td>
<td>19.1</td>
<td>728.8</td>
</tr>
<tr>
<td>Cranberry</td>
<td>200.0</td>
<td>20.5</td>
<td>1048.7</td>
</tr>
<tr>
<td>Blackberry</td>
<td>199.5</td>
<td>18.1</td>
<td>939.9</td>
</tr>
<tr>
<td>Raspberry</td>
<td>200.1</td>
<td>14.2</td>
<td>496.5</td>
</tr>
<tr>
<td>Noble grape</td>
<td>200.6</td>
<td>24.5</td>
<td>1318.9</td>
</tr>
</tbody>
</table>

### Table 3-2. Sugar concentrations in crude and sugar-free extracts.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Samples</th>
<th>Sucrose (%)</th>
<th>Glucose (%)</th>
<th>Fructose (%)</th>
<th>Total Sugar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extracts</td>
<td>Raspberries</td>
<td>13±0.2</td>
<td>16±0.4</td>
<td>24±0.04</td>
<td>53±0.9</td>
</tr>
<tr>
<td></td>
<td>Cranberries</td>
<td>ND</td>
<td>40±0.0</td>
<td>15±0.0</td>
<td>55±0.0</td>
</tr>
<tr>
<td>Sugar-free Extracts</td>
<td>Blueberries</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Strawberries</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Raspberries</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Cranberries</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Blackberries</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Noble grapes</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of three tests.

### Table 3-3. Total phenolic, antioxidant capacity, total anthocyanins and total procyanidins in sugar-free extracts.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Total phenolics (µg GAE/ µg)</th>
<th>Antioxidant capacity (µmol Trolox/mg)</th>
<th>Total anthocyanins (µg Cy-G/mg)</th>
<th>Total procyanidins (µg epicatechin/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blueberries</td>
<td>0.5±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.9±0.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>153.5±3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.9±0.2&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Strawberries</td>
<td>0.4±0.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.1±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43.2±0.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27.7±1.7&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cranberries</td>
<td>0.4±0.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.6±0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.9±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.1±4.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Raspberries</td>
<td>0.4±0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.2±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20.9±0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>12.9±1.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blackberries</td>
<td>0.4±0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.0±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>163.5±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.9±3.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Noble grapes</td>
<td>0.6±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.6±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>118.9±5.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of three individual experiments for all the assays. Values in the same column with different letters denoted significantly differences (P ≤ 0.05) from each other.
Figure 3-1. Extraction and purification of sugar-free phytochemicals from berries and noble muscadine grapes.
Figure 3-2. HPLC chromatogram of sugars in (A) cranberry crude extract and (B) cranberry sugar-free extract. The peaks represent (1) sucrose, (2) glucose and (3) fructose.
Figure 3-3. Anti-glycation effects of berry extracts in bovine serum albumin-fructose model. Bars represent mean±standard deviation of triplicate tests. Bars with different letters denoted significant differences at $p \leq 0.05$. The concentrations of berry extracts, catechin and epicatechin were 0.05 mg/ml. Aminoguanidin (10 mM) served as the positive control.
Figure 3-4. Anti-glycation effects of berry extracts in bovine serum albumin-methylglyoxal model. Bars represent mean±standard deviation of triplicate tests. Bars with different letters denoted significant differences at p ≤ 0.05. The concentrations of berry extracts, catechin and epicatechin were 0.5 mg/ml. Aminoguanidin (10 mM) served as the positive control.
Figure 3-5. Anti-glycation effects of berry extracts in arginine-methylglyoxal model. Bars represent mean ± standard deviation of triplicate tests. Bars with different letters denoted significant differences at $p \leq 0.05$. The concentrations of berry extracts, catechin and epicatechin were 0.25 mg/ml. Aminoguanidin (10 mM) served as the positive control.
Figure 3-6. Chromatogram of methylglyoxal (A), methylglyoxal after reaction with blueberry sugar-free extract (B) and methylglyoxal after reaction with aminoguanidine (C). Methylglyoxal was detected as 1-methylquinoxaline after derivatization using o-phenylenediamine at 315 nm.
Figure 3-7. The percentage of remaining methylglyoxal after reacting with berry and grape extracts for 0.5, 1, 2, 4, 6h. The concentrations of berry extracts and catechin were 2.5 mg/ml and 5 mM, respectively. Data points represent the mean percentage of remaining methylglyoxal with the standard deviation for two independent experiments.
Sugar-free phytochemicals extracted from berries and noble grapes effectively inhibited the formation of AGEs in three model systems. Such effects were attributed to their antioxidant and carbonyl scavenging capacities. Florida is a major producer of blueberries in the US. Most of the Florida blueberries are the early season high-bush cultivars. They are harvested in late April and early May when blueberries from other parts of the US are not available (79). Blueberries have higher market values and per capita consumption compared to raspberries, blackberries, cranberries and muscadine grapes (80-81). The objective of this chapter was to fractionate blueberry phytochemicals, identify major phytochemicals in each fraction, and investigate their carbonyl scavenging capacities and antiglycation effects.

**Materials and Methods**

**Chemicals and Materials**

Sephadex LH 20 was a product from Sigma-Aldrich (Saint Louis, MO). Other chemicals and materials were purchased from different companies as described in *Chapter 2, Chemicals* and *Chapter 3, Chemicals and Materials*.

**Fractionation of Sugar-free Blueberry Phytochemicals**

Procedure for fractionation of sugar-free blueberry extracts is depicted in Figure 4-1. Frozen blueberries (200 g) were used to prepare sugar-free phytochemicals using a procedure described in *Chapter 3, Extraction and Purification of Sugar-free Phytochemicals*. The resultant dried extract was homogenized in water (10 ml) and loaded into Sephadex LH20 (80 g) through a column (2.8×55 cm, 500 ml). Fractions I to V were collected by eluting the column with distilled water (500 ml), methanol (20%, 700
ml), methanol (50%, 700 ml), methanol (70%, 700 ml) and acetone (80%, 500 ml). All solvents used to wash the Sephadex LH-20 column were acidified with 1% formic acid. Solvents in all the fractions were removed and the remaining solids were weighed.

**Phytochemical Identification on HPLC-ESI-MS**

Compound identification for blueberry fractions were performed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector, a fluorescence detector and a HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Compounds were separated on a Zorbax stablebond analytical SB-C18 column (4.6x250 mm, 5 µm, Agilent Technologies, Palo Alto, CA). Millipore water with 0.5% formic acid and pure methanol was used as the mobile phase A and the mobile phase B for elution. Flow rate was 1 ml/min. The linear gradient for elution was: 0-2 min, 5% B; 2-10 min, 5-20% B; 10-15 min, 20-30% B; 15-20 min, 30-35% B; 20-60 min, 35-80% B; 60-65 min, 80-85% B; 65-70 min, 85-5% B; followed by 5 min re-equilibration of the column. Wavelengths were set at 280, 360 and 520 nm for diode array detection. Excitation and emission of the fluorescent detector were set at 230 and 320 nm, respectively. Electrospray ionization at both negative and positive mode (alternative) at the same run was performed using nebulizer 45 psi, drying gas 11 L/min, and drying temperature 350 ºC. A full scan was obtained from m/z 100 to 2200. The most abundant ion in the full scan spectrum was isolated, and the product ion spectra (MS^2) were recorded.

**Total Phenolic Contents, Antioxidant Capacity, Total Anthocyanin Content and Total Procyanidin Content**

Dried blueberry fractions were re-dissolved in phosphate buffer (50 mM, pH 7.4) with 5% dimethyl sulfoxide (DMSO) to a concentration of 15 mg/ml. The blueberry
fractions in the phosphate buffer were further diluted to appropriate concentration for Folin-Ciocalteu assay, ORAC assay, total anthocyanin assay and total procyanidin assay whose procedures were described in Materials and Methods in Chapter 3, Folin Ciocalteu Assay, Antioxidant Capacity, Total Anthocyanin assay and Total procyanidin assay.

**Anti-glycation Effect in Selected Models**

The anti-glycation effects for five blueberry fractions were examined. All fractions were diluted to 0.15 mg/ml for BSA-fructose assay, 1.5 mg/ml for BSA-methylglyoxal assay and 0.75 mg/ml for arginine-methylglyoxal assay. Other procedures to determine anti-glycation effect were described in Materials and Methods in Chapter 3, Anti-glycation Effect in Selected Models. The percentage of inhibited fluorescent AGEs was calculated.

**Direct Methylglyoxal Trapping Assay**

Blueberry fractions were diluted to 2.5 mg/ml for the direct methylglyoxal trapping assay. The procedures of this assay were described in Materials and Methods in Chapter 3, Direct Methylglyoxal Trapping Assay.

**Data Expression, Grafting and Statistics**

Samples were analyzed in triplicate and data was expressed as mean ± standard deviation unless otherwise noted. One-way analyses of variance and Tukey-Kramer HSD pair-wise comparison of the means were performed using JMP software (Version 8.0, SAS Institute Inc. Cary, NC). A difference of p ≤ 0.05 was considered as significant. Data grafting was done with SigmaPlot (Version 11.0, Systat Software Inc, San Jose, CA).
Results

Phytochemical Fractionation

Weights of blueberry extracts and fractions are shown in Table 4-1. Frozen blueberries (201.1 g) were used for the fractionation. The amount of crude extract and sugar-free extract from blueberries were similar to the results in Chapter 3, Extraction and Column Separation. The water fraction (fraction I) yielded 272.1 mg, while fraction II to fraction V weighed from 78.2 to 166.6 mg.

Phytochemical Identification on HPLC-ESI-MS^n

Phenolic compounds in five blueberry fractions were identified based on mass spectra, UV/Vis and fluorescent spectra. HPLC chromatograms of fraction I to fraction V at 280 nm and 520 nm are shown in Figure 4-2 to Figure 4-6. Compounds identified from the different fractions were marked with numbers on the chromatograms and listed in Table 4-2. Fraction I and II contained phenolic acids. The major phenolic acid found in Fraction I and II was chlorogenic acid. Fraction II and III contained anthocyanin monoglycosides. Flavonols and proanthocyanidin monomers and dimers were identified in Fraction IV. Procyanidin oligomers (from monomer to tetramer) were identified in Fraction V. Most of the anthocyanin monoglycosides were eluted with 20% methanol (Fraction II) and 50% methanol (Fraction III). However, a small amount of anthocyanin monoglycosides were detected from Fraction I, IV and V. Anthocyanin diglycoside was not detected in any of the fractions.

The molecular ion at m/z 191 [M-H]^- (compound 1) produced a fragment at m/z 111, which was tentatively identified as quinic acid (82). Compound 2 had m/z 481 [M-H]^- which fragmented into m/z 301, 239 and 175. This compound was tentatively identified as hexahydroxydiphenoyl (HHDP)-glucose (48). The MS spectrum for
compound 3 showed m/z 169 [M-H]⁻ and a fragment at m/z 125 [M-H]⁻, which corresponds to gallic acid (48, 83). The molecular ion at m/z 331 [M-H]⁻ (compound 5) and its fragment at m/z 169 [M-H-162]⁻ were consistent with a gallic acid hexoside. Compound 10 showed m/z 325 [M-H]⁻ and fragment m/z 163 [M-H-162]⁻. It was identified as a coumaric acid hexoside (62). Compound 11 had m/z 353 [M-H]⁻ and fragment m/z 191. It was identified as chlorogenic acid (83). Molecular ions at m/z 463 [M-H]⁻ (compound 13), m/z 447 [M-H]⁻ (compound 15) and m/z 433 [M-H]⁻ (compound 18) produced fragments at m/z 301, 299 and 285, respectively. They were identified as ellagic acid hexoside, ellagic acid rhamnoside and ellagic acid xyloside, respectively (62).

Compounds 6, 7, 8, 9, and 14 were identified as flavan-3-ols and procyanidin oligomers (44). Compounds 8 and compound 14 had m/z 289 [M-H]⁻ and yielded fragments at m/z 245 and m/z 187. They were identified as catechin and epicatechin. Compound 6 had a molecular ion at 577 [M-H]⁻ and fragments at m/z 407 and 287, which was consistent with a procyanidin dimer. The molecular ion at m/z 865 [M-H]⁻ (compound 7) produced fragments at m/z 847, 694 and 575. It was identified as a procyanidin trimer. Compound 9 had m/z 1153 [M-H]⁻ and fragments at m/z 1027, 693 and 576, which was consistent with a procyanidin tetramer. Compound 4 had m/z 305 [M-H]⁻ and produced fragment ions at m/z 261, 247 and 179. This compound was tentatively identified as (epi)gallicatechin (48).

Compounds 12, 16, 17, 19, 20, 21, 22, 23, and 24 were identified as anthocyanins based on UV absorbance (λ_max=520 nm), their fragmentation patterns and previous studies (44, 83). Compounds 12 and 17 had m/z at 465 [M]+ and 435 [M]+. Both of them
fragmented and yielded an ion in the MS² at m/z 303 [M-162]⁺ and [M-132]⁺. They were tentatively identified as delphinidin hexoside and delphinidin xyloside. Compound 16 with m/z 449 [M]⁺ and compound 20 with m/z 419 [M]⁺ fragmented and produced ion with m/z 287 ([M-162]⁺ and [M-132]⁺). They were identified as cyanidin hexoside and cyanidin xyloside. Molecular ions at m/z 479 [M]⁺ (compound 19) and m/z 449 [M]⁺ (compound 21) fragmented to product ions at m/z 317 ([M-162]⁺ and [M-132]⁺), which were consistent with petunidin hexoside and petunidin xyloside. Compound 23 (m/z 493 [M]⁺) and compound 24 (m/z 463 [M]⁺) produced fragment ion at m/z 331 ([M-162]⁺ and [M-132]⁺), which corresponded to malvidin hexoside and malvidin xyloside. Compound 22 had molecular ion at m/z 463 [M]⁺ and fragment ion at m/z 301 [M-162]⁺. It was tentatively identified as peonidin hexoside.

Compound 25 to compound 31 were identified as flavonols (44, 62). Compound 25 (m/z 479 [M-H]-) and compound 26 (m/z 463 [M-H]-) produced a fragment at m/z 317 ([M-H-162]- and [M-H-146]-), which corresponded to myricetin hexoside and myricetin rhamnoside (48). Compound 27 to 29 had molecular ions at m/z 463 [M-H]-, 433 [M-H]- and 447 [M-H]-. These three compounds yielded fragments at m/z 301 ([M-H-162]-, [M-H-132]- and [M-H-146]-). They were identified as quercetin hexoside, quercetin xyloside and quercetin rhamnoside, respectively. Molecular ions m/z 447 [M-H]- (compound 30) and m/z 431 [M-H]- (compound 31) produced fragment at m/z 285 ([M-H-162]- and [M-H-146]-). They were identified as kaempferol hexoside and kaempferol rhamnoside, respectively (48).

**Total Phenolic Content**

The values of the total phenolic content of five blueberry fractions (Table 4-3) were closed to that of blueberry sugar-free extract. Fraction IV showed the highest value of
total phenolic content (0.63±0.02 µg gallic acid equivalents/µg dried fractions). It was significantly higher than that of the blueberry extract (p < 0.0001). Fraction V showed the lowest total phenolic content (0.31±0.01 µg gallic acid equivalents/µg dried fractions). The phenolic content of fraction I and fraction V were significantly lower than that of the blueberry extract (p < 0.0001). Fraction II and III had similar phenolic content to the original blueberry extract.

**Antioxidant Capacity**

Similar to the results of total phenolic content, the antioxidant capacities of the five fractions (Table 4-3) were close to that of the original blueberry extract. Fraction II and fraction IV showed the highest antioxidant capacities (8.20±1.02 and 8.30±0.37 µmol Trolox equivalents/mg dried fraction). Fraction II, III and IV had significantly higher antioxidant capacities than the original extract (P=0.041 for fraction III and P < 0.0001 for fraction II and fraction IV). Fraction V showed the lowest antioxidant capacity (2.53±0.26 µmol Trolox equivalents/mg dried fraction) and was significantly lower than the original extract (p = 0.002).

**Total Anthocyanin Content**

Total anthocyanins of blueberry fractions ranged from 14.8-367.8 µg Cyanidin-3-glucoside per mg dried fraction (Table 4-3). Fraction I had the highest total anthocyanin value (367.7±4.83 µg Cy-G/mg). It was the only fraction that had a significantly higher total anthocyanin value than blueberry extract. Fraction V showed the lowest total anthocyanin values (14.8±0.39 µg Cy-G/mg).

**Total Procyanidin Content**

Total procyanidins of blueberry fractions ranged from 1.3-35.0 µg epicatechin per mg dried fraction (Table 4-3). Total procyanidins in fraction III, IV and V were
significantly higher than blueberry extract. Fraction V had the highest total procyanidin value (35.0±1.21 µg epicatechin/mg). Fraction I showed the lowest total procyanidin values (1.3 ±0.08 µg epicatechin/mg).

**Anti-glycation Effect in Selected Models**

Results of the BSA-fructose model showed that all five fractions inhibited the generation of fluorescent AGES in six days (Figure 4-7). Fraction IV showed the highest anti-glycation effect in this model with 88.7±0.6% of the fluorescent AGES inhibited. Fraction I had the lowest anti-glycation effect (58.5%). They both significantly differed from the original extract (p < 0.0001 for fraction IV and p = 0.021 for fraction I). The anti-glycation effect of fraction II, III and V fell in between and were not significantly different from the blueberry sugar-free extract.

In the BSA-methylglyoxal model, blueberry extract inhibited more than 90% of the formation of fluorescent AGES. Five blueberry fractions were able to inhibit 27% to 65% of the fluorescent AGE generation (Figure 4-8). Among these fractions, fraction III showed the highest anti-glycation effect (64.9%), but it was not significantly different from the blueberry extract. Fraction V showed the lowest anti-glycation effect (27.6%) among all the blueberry fractions.

In the arginine-methylglyoxal model, all fractions inhibited more than 32% of the fluorescent AGE generation (Figure 4-9). Fraction II, III and IV showed 66.2, 62.2 and 69.4% inhibition, respectively. They had significantly higher anti-glycation effects when compared to the original sugar-free extract. Similar to the result in the BSA-methylglyoxal model, fraction V showed the lowest anti-glycation effect (32.4%).
Direct Methylglyoxal Trapping

The chromatograms of methylglyoxal, methylglyoxal with blueberry fraction III and methylglyoxal with aminoguanidine are shown in Figure 4-10. The amount of methylglyoxal decreased gradually when incubated with the blueberry fractions (Figure 4-11). All the fractions scavenged more than 45% of the methylglyoxal within 6 h. Fraction I and Fraction III had the strongest capacity to reduce methylglyoxal (75.7 and 80.6, respectively), while fraction II and fraction V were not as effective (48.5 and 50.4, respectively).

Discussion

The separation mechanism of phytochemicals on Sephadex LH-20 is primarily a normal phase partition with gel permeation also involved. Phenolic acids are eluted with water, whereas most of the anthocyanin monoglycosides are eluted with 20% and 50% methanol.

The total phenolic content, antioxidant capacities as well as the antiglycation effects in selected models varied among the different fractions. Blueberry phytochemical fractions acted either as antioxidants or scavengers of reactive carboxylics. Flavonoids in blueberries are a class of plant phenolics with powerful antioxidant capacity. The structures of flavonoids influence their radical scavenging activity. The radical scavenging activity of flavonoids is enhanced by the high degree of hydroxyl substitution (84-86). Flavonoids may also inhibit AGE generated by chelating transitional metal ions. A study from Urios et al. showed flavonoids prevented metal-catalysed formation of hydroxyl radicals, thus inhibiting protein glycation (86).

The five blueberry fractions contained different types of flavonoids. Fraction I contained phenolic acids, including chlorogenic acid, with a small amount of
anthocyanin monoglycosides. The majority of compounds in fraction II were chlorogenic acid and anthocyanin monoglycosides. Fraction III contained large amount of anthocyanin monoglycosides and procyanidins oligomers (monomer and dimer). Fraction IV was found to have flavonols and procyanidin oligomers (monomer to tetramer). Fraction V contained a small amount of procyanidins.

Various phenolic compounds possess carbonyl trapping ability. Anthocyanins may have potential carbonyl quenching abilities. Anthocyanins react with pyruvic acid and acetaldehyde to form pyranoanthocyanins during red wine aging, contributing to wine color, aroma and taste (87-89). Procyanidin monomer, catechin and epicatechin were potent carbonyl scavengers (90-91). Procyanidin B-type dimer was shown to have methylglyoxal trapping ability as well as anti-glycation property (51, 92). The different antioxidant capacity, anti-glycation effects, and methylglyoxal scavenging ability in the five fractions may be explained by differences in phenolic composition.

In conclusion, five fractions were obtained from a sugar-free blueberry extract. The major compounds in those fractions were phenolic acids, procyanidins, anthocyanins and flavonols. Blueberry fractions inhibited 28%-88% of AGE generation in BSA-fructose, BSA-methylglyoxal and arginine-methylglyoxal models. They scavenged 49%-80% of methylglyoxal in 6 h. Fraction II, fraction III and fraction IV showed higher AGE inhibitory effects, and methylglyoxal trapping capacities than the original blueberry extracts.
### Table 4.1. Weights of blueberry extracts and fractions.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Weight</th>
<th>Major phytochemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen berries</td>
<td>201.1 g</td>
<td>-</td>
</tr>
<tr>
<td>Crude Extract</td>
<td>25.3 g</td>
<td>-</td>
</tr>
<tr>
<td>Sugar-free Extract</td>
<td>925.3 mg</td>
<td>Phenolic acids</td>
</tr>
<tr>
<td>Fraction I</td>
<td>272.2 mg</td>
<td>Chlorogenic acid, anthocyanins</td>
</tr>
<tr>
<td>Fraction II</td>
<td>132.4 mg</td>
<td>Procyanidins, flavonols</td>
</tr>
<tr>
<td>Fraction III</td>
<td>78.2 mg</td>
<td>Procyanidins, flavonols</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>166.6 mg</td>
<td>Procyanidins</td>
</tr>
<tr>
<td>Fraction V</td>
<td>106.4 mg</td>
<td>Procyanidins</td>
</tr>
</tbody>
</table>

### Table 4.2. Tentatively identified compounds in blueberry fractions.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Retention time (min)</th>
<th>MS (m/z)</th>
<th>MS2 (m/z)</th>
<th>MS3 (m/z)</th>
<th>Identified compound</th>
<th>Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.7</td>
<td>191 [M-H]-</td>
<td>111</td>
<td>HHDP-glucose</td>
<td>Quinic acid</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
<td>481 [M-H]-</td>
<td>301, 239, 175</td>
<td>Gallic acid (Epi)galocatechin</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.2</td>
<td>169 [M-H]-</td>
<td>125</td>
<td>Procyandin dimer</td>
<td>III, IV, V</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>11.8</td>
<td>305 [M-H]-</td>
<td>261, 247, 219, 179, 165</td>
<td>Procyandin trimer</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>12.9</td>
<td>331 [M-H]-</td>
<td>169, 125 125</td>
<td>Monogalloyl glucose</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>15.1</td>
<td>865 [M-H]-</td>
<td>847, 740, 694, 575</td>
<td>(Epi)catechin</td>
<td>III, IV, V</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>16.7</td>
<td>289 [M-H]-</td>
<td>245, 203, 187</td>
<td>Procyandin tetramer</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>17.0</td>
<td>1153 [M-H]-</td>
<td>1027, 983, 693, 576</td>
<td>Coumaric acid 4-glucoside</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>18.7</td>
<td>325 [M-H]-</td>
<td>163, 263, 145</td>
<td>Chlorogenic acid</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>19.0</td>
<td>353 [M-H]-</td>
<td>191</td>
<td>Delphiniin hexoside</td>
<td>I, II, III, IV, V</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>20.2</td>
<td>463 [M-H]-</td>
<td>301, 299, 284 256, 176, 125</td>
<td>(Epi)catechin</td>
<td>III, IV, V</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>22.1</td>
<td>435 [M]+</td>
<td>303 257, 229, 151, 257</td>
<td>Delphiniin xylloside</td>
<td>II, III, IV, V</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>22.4</td>
<td>433 [M-H]-</td>
<td>301, 299, 285 257</td>
<td>Ellagic acid xylloside</td>
<td>I, II, III, IV, V</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>22.7</td>
<td>479 [M]+</td>
<td>317 302, 274, 261, 229</td>
<td>Petunidin hexoside</td>
<td>I, II, IV, V</td>
<td></td>
</tr>
<tr>
<td>Compound number</td>
<td>Retention time (min)</td>
<td>MS (m/z)</td>
<td>MS2 (m/z)</td>
<td>MS3 (m/z)</td>
<td>Identified compound</td>
<td>Fractions</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------</td>
<td>----------</td>
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<td>-----------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>20</td>
<td>23.8</td>
<td>419 [M]+</td>
<td>287</td>
<td>213, 187, 149</td>
<td>Cyanidin xyloside</td>
<td>II, III</td>
</tr>
<tr>
<td>22</td>
<td>25.3</td>
<td>463 [M]+</td>
<td>301</td>
<td>286</td>
<td>Peonidin hexoside</td>
<td>II</td>
</tr>
<tr>
<td>26</td>
<td>30.7</td>
<td>463 [M-H]</td>
<td>317, 271</td>
<td>271</td>
<td>Myricetin Rhamnoside</td>
<td>III, IV</td>
</tr>
<tr>
<td>27</td>
<td>32.1</td>
<td>463 [M-H]</td>
<td>301</td>
<td>271, 255, 179, 151</td>
<td>Quercetin hexoside</td>
<td>IV</td>
</tr>
<tr>
<td>28</td>
<td>33.9</td>
<td>433 [M-H]</td>
<td>301</td>
<td>271, 255, 179, 107</td>
<td>Quercetin xyloside</td>
<td>IV</td>
</tr>
<tr>
<td>29</td>
<td>35.6</td>
<td>447 [M-H]</td>
<td>301</td>
<td>275, 257, 229, 165, 137</td>
<td>Quercetin Rhamnoside</td>
<td>IV</td>
</tr>
<tr>
<td>30</td>
<td>36.2</td>
<td>447 [M-H]</td>
<td>327, 285, 256</td>
<td>95.1±4.2c</td>
<td>46.4±0.7e</td>
<td>27.5±1.8b</td>
</tr>
<tr>
<td>31</td>
<td>39.8</td>
<td>431 [M-H]</td>
<td>285, 255</td>
<td>14.8±0.4d</td>
<td>3.8±0.1d</td>
<td>29.1±3.1b</td>
</tr>
</tbody>
</table>

Table 4-3. Total phenolic, antioxidant capacity, total anthocyanins and total procyanidins in blueberry extract and fractions.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total phenolics (µg GAE/µg)</th>
<th>Antioxidant capacity (µmol Trolox/mg)</th>
<th>Total anthocyanins (µg Cy-G/mg)</th>
<th>Total procyanidins (µg epicatechin/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blueberries</td>
<td>0.5±0.0bc</td>
<td>4.9±0.0c</td>
<td>153.5±3.2b</td>
<td>20.9±0.2c</td>
</tr>
<tr>
<td>I</td>
<td>0.4±0.0d</td>
<td>6.0±0.5bc</td>
<td>367.8±4.8a</td>
<td>1.3±0.1d</td>
</tr>
<tr>
<td>II</td>
<td>0.6±0.0b</td>
<td>8.2±1.0a</td>
<td>95.1±4.2c</td>
<td>3.8±0.1d</td>
</tr>
<tr>
<td>III</td>
<td>0.5±0.0c</td>
<td>6.4±0.5b</td>
<td>68.2±2.2d</td>
<td>29.1±3.1b</td>
</tr>
<tr>
<td>IV</td>
<td>0.6±0.0a</td>
<td>8.3±0.4a</td>
<td>46.4±0.7e</td>
<td>27.5±1.8b</td>
</tr>
<tr>
<td>V</td>
<td>0.3±0.0e</td>
<td>2.5±0.3d</td>
<td>14.8±0.4d</td>
<td>35.0±1.2a</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of three individual experiments for all the assays. Values in the same column with different letters denoted significantly differences (P ≤ 0.05) from each other.
Figure 4-1. Fractionation flow chart of blueberry sugar-free phytochemicals.
Figure 4-2. HPLC chromatograms of blueberry fraction I at 280 nm (A) and 520 nm (B). Peaks marked with numbers were tentatively identified. Mass spectral data of identified compounds are listed in Table 4-2.

Figure 4-3. HPLC chromatograms of blueberry fraction II at 280 nm (A) and 520 nm (B). Peaks marked with numbers were tentatively identified. Mass spectral data of identified compounds are listed in Table 4-2.
Figure 4-4. HPLC chromatograms of blueberry fraction III at 280 nm (A), 360 nm (B) and 520 nm (C). Peaks marked with numbers were tentatively identified. Mass spectral data of identified compounds are listed in Table 4-2.

Figure 4-5. HPLC chromatograms of blueberry fraction IV at 280 nm (A) and 520 nm (B). Peaks marked with numbers were tentatively identified. Mass spectral data of identified compounds are listed in Table 4-2.
Figure 4-6. HPLC chromatograms of blueberry fraction V at 280 nm (A) and 520 nm (B). Peaks marked with numbers were tentatively identified. Mass spectral data of identified compounds are listed in Table 4-2.
Figure 4-7. Anti-glycation effects of blueberry extract and fractions in bovine serum albumin-fructose model. The bars showed the mean percentage of fluorescent advanced glycation end product inhibition with their standard deviation for three independent experiments. Bars with different letters denoted significant differences (p ≤ 0.05) from each other. The concentration of fractions, catechin and epicatechin were 0.05 mg/ml. Aminoguanidine (10 mM) served as the positive control.
Figure 4-8. Anti-glycation effects of blueberry extract and fractions in bovine serum albumin-methylglyoxal model. The bars showed the mean percentage of fluorescent advanced glycation end product inhibition with their standard deviation for three independent experiments. Bars with different letters denoted significant differences (p ≤ 0.05) from each other. The concentration of fractions, catechin and epicatechin were 0.5 mg/ml. Aminoguanidine (10 mM) served as the positive control.
Figure 4-9. Anti-glycation effects of blueberry extract and fractions in arginine-methylglyoxal model. The bars showed the mean percentage of fluorescent advanced glycation end product inhibition with their standard deviation for three independent experiments. Bars with different letters denoted significant differences ($p \leq 0.05$) from each other. The concentration of fractions, catechin and epicatechin were 0.25 mg/ml. Aminoguanidine (10 mM) served as the positive control.
Figure 4-10. Chromatogram of methylglyoxal (A), methylglyoxal after reaction with blueberry fraction III (B) and methylglyoxal after reaction with aminoguanidine (C). Methylglyoxal was detected as 1-methylquinoxaline after derivatization using o-phenylenediamine at 315 nm.
Figure 4-11. The percentage of remaining methylglyoxal after been incubated with blueberry extract and fractions for 0, 0.5, 1, 2, 4, 6h. The concentrations of blueberry fractions and catechin were 2.5 mg/ml and 5 mM, respectively. Scattered dots represented the mean percentage of remaining methylglyoxal with the standard deviation for two independent experiments.
CHAPTER 5
REACTIONS BETWEEN PHYTOCHEMICALS AND REACTIVE CARBONYL SPECIES

Sugar-free phytochemicals from berries and noble grapes inhibited AGE formation partly by scavenging reactive carbonyls. Previous research demonstrated that a wide range of phytochemicals exist in berries and grapes (62-63, 93). Flavan-3-ols, flavonols, anthocyanins and chlorogenic acid were identified from fractions of blueberry extract using HPLC-ESI-MS\textsuperscript{n}. No research has been conducted to systematically evaluate their scavenging capacity to different reactive carbonyls. The objective of this chapter was to investigate the reaction kinetics between pure phytochemicals and reactive carbonyls and tentatively identify phytochemical-carbonyl adducts on HPLC-ESI-MS\textsuperscript{n}.

Materials and Methods

Chemicals

Resveratrol was a product from Quality Phytochemicals LLC. (Edison, NJ). (+)-Catechin and (-)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO). Glyoxal (40% solution in water) and quercetin (95% hydrate) were products from Acros Organics (Morris Plains, NJ). Methylglyoxal (40% aqueous solution) and chlorogenic acid were purchased from MP Biomedicals, LLC (Solon, OH). Acrolein (97% stabilized with hydroquinone) was purchased from Alfa Aesar (Ward Hill, MA). Other chemicals and materials were described in previous chapters.

Preparation of Malondialdehyde

Malondialdehyde was produced by hydrolysis of malonaldehyde bis (diethyl acetal or 1,1,3,3-tetraethoxypropane). Malonaldehyde bis (diethyl acetal, 166 µl) was homogenized with hydrogen chloride (90 µl, 1.0 M) at 40 °C for 2 min. Then distilled water (729 µl) was added and the solution was incubated in 40 °C water bath for 1 h for
complete hydrolysis. After incubation, the pH of the solution was adjusted to 7.0 by adding NaOH (15 µl, 6.0 M). This solution was diluted by $10^5$ times and the concentration of malondialdehyde was measured immediately on a spectrophotometer at 267 nm ($\varepsilon_{\text{MDA}}=31500/M\cdot\text{cm}$) (DUseries 700 UV/Vis Scanning Spectrophotometer, Beckman Coulter, Brea, CA) (94). The concentration of malondialdehyde was calculated by the following equation:

$$Concentration\ (\text{mole/L}) = \frac{A}{31500}$$

**Preparation of Phytochemical and Carbonyl Solutions**

Solutions of phytochemicals and reactive carbonyl compounds were freshly prepared before the experiment. In the studies for catechin, epicatechin and chlorogenic acid, chemicals (catechin, epicatechin, chlorogenic, glyoxal, methylglyoxal, malondialdehyde and acrolein) were dissolved or diluted in a 50 mM phosphate buffer (pH 7.4) to a concentration of 10 mM. In the studies for quercetin and resveratrol, these two phytochemicals and other reactive carbonyl compounds were dissolved or diluted in a DMSO: phosphate buffer (50:50, v/v) to a concentration of 10 mM.

**Preparation of Derivatization Reagents**

The derivatization agent was $\alpha$-phenylenediamine for glyoxal and methylglyoxal and 2,4-dinitrophenylhydrazine for acrolein. $\alpha$-phenylenediamine (50 mM) was prepared fresh daily in phosphate buffer (50 mM, pH 7.4). 2,4-dinitrophenylhydrazine solution (6 mM) was prepared weekly by dissolving 62.5 mg of 2,4-dinitrophenylhydrazine crystal and hydrogen chloride (1.0 M, 3 ml) in acetonitrile to a total volume of 50 ml. Trichloroacetic acid buffer and thiobarbituric acid solution were prepared for the detection of malondialdehyde. The trichloroacetic acid buffer (0.61 M)
was produced by dissolving 10 g of trichloroacetic acid in 20 ml distilled water, followed by adding 80 ml of phosphate buffer (50 nM) containing 0.1% ethylenediaminetetraacetic acid (EDTA). The thiobarbituric acid solution (20 mM) was prepared by dissolving 1.441 g thiobarbituric acid in 500 ml distilled water. All solutions were sealed and kept at 4 °C before use.

**Catechin-carbonyl reaction study**

Catechin (10 mM, 1 ml) was incubated with glyoxal, methylglyoxal, malondialdehyde, acrolein (10 mM, 1 ml) or phosphate buffer (50 mM, pH 7.4, 1 ml, control) separately for 0-48 h. Carbonyls (10 mM, 1 ml) were also incubated with phosphate buffer (50 mM, pH 7.4, 1 ml). Incubation was conducted in duplicate. At 0, 0.5, 1, 2, 4, 6, 12, 24 and 48 h of incubation, o-phenylenediamine (50 mM, 200 µl) was mixed with 200 µl of catechin-glyoxal or catechin-methylglyoxal media to terminate the reaction. 2,4-Dinitrophenylhydrazine (6 mM, 300 µl) was mixed with 50 µl of catechin-acrolein media to terminate the reaction. An aliquot of 200 µl catechin control solution, catechin-malondialdehyde media and other catechin-carbonyl media with derivatization agents were transferred into vials for HPLC-ESI-MS\textsuperscript{n} analysis.

Detection of catechin and catechin-carbonyl adducts were performed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector, a fluorescence detector and HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). A zorbax stable-bond analytical SB-C18 column (4.6×250 mm, 5 µm, Agilent Technologies, Palo Alto, CA) was used for compound separation. Mobile phases were composed of 0.1% formic acid in Millipore water (mobile phase A) and pure methanol (mobile phase B). The linear gradient was: 0-5 min, 10-22.5% B; 5-15 min, 22.5-27.5% B; 15-18 min, 27.5-70% B; 18-21 min, 70-90% B; 21-23 min, 90-10%
B, followed by 2 min of re-equilibration. The injection volume was 5 µl and flow rate was 1 ml/min. Peak areas of catechin in catechin-carbonyl samples were integrated and compared to the peak areas of a catechin control. A mass spectrometer with electrospray ionization interface was operated at both positive and negative modes using nebulizer 50 psi, drying gas 10 L/min and drying temperature 300 °C. Retention time for catechin was 12.4 min on a fluorescent chromatogram (excitation 231 nm, emission 320 nm). Mass spectra were scanned from 50 to 800 m/z. The most abundant ions in the full scan spectrum were isolated and their product ion spectra (MS²) were recorded.

Detection of glyoxal and methylglyoxal were performed using the same HPLC system and column. The flow rate was 1 ml/min and the injection volume was 15 µl. The linear gradient for elution was: 0-3 min, 5-50% B; 3-16 min, 50-50% B; 16-17 min, 50-90% B; 17-19 min, 90-90% B; 19-19.5 min, 90-5% B; followed by 1 min of re-equilibration. Glyoxal and methylglyoxal were detected in the form of quinoxaline and 1-methylquinoxaline at 315 nm at 11.7 min and 12.9 min, respectively. The peak areas of quinoxaline in the catechin-glyoxal incubations were integrated and compared with the peak areas in the glyoxal control. Peak areas of 1-methylquinoxaline in the catechin-methylglyoxal incubations were integrated and compared with the peak areas in the methylglyoxal control.

Detection of the remaining acrolein was also completed by the same HPLC system. The mobile phases were 0.1% formic acid in Millipore water (mobile phase A) and pure acetonitrile (mobile phase B). An isocratic elution (1 ml/min) was used containing 40% A and 60% B. An aliquot of 15 µl of catechin-acrolein mixture with
derivatization agent was injected. Acrolein was detected in the form of acrolein-2,4-dinitrophenylhydrazine adduct at 372 nm at 13.8 min. The peak areas of acrolein in the catechin-acrolein incubations was integrated and compared with the peak areas in the acrolein control.

Detection of the remaining malondialdehyde was modified from the method of thiobarbituric acid reactive substances (TBARS). An aliquot of 3 µl of catechin-malondialdehyde media or malondialdehyde control was mixed with trichloroacetic acid buffer (497 µl) and thiobarbituric acid solution (500 µl). A blank was prepared by adding distilled water instead of incubation media. Mixtures were heated in 80 °C water bath for 1 h and cooled in ice. Mixtures were diluted by half with distilled water. An aliquot of 200 µl from each mixture were pipetted into a 96-well plate with clear flat bottom and the absorbance at 530 nm were recorded. Absorbance readings from the catechin-malondialdehyde samples were compared with the malondialdehyde control. Absorbance was measured at the linear range of the plate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA).

**Epicatechin-carbonyl reaction and chlorogenic acid-carbonyl reaction studies**

Procedures followed the *Catechin-carbonyl reaction study*. Epicatechin eluted at 19.4 min and was detected using a fluorescent detector (230 nm excitation and 320 nm emission). Peak of chlorogenic acid eluted at 15.0 min using 330 nm for detection.

**Resveratrol-carbonyl reaction and quercetin-carbonyl reaction studies**

Incubation procedures were the same as *Catechin-carbonyl reaction study*. Detection of resveratrol, quercetin and their carbonyl adducts were performed on the same HPLC-ESI-MS<sup>n</sup> system. The mobile phases were composed of 0.1% formic acid in Millipore water (mobile phase A) and pure acetonitrile (mobile phase B). The linear
gradient was: 0-10 min, 5-30% B; 10-17 min, 30-40% B; 17-20 min, 40-60% B; 20-23 min, 60-5% B, followed by 2 min of re-equilibration. A resveratrol peak appeared at 16.7 min on the fluorescent chromatogram (330 nm excitation/374 nm emission).

Identification of quercetin was done at 360 nm by diode array detector. Detection of other carbonyl compounds, resveratrol-carbonyl adducts and quercetin-carbonyl adducts followed the methods described in *Catechin-carbonyl reaction study*.

**Data Calculation, Grafting and Statistics**

The peak area of each phytochemical and carbonyl compound at different incubation time was integrated. The remaining percentage of phytochemical or carbonyl compound was calculated by the following equation:

\[
\text{remaining percentage} = \frac{\text{Peak area at different incubation time}}{\text{Peak area at time zero}} \times 100\%
\]

The remaining percentage of malondialdehyde was calculated by the following equation:

\[
\text{remaining percentage} = \frac{\text{Absorbance at different incubation time}}{\text{Absorbance at time zero}} \times 100\%
\]

The remaining percentage of the phenolic compounds or carbonyls was plotted against incubation time. Samples were analyzed in duplicate and data was expressed as mean ± standard deviation. One-way analyses of variance with Tukey-Kramer HSD pair-wise comparison of the means were performed using JMP software (Version 8.0, SAS Institute Inc. Cary, NC) for mean comparison at 24 h. A difference of \( p \leq 0.05 \) was considered significant. Empirical degradation half time for phytochemicals and reactive carbonyl compounds were calculated using Excel software (Version 2007, Microsoft Cooperation. Redmond, WA). Data grafting was done using SigmaPlot (Version 11.0, Systat Software Inc, San Jose, CA).
Results

Reaction Kinetics between Phenolics and Carbonyl Compounds

Phenolic compounds were unstable in pH-7.4 buffer. After 48-hour incubation, 90% of catechin, 63% of epicatechin and 44% of chlorogenic acid remained. Quercetin and resveratrol were the least stable compounds among the five. They were not detected at 48 h. The empirical degradation half time of the phytochemicals are listed in Table 5-1.

HPLC analysis with fluorescent detection showed that catechin was more susceptible to glyoxal and acrolein, than to methylglyoxal and malondialdehyde. The empirical degradation half times for catechin were 3.6, 3.6, 7.0 and >48 h when reacted with glyoxal, acrolein, methylglyoxal and malondialdehyde, respectively (Table 5-1). At 48 h, catechin was reduced to 1%, 12%, 27% and 72% of its original level by glyoxal, acrolein, methylglyoxal and malondialdehyde, respectively (Figure 5-1). Similar to the result found in catechin, epicatechin required 4.3, 4.6, 11.7 and >48 h for half reduction when reacted with glyoxal, acrolein, methylglyoxal and malondialdehyde, respectively (Table 5-1). Epicatechin was decreased to 1%, 12%, 24% and 68% of its original level after 48 h incubation with glyoxal, acrolein, methylglyoxal and malondialdehyde, respectively (Figure 5-2).

Chlorogenic acid was decreased by 75% after reacting with glyoxal and methylglyoxal for 48 h. The decrease of chlorogenic acid in the presence of glyoxal and methylglyoxal was more rapid than in the control, with an empirical degradation half time of 9.4 and 9.2 h, respectively (Table 5-1). However, acrolein and malondialdehyde were similar to the buffer control in decreasing chlorogenic acid content (Figure 5-3).
The decrease of quercetin by methylglyoxal and glyoxal was significantly faster than that in the control, with an empirical degradation half time of 5.5 and 6.4 h (Table 5-1). Acrolein slightly sped up the degradation of quercetin. On the other hand, malondialdehyde delayed the degradation of quercetin. At the end of the 48 h, quercetin degraded completely with or without the presence of reactive carbonyl compounds (Figure 5-4).

Resveratrol content decreased after incubation with glyoxal, methylglyoxal and acrolein. It required 3.8, 7.4 and 7.4 h for half reduction when reacted with acrolein, glyoxal and methylglyoxal, respectively (Table 5-1). But the addition of malondialdehyde did not reduce the resveratrol amount compared to the control. After 48 h incubation, resveratrol degraded completely in all tested incubation systems including the controls (Figure 5-5).

The empirical degradation half time for reactive carbonyl compounds are listed in Table 5-2. Catechin, epicatechin, quercetin and resveratrol scavenged more than 90% of glyoxal in 48 hours (Figure 5-6). Glyoxal decreased by half at 3.5, 2.7, 3.6 and 4.3 h when reacted with catechin, epicatechin, quercetin and resveratrol, respectively (Table 5-2). Chlorogenic acid also significantly reduced the amount of glyoxal when compared to the control.

Similar to the results of glyoxal, methylglyoxal showed rapid reduction when incubated with phenolic compounds. Its empirical degradation half time was shorter than 1 h when reacted with catechin, epicatechin, quercetin and resveratrol. Epicatechin was the most efficient agent for methylglyoxal scavenging, followed by catechin,
quercetin and resveratrol. Chlorogenic acid was less effective than resveratrol. (Figure 5-7).

Catechin and epicatechin were the most efficient agents to scavenge malondialdehyde, followed by chlorogenic acid and quercetin. However, resveratrol did not significantly reduce the amount of malondialdehyde. (Figure 5-8).

Although acrolein gradually decreased to 16% in the control solution at 48 h, the addition of phenolic compounds efficiently scavenged 70% of it in 6 h and more than 95% in 48 h compared to the control (Figure 5-9). Catechin and epicatechin were the most efficient agents at lowering the amount of acrolein in solution, followed by quercetin and resveratrol. Chlorogenic acid did not significantly lower the amount of acrolein.

In general, the decrease of phenolic compounds occurred simultaneously with the decrease of carbonyl compounds. The percentage remaining decreased with reaction time.

**Adduct Identification**

HPLC-ESI-MS” analysis was performed to identify adducts that were formed during phytochemical-carbonyls incubation. Adducts were tentatively identified on the basis of [M-H]- and their product ion spectra. Identification was confirmed by comparing part of adducts to those previously identified in research. Phytochemical-carbonyl adducts identified in this thesis are listed in Table 5-3.

**Catechin-carbonyl adducts**

Figure 5-10 shows the HPLC fluorescent chromatogram of catechin control (A), catechin with glyoxal (B), catechin with methylglyoxal (C), catechin with malondialdehyde (D) and catechin with acrolein (E) after six hours of incubation. Three
catechin-glyoxal adducts were tentatively identified (Figure 5-10 B) and their mass spectra are showed in Figure 5-11. The first adduct gave rise to m/z 619 [M-H] and fragment ions with m/z 468, 329, 289 and 245. This was consistent with an adduct that composed of two catechin molecules and a glyoxal molecule in between. The fragment m/z 289 [M-H] was catechin, which lost a –CH2-CHOH- group in the benzofuran skeleton to yield m/z 245. This adduct lost a catechin molecule to yield a fragment m/z 329 [M-catechin-H]. This compound was tentatively identified as a dicatechin-monoglyoxal adduct. The second adduct gave [M-H] at m/z 347 [M-H] and fragment ions of m/z 329, 289, 245, and 205. It was identified as a monocatechin-monoglyoxal adduct. The fragments at m/z 289 were from catechin. Ions 245, 167 and 205 resulted from catechin fragmentation which has been reported previously (95). The third adduct gave [M-H] m/z 637 and a product ion m/z 289 [M-H] formed by catechin fragment. It was tentatively identified as a dicatechin- monoglyoxal adduct. Figure 5-12 shows the proposed structures of catechin-glyoxal adduct and their fragments. Reactive carbonyls attacked the C-6 or C-8 positions on the A-ring of catechin to form adducts (91). Because the positive carbonyl moieties on catechin cannot be confirmed by mass spectrometry, only one isomer of possible adducts are shown in this figure.

Two catechin-methylglyoxal adducts were identified. Their mass spectra are shown in Figure 5-13. The first adduct yielded m/z 361 [M-H] and fragments at m/z 343, 289, 165 and 137. Ion m/z 343 [M-H] was due to water elimination of m/z 361 [M-H]. Ion m/z 289 resulted from catechin. The cleavage of the C-ring of catechin yielded fragments with m/z 165 and 137 (96). This compound was tentatively identified as monocatechin-monomethylglyoxal adduct. The second adduct with m/z 433 [M-H] was
tentatively identified as a monocatechin-dimethylglyoxal adduct. It yielded \textit{m/z} 415 due to water elimination, \textit{m/z} 362 after losing a methylglyoxal moiety and \textit{m/z} 253 after the cleavage of the C-ring of catechin. Figure 5-14 shows the proposed structures of catechin-methylglyoxal adduct and their fragments.

Two catechin-malondialdehyde adducts were identified and their mass spectra are shown in Figure 5-15. An adduct with \textit{m/z} 361 \([\text{M-H}]^\text{−}\) was tentatively identified as a monocatechin-monomalondialdehyde adduct. It yielded a catechin fragment at \textit{m/z} 289 and \textit{m/z} 327 due to water elimination. The second adduct with \textit{m/z} 433 \([\text{M-H}]^\text{−}\) was tentatively identified as a monocatechin-dimalondialdehyde adduct. It produced a fragment at \textit{m/z} 326 \([\text{M}]^\text{−}\) after losing a malondialdehyde moiety. Catechin (\textit{m/z} 289) fragmented to generate \textit{m/z} 163. Proposed structures of adducts and fragments are shown in Figure 5-16.

One catechin-acrolein adduct was identified and its MS/MS\textsuperscript{2} spectra are shown in Figure 5-17. It yielded \textit{m/z} 345 \([\text{M-H}]^\text{−}\) and a catechin fragment at \textit{m/z} 289. Fragments at \textit{m/z} 245 and 215 were generated from catechin, which has been reported \((97)\). Proposed structures of adduct and fragments are shown in Figure 5-18.

\textbf{Epicatechin-carbonyl adducts}

Figure 5-19 shows the HPLC fluorescent chromatogram of epicatechin control (A), epicatechin with glyoxal (B), methylglyoxal (C), malondialdehyde (D) and acrolein (E) after six hours of incubation. Three epicatechin-glyoxal adducts were identified and their mass spectra are shown in Figure 5-20. The first adduct gave \textit{m/z} 347 \([\text{M-H}]^\text{−}\) and was tentatively identified as a monoepicatechin-monoglyoxal adduct. The fragment at \textit{m/z} 329 was due to water elimination. Fragments of \textit{m/z} 289 and 245 were resulted from epicatechin. The second adduct with \textit{m/z} 619 \([\text{M-H}]^\text{−}\) was tentatively identified as a
diepicatechin-monoglyoxal adduct. Its fragmentation pattern was the same as the catechin-glyoxal adduct. The third adduct yielded \( m/z \) 677 [M-H] and was tentatively identified as a diepicatechin-diglyoxal adduct. It produced fragment at \( m/z \) 388 after losing an epicatechin moiety. Fragment at \( m/z \) 347 was generated after losing one glyoxal moiety and one epicatechin moiety. The fragment at \( m/z \) 289 [M-H] was epicatechin. Proposed structures of adducts and fragments are shown in Figure 5-21.

Two epicatechin-methylglyoxal adducts were tentatively identified. Figure 5-22 shows their mass spectra. An adduct gave \( m/z \) 361 [M-H] and was tentatively identified as a monoepicatechin-monomethylglyoxal adduct. Its fragmentation pattern was the same as the monocatechin-monomethylglyoxal adduct. The second adduct yielded \( m/z \) 433 [M-H] and was tentatively identified as a monoepicatechin-dimethylglyoxal adduct. It yielded an epicatechin fragment at \( m/z \) 289 and \( m/z \) 416 due to water elimination. The fragment at \( m/z \) 343 was yielded after losing a methylglyoxal moiety and a water molecule. The fragment \( m/z \) 181 was deduced to be a methylglyoxal linked with a phlorogluclinol molecule after water elimination. Proposed structures for adducts and fragments are shown in Figure 5-23.

One epicatechin-malondialdehyde adduct at \( m/z \) 433 [M-H] was identified as a monoepicatechin-dimalondialdehyde adduct. Its mass spectra are shown in Figure 5-24. It produced a fragment at \( m/z \) 325 after losing a malondialdehyde moiety and two water molecules. Epicatechin (\( m/z \) 289) fragmentated to produce \( m/z \) 245. Figure 5-25 shows the proposed structures of adduct and fragments.

A monoepicatechin-monoacrolein adduct at \( m/z \) 345 [M-H] was identified. Figure 5-26 shows its MS and MS\(^2\) spectra. It yielded fragments at \( m/z \) 289 and 245, which
resulted from epicatechin. A fragment at \( m/z \) 193 was due to the cleavage of C-ring on the epicatechin skeleton (Figure 5-27).

**Quercetin-carbonyl adducts**

Figure 5-28 shows the HPLC chromatogram of quercetin control (A), quercetin with glyoxal (B), methylglyoxal (C) and acrolein (D). The mass spectra for monoquercetin-monoglyoxal adduct (\( m/z \) 341 [M-H]⁻), monoquercetin-monomethylglyoxal adduct (\( m/z \) 373 [M-H]⁻) and monoquercetin-monoacrolein adduct (\( m/z \) 356 [M-H]⁻) are shown in Figure 5-29. They fragmented and produced the quercetin fragment at \( m/z \) of 301 after losing a glyoxal moiety, a methylglyoxal moiety and an acrolein moiety (Figure 5-30).

**Resveratrol-carbonyl adducts**

Figure 5-31 shows the HPLC fluorescent chromatogram of resveratrol control (A), resveratrol with glyoxal (B), methylglyoxal (C), malondialdehyde (D) and acrolein (E). Adducts yielded \( m/z \) 285 [M-H]⁻, \( m/z \) 299 [M-H]⁻, \( m/z \) 299 [M-H]⁻, \( m/z \) 283 [M-H]⁻ (Figure 5-32) and were tentatively identified as a monoresveratrol-monoglyoxal adduct, a monoresveratrol-monomethylglyoxal adduct, a monoresveratrol-monomalondialdehyde adduct and a monoresveratrol-monoacrolein adduct. They produced resveratrol fragments at \( m/z \) 227 after losing a glyoxal, methylglyoxal, malondialdehyde or acrolein moiety. An adduct at \( m/z \) 339 [M-H]⁻ from resveratrol-acrolein incubation was tentatively identified as a monoresveratrol-diacrolein adduct, because it produced a fragment at \( m/z \) 227 (resveratrol) and a fragment at \( m/z \) 283 (monoresveratrol-monoacrolein). Figure 5-33 shows the proposed structures of adduct and fragments.
Discussion

Emerging research suggests plant extracts with high phenolic contents effectively inhibit the generation of AGEs. Phenolic compounds were considered as major candidates for AGE inhibition due to their antioxidant and chelating properties. However, a limited number of studies have investigated their chemical reaction between phenolic compounds and reactive carbonyls. In this study, catechin, epicatechin, chlorogenic acid, quercetin and resveratrol were selected to represent various classes of phenolic compounds in berries and grapes. They were used to react with dicarbonyl compounds and α,β-unsaturated aldehydes. This was the first study that compared the carbonyl scavenging capacity of selected phenolic compounds and the relative stability of phenolic compounds in the presence of carbonyls. Mass spectra data demonstrated that phenolic compounds scavenged reactive carbonyls by forming adducts of different structures.

Catechin and epicatechin were reported to react with glyoxal or methylglyoxal at the C-6 or C-8 position (91). Additional research showed their carbonyl trapping capacity was achieved under physiological conditions. It was also suggested that catechin-methylglyoxal adduct might undergo further metabolisms by enzymes in vivo (71). Our result, for the first time, showed that carbonyls can cross-line two catechins or epicatechins to form dimers.

Stilbene glucosides, including 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside and resveratrol, had been tested for their efficiency in trapping methylglyoxal. Resveratrol was less efficient than other stilbenes (56). In our study, adducts formed between resveratrol and glyoxal, malondialdehyde and acrolein were detected for the first time.
Previous research found that plant extracts high in quercetin exhibited high inhibitory effects on the formation of AGEs (73). Our study confirmed that quercetin quenched carbonyls by forming adducts. Unlike catechin, no carbonyl cross-linked quercetin dimers was identified.

It was reported that chlorogenic acid from *Ilex paraguariensis* was the major anti-glycation agent for BSA and histones (98). Another study indicated *Ilex paraguariensis* extracts had higher inhibitory effects on the formation of AGEs than green tea (99). Adducts formed between chlorogenic acid and carbonyl compounds were not detected in our study. Except for antioxidant effects, mechanisms for chlorogenic acid inhibition AGE formation remain unclear.

The A ring of flavonoids was suggested to be the trapping site for reactive carbonyls, including dicarbonyls and α,β-unsaturated aldehydes. The A rings of epigallocatechin-3-gallate, catechin, phloretin, phloridzin and naringenin were able to undergo nucleophilic substitution by reactive carbonyl compounds (50, 52, 54, 90, 100-102). The carbonyl group of both dicarbonyl compounds and α,β-unsaturated aldehydes can undergo electrophilic substitution to the A ring of flavonoids.

In conclusion, phytochemicals (catechin, epicatechin, quercetin, resveratrol) rapidly reacted with carbonyls and resulted in the formation of phytochemical-carbonyl adducts of different structures.
Table 5-1. Empirical degradation half time of phytochemicals in different incubation mixtures.

<table>
<thead>
<tr>
<th>Time (h) Phytochemicals</th>
<th>Reactive carbonyl compounds</th>
<th>Glyoxal</th>
<th>Methylglyoxal</th>
<th>Malondialdehyde</th>
<th>Acrolein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>--</td>
<td>3.7</td>
<td>7.0</td>
<td>&gt;48</td>
<td>3.6</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>&gt;48</td>
<td>4.3</td>
<td>11.7</td>
<td>&gt;48</td>
<td>4.7</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
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<td>9.4</td>
<td>9.2</td>
<td>&gt;48</td>
<td>47.7</td>
</tr>
<tr>
<td>Quercetin</td>
<td>11.9</td>
<td>6.4</td>
<td>5.5</td>
<td>20.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>34.0</td>
<td>7.4</td>
<td>7.4</td>
<td>35.3</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Table 5-2. Empirical degradation half time of reactive carbonyl compounds in different incubation mixtures.

<table>
<thead>
<tr>
<th>Time (h) Carbonyls</th>
<th>Control</th>
<th>Catechin</th>
<th>Epicatechin</th>
<th>Chlorogenic acid</th>
<th>Quercetin</th>
<th>Resveratrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyoxal</td>
<td>&gt;48</td>
<td>3.5</td>
<td>2.7</td>
<td>28.2</td>
<td>3.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Methylglyoxal</td>
<td>&gt;48</td>
<td>0.4</td>
<td>0.4</td>
<td>11.1</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>&gt;48</td>
<td>28.9</td>
<td>24.5</td>
<td>30.1</td>
<td>&gt;48</td>
<td>&gt;48</td>
</tr>
<tr>
<td>Acrolein</td>
<td>9.5</td>
<td>0.6</td>
<td>0.5</td>
<td>3.7</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Figure 5-1. Percentage of remaining catechin (CAT) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR). Scattered dots represented the mean ± standard deviation of remaining catechin for two independent experiments. Different letters denoted significant differences ($p \leq 0.05$) from each other at 24 h.
Figure 5-2. Percentage of remaining epicatechin (EPI) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR). Scattered dots represented the mean± standard deviation of remaining epicatechin for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
Figure 5-3. Percentage of remaining chlorogenic acid (CGA) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR). Scattered dots represented the mean± standard deviation of remaining chlorogenic acid for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
Figure 5-4. Percentage of remaining quercetin (QUE) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR). Scattered dots represented the mean± standard deviation of remaining quercetin for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
Figure 5.5. Percentage of remaining resveratrol (RES) during 37 °C incubation with phosphate buffer (blank), glyoxal (GO), methylglyoxal (MGO), malondialdehyde (MDA) and acrolein (ACR). Scattered dots represented the mean± standard deviation of remaining resveratrol for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
Figure 5-6. Percentage of remaining glyoxal (GO) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA). Scattered dots represented the mean± standard deviation of remaining glyoxal for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
Figure 5-7. Percentage of remaining methylglyoxal (MGO) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA). Scattered dots represented the mean± standard deviation of remaining methylglyoxal for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
Figure 5-8. Percentage of remaining malondialdehyde (MDA) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA). Scattered dots represented the mean± standard deviation of remaining malondialdehyde for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
Figure 5-9. Percentage of remaining acrolein (ACR) during 37 °C incubation with phosphate buffer (blank), catechin (CAT), epicatechin (EPI), quercetin (QUE), resveratrol (RES) and chlorogenic acid (CGA). Scattered dots represented the mean± standard deviation of remaining acrolein for two independent experiments. Different letters denoted significant differences (p ≤ 0.05) from each other at 24 h.
<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Molecular Weight</th>
<th>MS1</th>
<th>MS2</th>
<th>Adduct Composition</th>
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<td>Catechin-carbonyl systems</td>
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<td></td>
</tr>
<tr>
<td>9.8</td>
<td>348</td>
<td>347 [M-H]^−</td>
<td>329, 289, 245, 205,</td>
<td>Monocatechin + monoglyoxal</td>
</tr>
<tr>
<td>8.2</td>
<td>620</td>
<td>619 [M-H]^−</td>
<td>468, 329, 289, 245</td>
<td>Diccatechin + monoglyoxal</td>
</tr>
<tr>
<td>9.5</td>
<td>638</td>
<td>638 [M]</td>
<td>289</td>
<td>Diccatechin + monoglyoxal</td>
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<tr>
<td>12.4</td>
<td>362</td>
<td>361 [M-H]^−</td>
<td>343, 289, 245, 181</td>
<td>Monocatechin + monomethylglyoxal</td>
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<tr>
<td>20.2</td>
<td>434</td>
<td>433 [M-H]^−</td>
<td>415, 361, 343, 289</td>
<td>Monocatechin + dimethylglyoxal</td>
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<tr>
<td>21.8</td>
<td>362</td>
<td>361 [M-H]^−</td>
<td>327, 289</td>
<td>Monocatechin + monomalondialdehyde</td>
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<td>20.4</td>
<td>434</td>
<td>433 [M-H]^−</td>
<td>289, 163, 326</td>
<td>Monocatechin + dimalondialdehyde</td>
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<td>Epicatechin-carbonyl systems</td>
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<tr>
<td>9.4</td>
<td>348</td>
<td>347 [M-H]^−</td>
<td>329, 289, 245</td>
<td>Monoeipicatechin + monoglyoxal</td>
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<td>620</td>
<td>619 [M-H]^−</td>
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<td>20.0</td>
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<td>677 [M-H]^−</td>
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<td>361 [M-H]^−</td>
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<td>Monoepicatechin + dimethylglyoxal</td>
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<td>25.3</td>
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<td>289, 245</td>
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<td>Chlorogenic acid-carbonyl systems</td>
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<td>ND</td>
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<td>Quercetin-carbonyl systems</td>
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<tr>
<td>12.6</td>
<td>374</td>
<td>373 [M-H]^−</td>
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<td>16.0</td>
<td>358</td>
<td>357 [M-H]^−</td>
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<td>Monoquercetin + monoacrolein</td>
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<td>Resveratrol-carbonyl systems</td>
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<tr>
<td>13.6</td>
<td>286</td>
<td>285 [M-H]^−</td>
<td>267, 227, 161</td>
<td>Monoresveratrol + monoglyoxal</td>
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<td>14.9</td>
<td>300</td>
<td>299 [M-H]^−</td>
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<td>16.0</td>
<td>300</td>
<td>299 [M-H]^−</td>
<td>227</td>
<td>Monoresveratrol + monomalondialdehyde</td>
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<td>283 [M-H]^−</td>
<td>228</td>
<td>Monoresveratrol + monoacrolein</td>
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<tr>
<td>20.7</td>
<td>340</td>
<td>339 [M-H]^−</td>
<td>228, 283</td>
<td>Monoresveratrol + diacrolein</td>
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Figure 5-10. HPLC-FLD (Ex=231nm, Em=320nm) chromatogram of the reaction products in the incubation of catechin with phosphate buffer (A), catechin with glyoxal (B), catechin with methylglyoxal (C), catechin with malondialdehyde (D) and catechin with acrolein (E). Emerging peaks were reaction adducts and labeled with their molecular weight.
Figure 5-11. MS and MS² spectra of the precursor ions of \( m/z=619[M-H]^- \) (A), \( m/z=347[M-H]^- \) (B) and \( m/z=638[M]^- \) (C) in the incubation of catechin and glyoxal in negative electrospray mode.
Figure 5-12. The proposed structures of reaction adducts formed by catechin and glyoxal.
Figure 5.13. MS and MS$^2$ spectra of the precursor ions of $m/z=433$[M-H]$^-$ (A) and $m/z=361$[M-H]$^-$ (B) in the incubation of catechin and methylglyoxal in negative electrospray mode.

Figure 5.14. The proposed structures of reaction adducts formed by catechin and methylglyoxal.
Figure 5-15. MS and MS$^2$ spectra of the precursor ions of $m/z=433$[M-H]$^-$ (A) and $m/z=361$[M-H]$^-$ (B) in the incubation of catechin and malondialdehyde in negative electrospray mode.

Monocatechin+Dimalondialdehyde, MW=434
$m/z=433$ [M-H]$^-$

Monocatechin+Monomalondialdehyde, MW=362
$m/z=361$ [M-H]$^-$

Figure 5-16. The proposed structures of reaction adducts formed by catechin and malondialdehyde.
Figure 5-17. MS and MS² spectra of the precursor ion of $m/z=345$ [M-H]⁻ in the incubation of catechin and acrolein in negative electrospray mode.

Monocatechin+Monoacrolein, MW = 346
$m/z=345$ [M-H]⁻

Figure 5-18. The proposed structure of reaction adduct formed by catechin and acrolein.
Figure 5-19. HPLC-FLD (Ex=231nm, Em=320nm) chromatogram of the reaction products in the incubation of epicatechin with phosphate buffer (A), epicatechin with glyoxal (B), epicatechin with methylglyoxal (C), epicatechin with malondialdehyde (D) and epicatechin with acrolein (E). Emerging peaks were reaction adducts and labeled with their molecular weight.
Figure 5-20. MS and MS\(^2\) spectra of the precursor ions of \(m/z=347\)\([M-H]\) \(\text{(A)}, \ m/z=619\)\([M-H]\) \(\text{(B)}\) and \(m/z=677\)\([M-H]\) \(\text{(C)}\) in the incubation of epicatechin and glyoxal in negative electrospray mode.
Figure 5-21. The proposed structures of reaction adducts formed by epicatechin and glyoxal.
Figure 5-22. MS and MS² spectra of the precursor ions of m/z=361 [M-H]⁻ and m/z 433 [M-H]⁻ in the incubation of epicatechin and methylglyoxal in negative electrospray mode.

Monoepicatechin+monomethylglyoxal, MW = 362 m/z=361 [M-H]⁻

Monoepicatechin+Dimethylglyoxal, MW = 434 m/z=433 [M-H]⁻

Figure 5-23. The proposed structures of reaction adducts formed by epicatechin and methylglyoxal.
Figure 5-24. MS and MS\(^2\) spectra of the precursor ion of \(m/z=433\)[M-H]\(^-\) in the incubation of epicatechin and malondialdehyde in negative electrospray mode.

Monoepicatechin+Dimalondialdehyde, MW=434
\(m/z=433\) [M-H]\(^-\)

Figure 5-25. The proposed structure of reaction adduct formed by epicatechin and malondialdehyde.
Figure 5-26. MS and MS\(^2\) spectra of the precursor ions of \(m/z=345\) [M-H]\(^{-}\) in the incubation of epicatechin and acrolein in negative electrospray mode.

Monoepicatechin+Monoacrolein, MW = 346
\(m/z=345\) [M-H]\(^{-}\)

Figure 5-27. The proposed structures of reaction adducts formed by epicatechin and acrolein.
Figure 5-28. HPLC-DAD (360nm) chromatogram of the reaction products in the incubation of quercetin with phosphate buffer (A), quercetin with glyoxal (B), quercetin with methylglyoxal (C) and quercetin with acrolein (D). Emerging peaks were reaction adducts and labeled with their molecular weight.
Figure 5-29. MS and MS\(^2\) spectra of the precursor ions of \(m/z=341\) [M-H]\(^-\) (A, quercetin and glyoxal), \(m/z=373\) [M-H]\(^-\) (B, quercetin and methylglyoxal) and \(m/z=357\) [M-H]\(^-\) (C, quercetin and acrolein) in the incubations of quercetin and reactive carbonyl compounds in negative electrospray mode.
Monoquercetin+monoglyoxal, MW=360
m/z =341 [M-H-H\textsubscript{2}O]\textsuperscript{-}

Monoquercetin+monomethylglyoxal, MW=374
m/z =373 [M-H]\textsuperscript{-}

Monoquercetin+monoacrolein, MW=358
m/z =357 [M-H]\textsuperscript{-}

Figure 5-30. The proposed structures of reaction adducts formed by quercetin with glyoxal, methylglyoxal, malondialdehyde and acrolein.
Figure 5-31. HPLC-FLD (Ex=330nm, Em=374nm) chromatogram of the reaction products in the incubation of resveratrol with phosphate buffer (A), resveratrol with glyoxal (B), resveratrol with methylglyoxal (C), resveratrol with malondialdehyde (D) and resveratrol with acrolein (E). Emerging peaks were reaction adducts and labeled with their molecular weight.
Figure 5-32. MS and MS\(^2\) spectra of the precursor ions of \(m/z=285\) [M-H]\(^-\) (A, resveratrol and glyoxal), \(m/z=299\) [M-H]\(^-\) (B, resveratrol and methylglyoxal), \(m/z=299\) [M-H]\(^-\) (C, resveratrol and malondialdehyde), \(m/z=283\) [M-H]\(^-\) and \(m/z=339\) [M-H]\(^-\) (D and E, resveratrol and acrolein) in the incubation of resveratrol and reactive carbonyl compounds in negative electrospray mode.
Monoresveratrol+monoglyoxal, MW=286
m/z = 285 [M-H]⁻

Monoresveratrol+monomethylglyoxal, MW=300
m/z = 299 [M-H]⁻

Monoresveratrol+monomalondialdehyde, MW=300
m/z = 299 [M-H]⁻

Monoresveratrol+monoacrolein, MW=284
m/z = 283 [M-H]⁻

Monoresveratrol+diacrolein, MW=340
m/z = 339 [M-H]⁻

Figure 5-33. The proposed structures of reaction adducts formed by resveratrol with glyoxal, methylglyoxal, malondialdehyde and acrolein.
In conclusion, sugar-free phytochemicals extracted from berries and grapes effectively scavenged methylglyoxal. They had potent anti-glycation capacities in BSA-fructose, BSA-methylglyoxal and arginine-methylglyoxal assays. Phenolic compounds from berries and grapes scavenged reactive carbonyls by forming adducts of different structures.


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BIOGRAPHICAL SKETCH

Wei Wang was originally from Guangzhou, China. She received her bachelor’s degree in biotechnology from Sun Yat-sen University in 2007. After that, she entered a nutritional PhD program at the Ohio State University in Columbus, Ohio. Eager to explore her interest in food science, she transferred to the University of Florida and entered the Food Science and Human Nutrition Department in 2008 under the supervision of Dr. Liwei Gu. While in graduate school, Wei had one publication in the Journal of Food Chemistry. Furthermore, she received the William L. and Agnes F. Brown Graduate Scholarship and Outstanding Academic Achievement Certificate from UF in 2010. Upon her completion of the master’s degree in 2010, Wei plans to have a career in the food industry and is looking forward to any challenge that life will bring.