Discovery and dissemination of new knowledge in food science: Analytical methods for quantification of polyphenols and amino acids in fruits and the use of mobile phone-based instructional technology in food science education

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Food Science and Technology

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April 30, 2019

Blacksburg, VA

Keywords: flavanols, Folin-Ciocalteu, Lowenthal permanganate, Prussian blue, DMAC, BSA precipitation, apple juice chemistry, fermentation, engagement, student performance

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ABSTRACT

The discovery and dissemination of new knowledge are essential in food science. To advance our understanding of fruit chemistry, analytical methods were compared and applied. Polyphenols are secondary metabolites in fruits of particular importance in food science, as they contribute to the sensory attributes and health benefits of the products. Evaluation of common analytical methods for the quantification of polyphenols, including the Folin-Ciocalteu (F-C), Lowenthal permanganate (L-P), 4-dimethylaminocinnamaldehyde (DMAC) and the bovine serum albumin (BSA) precipitation methods, was conducted using analytical method validation procedures. The F-C method was not specific to polyphenols, and the L-P method had the widest working range but lacked accuracy. The DMAC method was the most specific to flavanols, and the BSA method was not suitable for quantification of smaller flavanols. Quantitative performance of these four methods was evaluated using a broad range of fruit-derived samples. Variation in quantitative results obtained using these four methods was explained by differences in polyphenol and matrix composition of these samples and differences in operating principles of the methods.

The reactivity of individual polyphenol compounds (catechin, epicatechin, PC B2, PC pentamer, chlorogenic acid, phloretin, and quercetin) to the polyphenol and flavanol quantification results using Prussian blue (P-B), F-C, DMAC and BSA precipitation methods were also assessed and determined to differ by up to thirteen-fold, depending on the assay. Furthermore, the contribution and interactions of polyphenol compounds (catechin, PC B2, and chlorogenic acid) and potentially interfering compounds likely to be found in fruit and fruit products (ascorbic acid, glucose, and SO_2) to the quantitative results of these methods were evaluated using a full factorial design. Significant interactions among polyphenol compounds, and among the interfering compounds were found. The standardized coefficient (β) for all factors and interactions of polyphenol compounds varied from 0.347 to 129, and from near 0 to -46.8 for all factors and interactions of interfering compounds. Our findings indicate that the choice of standards, polyphenol and matrix composition of the sample may cause disparity among the quantitative results of these methods.

Amino acids in apple (*Malus* × *domestica* Borkh.) juice not only influence the quality of fermented cider through fermentation kinetics, but also impact the flavor of the cider through yeast metabolism. Due to recent advances in analytical instrumentation, amino acids profiles in apple juice were determined much faster and more accurately than by previously applied methods. Twenty amino acids were quantified by UPLC-PDA in juices from 13 apple cultivars grown in Virginia. The relative amino acid profile was significantly different among the apple juices evaluated. The total amino acid concentration ranged from 18 mg/L in Blacktwig juice to 57 mg/L in Enterprise juice. L-Asparagine, L-aspartic acid and L-glutamine are the principal amino acids observed in most apple juices. These results will inform future research on yeast metabolism and nitrogen management during cider fermentation.

To better disseminate knowledge gained through research to the next generation of food scientists, the effectiveness of a new instructional technology—a cellphone-based personal response system—in food science education was evaluated. Students' academic performance was improved by the incorporation of this technology into lectures, and its use was well perceived by the students (easy to use and positively impacted their learning). This finding contributes to the scholarship of teaching and learning in food science by providing useful insight into the potential for application of such tools with improved student engagement and learning outcomes.

Advances in food chemistry research will enable the development of value-added food products, and the pedagogical advancement in food science education will better convey new and existing knowledge to students, who will apply this knowledge to promote a safe and nutritious food supply that enhances human health and increases the value of specialty crops.

Discovery and dissemination of new knowledge in food science: Analytical methods for quantification of polyphenols and amino acids in fruits and the use of mobile phone-based instructional technology in food science education

Sihui Ma

GENERAL AUDIENCE ABSTRACT

In food science, both the discovery and dissemination of new knowledge are essential. To advance our understanding in fruit chemistry, several analytical methods were compared and applied. Polyphenols are important bioactive compounds in fruits associated with health benefits, and they also contribute to the bitterness and astringency of the products such as chocolate and red wines. Systematic evaluation of common analytical methods used to quantify polyphenols was conducted. When different methods were used to evaluate a broad range of fruit-derived samples, different results were obtained for a given sample, depending on the method applied. This was explained by the difference in polyphenol composition of these samples. Furthermore, different individual polyphenol compounds contributed differently to quantitative results for these methods. Interactions among polyphenol compounds and interference from constituents of the juice samples other than polyphenols were also found. These findings demonstrate that when comparing fruit chemistry (polyphenol concentration) results obtained using the methods evaluated, it is necessary to consider the polyphenol composition as well as the sample matrix composition. This knowledge will improve our ability to interpret and compare existing data on polyphenol content in fruits, advancing the understanding the polyphenols and health and informing producers to improve their fruit products with optimized quality and sensory characters.

Secondly, amino acids in apple juice influence the quality of fermented cider, through not only controlling the fermentation rate, but also impacting the flavor of the cider through yeast metabolism. Twenty amino acids were quantified in juices from 13 apple cultivars grown in Virginia with potential use in cider making using a recently developed method in analytical chemistry. The relative amino acid profile was significantly different among the apple juices evaluated. L-Asparagine, L-aspartic acid and L-glutamine are the principal amino acids observed in most apple juices. This knowledge will help with the development of fermentation strategies for production of ciders with targeted sensory attributes.

To better disseminate new knowledge in food science to the next generation, the effectiveness of a new educational technology application—a cellphone-based personal response system (similar to clickers)—in food science education was evaluated. Using this application during lecture resulted in improved quiz grades, and students felt that it was easy to use and positively impacted their learning. This application has the potential to improve effectiveness of lectures in higher education classrooms.

Advances in food chemistry research will enable development of value-added food products, and the pedagogical advancement in food science education will better convey new and existing knowledge to students, who will apply this knowledge to promote a safe and nutritious food supply that enhances human health and increases the value of specialty crops.

Dedication

To the caring educators.

Acknowledgements

I would like to thank my advisor, Dr. Amanda Stewart for her encouragement, guidance, and the opportunities to explore different options throughout my Ph.D. study. I would also like to thank my committee members Drs. Sean O'Keefe, Gregory Peck, and Mellissa Chase for their support and advice.

I would like to thank the faculty and staff in the Department of Food Science and Technology at Virginia Tech, including Dr. Andrew Neilson for his assistance in experimental design and manuscript writing; Dr. Jacob Lahne for this his assistance in statistical analysis; Dr. Monica Ponder for her advising in the development and implementation of course Food and Beverage Fermentation; Drs. Susan Duncan and Joe Marcy for their assistance in academic job applications; Joell Eifert for her assistance in the development of course Food and Beverage Fermentation; Dr. Hengjian Wang, Kim Waterman, Trina Pauley, Terry Rakestraw, Ann Sandbrrok, and Ken Hurley, and Brian Wiersema for their generous assistance during my Ph.D. program.

I would like to thank Drs. Donna Westfall-Rudd, Carrie Kroehler, Amy Nelson, Shelly Schmidt, and Peter Doolittle for their encouragement and guidance in teaching and the scholarship of teaching and learning in food science.

I would like to thank my fellow graduate students for their support in Graduate School. Special thanks to Lily Yang, Evelien Schilder, Jennifer Acuff, Natalie Pulido, Razieh Farzad, Cameron Bardsley, Paulette Cairns, Yanhong He, Kathryn Racine, Amy Moore, Oumoule Ndiaye, J'Nai Phillips, Lester Schonberger, Kendall Fogler, Liyun Ye, and Laura Hanzly for their friendship.

I would like to thank my advisee Cathlean Kim for her assistance in data collection and friendship, and students enrolled in FST 4654 Food and Beverage Fermentation, Robert Gunter, Catie Pannill, J'Nai, Phillips, Merrick Seltz, Kyle Templeton, and Cameron Walton, for their engagement and support in this class.

I would like to thank the Virginia Tech community for their warm welcome and wonderful opportunities for my five-year stay in Blacksburg, Virginia. Special thanks to the Graduate School for cultural immersion events, Police Department for the Student Police Academy, Center for Communicating Science, Academy for Graduate Teaching Excellence.

My greatest gratitude goes to my husband Jiajun Li, my parents, and grandparents for the infinite love, support, and encouragement, allowing me to explore this beautiful world.

Finally, I would like to thank all the educators who claim the excellence and believed in me along the way. Special thanks to Mr. Rongguo Jin at Jiaozuo No.1 High School, Dr. Zhenyu Wang and Dr. Xiucheng Zhang at Northeast Forestry University.

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Attributions

Several colleagues contributed to chapters 3 to 6 of this dissertation. A brief description of their contributions are included here.

Chapter 3: Comparison of common analytical methods for the quantification of total polyphenols and flavanols in fruit juices and ciders

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Laura E. Griffin, Ph.D., a former doctoral student in the Department of Food Science and Technology at Virginia Tech and a current postdoctoral scholar at the Plants for Human Health Institute, Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, collected data in Supplementary Information Figure 2 and reviewed the manuscript.

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Gregory M. Peck, Ph.D., a current Assistant Professor in the School of Integrative Plant Science, Horticulture Section, Cornell University assisted with the study design and completion of the manuscript.

Sean F. O'Keefe, Ph.D., a current Professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, data interpretation and completion of the manuscript.

Amanda C. Stewart, Ph.D., a current Assistant Professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation and completion of the manuscript.

Chapter 4: Contribution of individual polyphenol compounds and potential interfering compounds to the overall results of polyphenol quantification by spectrometric methods

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Amanda C. Stewart, Ph.D., a current Assistant Professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation and completion of the manuscript.

Chapter 5: Free amino acid composition of apple juices with potential for cider making as determined by UPLC-PDA

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Gregory M. Peck, Ph.D., a current Assistant Professor in the School of Integrative Plant Science, Horticulture Section, Cornell University assisted with the study design and completion of the manuscript.

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Amanda C. Stewart, Ph.D., a current Assistant Professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation and completion of the manuscript.

Chapter 6: Improved academic performance and student perceptions of learning through use of a cell phone-based personal response system

Daniel G. Steger, a former technician in the Department of Food Science and Technology at Virginia Tech assisted with the study design and completion of the manuscript.

Peter E. Doolittle, Ph.D., a former Director of the Center for Excellence in Teaching and Learning at Virginia Tech and a current Director of the School of Education at Virginia Tech assisted with the study design, data analysis, and completion of the manuscript.

Amanda C. Stewart, Ph.D., a current Assistant Professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation and completion of the manuscript.

CHAPTER 1 Introduction and objectives

Advances in analytical instrumentation and methods in recent decades have resulted in substantial advances in the understanding of fruit chemistry, especially with regards to secondary metabolites such as polyphenols and amino acids. This knowledge has enabled development of targeted fermentation strategies for production of value-added products, with enhanced sensory properties and/or health benefits. Both polyphenols and amino acids are major groups of compounds with relevance not only to the quality of the food products, but also to human health. Studying the concentration and composition of polyphenols and amino acids in fruit, including the methods available for their identification and quantification, contributes to the understanding of how these bioactive compounds impact the quality and health benefits of fruits and beverages made from fruits via fermentation.

Polyphenols are plant secondary metabolites capable of bioactive functionality in humans. The dietary intake of polyphenols has been associated with human health benefits (Cory, Passarelli, Szeto, Tamez, & Mattei, 2018; Nash et al., 2018), and with the sensory properties of bitterness and astringency (Lesschaeve & Noble, 2005). The mechanisms underlying many modes of polyphenol bioactivity remain unknown, despite decades of research into these structure-function relationships (Chong, Macdonald, & Lovegrove, 2010; Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Neilson & Goodrich, 2017). Because of significant challenges including (1) the complex and diverse nature of polyphenol structure, (2) the different mechanisms of analytical methods used for the quantification of polyphenols in fruit juices and fruit beverages, and (3) the complex matrices of fruit samples being analyzed for polyphenol content causing interfering with the analytical methods, significant variability in the results of total polyphenols obtained from several common analytical methods for a given sample can be expected (Seddon & Downey, 2008). No single best method for polyphenol quantification is agreed upon by all scientists. These challenges persist as major contributors to the continuing difficulty in elucidating polyphenol structure-function relationships in terms of bioactivity (Neilson, O'Keefe, & Bolling, 2016). Common analytical methods for the quantification of total polyphenols in fruit should be validated for the intended purpose and the results should be critically evaluated, interpreted and compared. A systematic evaluation of these analytical methods will provide a basis for selection of the most appropriate method to fit the specific goal of a given research project.

The long-term objective of the studies presented in Chapters 3 and 4 of this dissertation is to advance the understanding and interpretation of results of the quantification of total polyphenols in fruit juices and fruit beverages in horticulture and food science research. Deeper comprehension of the relationship between polyphenol composition, matrix effects and the response of multiple analytical methods will also be gained. The overall objective of these studies is to evaluate and/or develop appropriate analytical methods for the quantification of total polyphenols and total flavanols in specific fruit juices and fruit beverages, taking into account polyphenol composition, matrix effects, and the intended use of the data obtained. The central hypothesis is that the complexity and variability of polyphenol composition and matrix components in fruit juices and fruit beverages will lead to significant variation in the quantitative results of polyphenol analysis by multiple common analytical methods. Quantification of polyphenols using a single standard and/or with interfering compounds present in the sample matrix will lead to inaccurate results and can in turn misinform interpretation of these results in terms of polyphenol bioactivity or sensory attributes imparted by polyphenols.

Yeast assimilable nitrogen (YAN) is the nitrogen consumed by the yeast during fermentation. Free amino acids represent the majority of YAN in apple juice (Boudreau, Peck, O'Keefe, & Stewart, 2017). The concentration and composition of free amino acids in apple juice not only affect the fermentation kinetics, but also influence the flavor of the hard cider (the alcoholic beverage made from apple juice) (Alberti et al., 2016). However, the concentration and composition of the amino acids in apple juice varied according to the apple cultivars, growing conditions and practices, such as fertilizer application, as well as post-harvest storage conditions and juice processing methods (Villière, Arvisenet, Bauduin, Le Quéré, & Sérot, 2015). Determination of the amino acid composition in apple juice will provide a basis for development of targeted fermentation strategies for cider.

The objective of the study described in Chapter 5 was to employ a state of the art analytical technique, UPLC-PDA, to characterize the amino acid composition in apples with potential use in cider making, and to assess the extent to which amino acid concentration and composition vary among juice samples. In this study, 20 amino acids were analyzed in 13 apple juice samples from cultivars grown in Virginia with potential for use in cider making. The hypothesis is that the concentration and composition of amino acids in these apple juice samples varies greatly, which would in turn impact the fermentation kinetics and aroma production during cider fermentation. Different nitrogen management strategies could then be developed to accommodate the variance in amino acid composition of apple juice for successful fermentation and intended flavor of the finished cider.

Education is an integral part of food science. As of 2017, 63% of the employees in the food industry hold their highest degree in Food Science/Technology (*2017 IFT employment & salary survey report*, 2017). Educational technology applications have the potential to improve the quality of higher education, including but not limited to the field of Food Science. Clickers have been used in in Food Science courses to engage students and promote active learning (Intemann, 2006). Recently, Shaw et al. demonstrated that incorporating clickers into a short course on Hazard Analysis Critical Control Points (HACCP) improved the pass rate of the course (Shaw, Mendonca, & Daraba, 2015). However, the effectiveness of using a cell phone-based personal response system in Food Science higher education has not been systematically evaluated and a better understanding of students' perceptions of cell phone-based personal response system used in Food Science education would provide useful insight into the potential for application of such tools. In order to advance the scholarship of teaching and learning in food science, and to address this gap specifically, the study reported in Chapter 6 of this dissertation aimed to evaluate the impact of using a cell phone-based personal response system on academic performance and students' perceptions of learning in an upper-level undergraduate Food Science course.

In summary, advances in technology contribute to the advancement of both research and education in Food Science. Relatively recent developments in analytical instrumentation have allowed more accurate, precise and inexpensive quantification of polyphenols and amino acids in fruits and related products. Deeper understanding of food chemistry will result in more suitable processing strategies, leading to food products with better quality and maximized health benefits. Development in educational technology will likewise facilitate and improve student learning in food science, leading to stronger and more skillful workforce for the food science industry. Taken together, the chapters in this dissertation characterize specific ways in which these technological advancements are furthering the food system, as a whole.

References

2017 IFT employment & salary survey report. (2017).

Alberti, A., Machado dos Santos, T. P., Ferreira Zielinski, A. A., Eleutério dos Santos, C. M., Braga, C. M., Demiate, I. M., & Nogueira, A. (2016). Impact on chemical profile in apple juice and cider made from unripe, ripe and senescent dessert varieties. LWT - Food Science and Technology, 65, 436–443. https://doi.org/http://dx.doi.org/10.1016/j.lwt.2015.08.045

Boudreau, T. F., Peck, G. M., O'Keefe, S. F., & Stewart, A. C. (2018). Free amino nitrogen concentration correlates to total yeast assimilable nitrogen concentration in apple juice. Food Science and Nutrition, 6(1), 119-123. https://doi.org/10.1002/fsn3.536

Chong, M. F.-F., Macdonald, R., & Lovegrove, J. A. (2010). Fruit polyphenols and CVD risk: a review of human intervention studies. British Journal of Nutrition, 104(S3), S28–S39. https://doi.org/10.1017/S0007114510003922

Cory, H., Passarelli, S., Szeto, J., Tamez, M., & Mattei, J. (2018). The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. Frontiers in Nutrition, 5, 87. https://doi.org/10.3389/fnut.2018.00087

Intemann, L. (2006). Clicking in class helps lecturers from appearing remote by using student remotes as instructional tool. Retrieved from http://www.news.cornell.edu/stories/2006/02/clicking-class-helps-lecturers-and-students-connect

Lesschaeve, I., & Noble, A. C. (2005). Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. The American Journal of Clinical Nutrition, 81(1), 330S–335S. https://doi.org/10.1093/ajcn/81.1.330S

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. The American Journal of Clinical Nutrition, 79(5), 727–747. Retrieved from http://ajcn.nutrition.org/content/79/5/727.abstract

Nash, V., Ranadheera, C. S., Georgousopoulou, E. N., Mellor, D. D., Panagiotakos, D. B., McKune, A. J., … Naumovski, N. (2018). The effects of grape and red wine polyphenols on gut microbiota – A systematic review. Food Research International, 113, 277–287. https://doi.org/10.1016/J.FOODRES.2018.07.019

Neilson, A. P., & Goodrich, K. M. (2017). Bioavailability and Metabolism of Bioactive Compounds From Foods. Nutrition in the Prevention and Treatment of Disease, 301–319. https://doi.org/10.1016/B978-0-12-802928-2.00015-1

Neilson, A. P., O'Keefe, S. F., & Bolling, B. W. (2016). High-molecular-weight proanthocyanidins in foods: Overcoming analytical challenges in pursuit of novel dietary bioactive components. Annual Review of Food Science and Technology, 7(1), 43–64. https://doi.org/10.1146/annurev-food-022814-015604

Seddon, T. J., & Downey, M. O. (2008). Comparison of analytical methods for the determination of condensed tannins in grape skin. Australian Journal of Grape and Wine Research, 14(1), 54–61. https://doi.org/10.1111/j.1755-0238.2008.00007.x

Shaw, A., Mendonca, A., & Daraba, A. (2015). "Clickers" and HACCP: Educating a Diverse Food Industry Audience with Technology. Journal of Extension, 53, 6.

Villière, A., Arvisenet, G., Bauduin, R., Le Quéré, J.-M., & Sérot, T. (2015). Influence of cider-making process parameters on the odourant volatile composition of hard ciders. Journal of the Institute of Brewing, 121(1), 95–105. https://doi.org/10.1002/jib.197

CHAPTER 2 Literature review

The following topics are discussed in this chapter: 1) the complex and diverse structures of polyphenols, 2) importance of polyphenols in food science, horticulture, and nutrition research, including the health benefits and the sensory impact of polyphenols in fruit and fruit-derived products, 3) factors influencing the concentration of polyphenols in fruit juices and fruit beverages, and 4) common analytical methods for the quantification of polyphenols in fruit juices and fruit beverages and analytical method validation.

Several existing analytical methods are commonly applied for the determination of total polyphenol concentration in fruit juices and fruit beverages, but each has its limitations. Choosing the appropriate analytical method for the quantification of total polyphenols for a specific research question, and understanding how the food matrix can influence these results will facilitate progress in research on bioactivity and sensory impacts of polyphenols in fruit juices and fruit beverages.

2.1 Complex structure of polyphenols

Polyphenols are secondary metabolites in plants with highly diverse chemical structures (Weber, Schulze-Kaysers, & Schieber, 2014). They are identified by their phenolic structures and more than 8000 polyphenol compounds have been identified in plants (Pandey & Rizvi, 2009). The concentration of more than 500 individual polyphenol compounds in more than 400 foods have been collected from more than 600 peer-reviewed journal articles (Rothwell JA, Pérez-Jiménez J, Neveu V, Medina-Ramon A, M'Hiri N, Garcia Lobato P, Manach C, Knox K, Eisner R, Wishart D, 2013). The main dietary sources of polyphenols are raw fruits and vegetables, such as grapes, apples, cocoa, and the products made from them, such as wine, hard cider, chocolate (Tsao, 2010).

Polyphenols are classified based on their chemical structures. Besides the diversity in the core polyphenol skeleton (aglycones), most polyphenols are present in foods as glycosides with a wide range of sugars and acylated sugars, such as monosaccharides, disaccharides, trisaccharides, and even tetrasaccharides attached to the aglycones at different positions contributing to the great diversity of the polyphenol structures (Harborne & Williams, 1982; Hollman & Arts, 2000). To simplify, polyphenols are classified based on their aglycone structures (Tsao, 2010) **(Figure 2.1).** Polyphenols can be divided into two major groups, flavonoids and phenolic acids (Tsao, 2010).

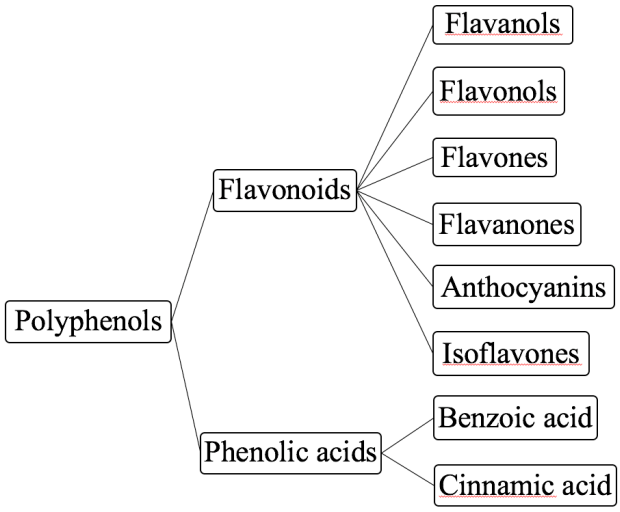


Figure 2. 1. Classification of major polyphenols based on aglycone structures

Within phenolic acids, there are two groups of compounds, the derivatives of benzoic acid [C1-C6 backbone, **Figure 2.2 (A)**] and the derivatives of cinnamic acid [C3-C6 backbone, **Figure 2.2 (B)**].

(A) Benzoic acid (B) Cinnamic acid

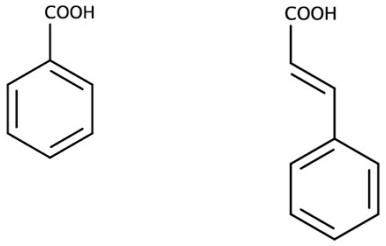
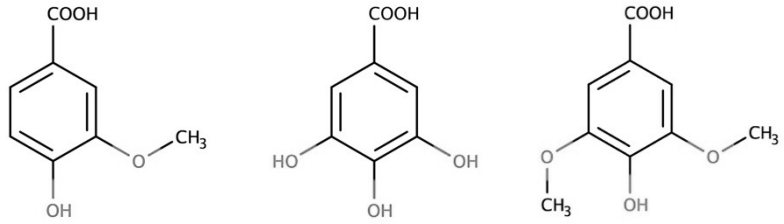


Figure 2. 2. Chemical structures of (A) Benzoic acid and (B) Cinnamic acid

The chemical structures of typical phenolic acids found in foods are shown in **Figure 2.3** with derivatives of benzoic acid on the top column and derivatives of cinnamic acid on the bottom column.

(A) Vanillic acid (B) Gallic acid (C) Syringic acid



(D) *p*-Coumaric acid (E) Caffeic acid (F) Ferulic acid (G) Sinapic acid (H) Chlorogenic acid

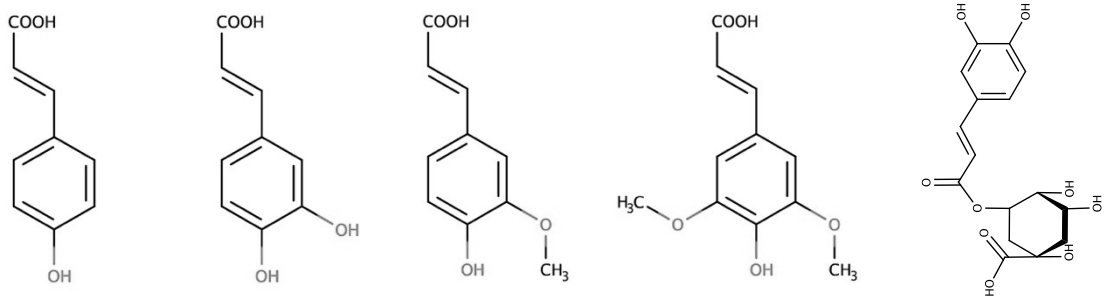


Figure 2. 3. Chemical structures of typical phenolic acids found in foods

Flavonoids are more diverse than phenolic acids and they share the backbone of C6-C3-C6 (**Figure 2.4**). Flavonoids can be future divided into six sub-groups: flavanols (flavan-3-ols), flavonols, flavones, flavanones, anthocyanins, and isoflavones (**Figure 2.5**) depending on the various hydroxylation in the C ring.

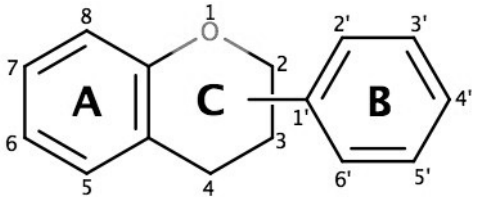


Figure 2. 4. Backbone skeleton of flavonoids

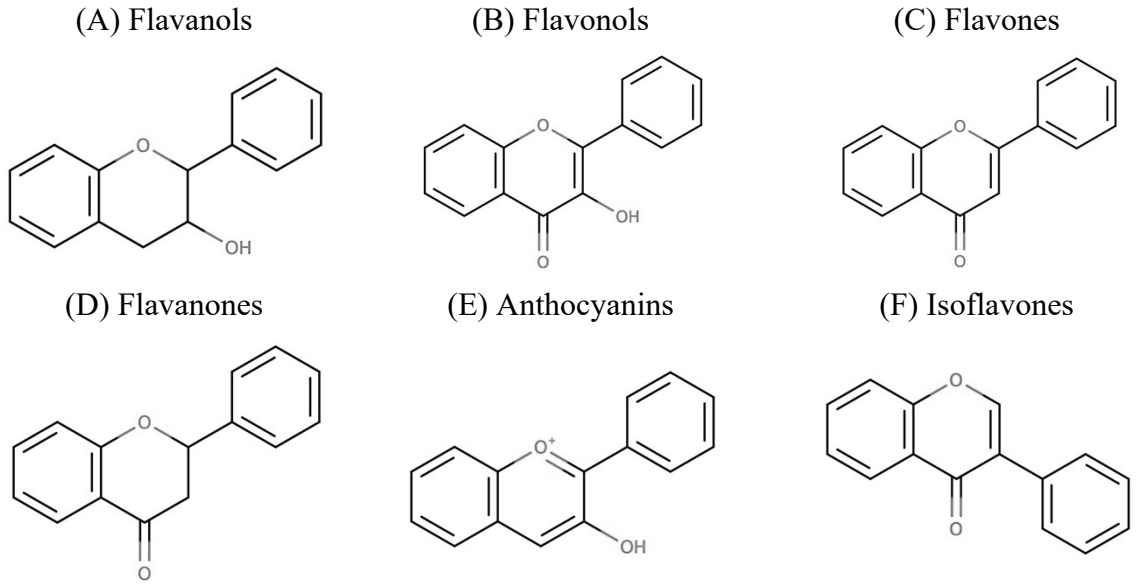


Figure 2. 5. Backbone skeleton of (A) flavanols, (B) flavonols, (C) flavones, (D) flavanones, (E) anthocyanins, and (F) isoflavones

Flavanols are present in food in monomeric and polymeric forms. Monomeric flavanols, such as catechin [**Figure 2.6 (A)**), epicatechin [**Figure 2.6 (B)**], and their derivatives, are often present in tea (Henning et al., 2004), cocoa beans, and chocolates (Cooper et al., 2007). Polymeric flavanols (procyanidins, or condensed tannins) are formed by catechins and epicatechins, and based on the degree of polymerization (DP, number of monomeric units), they can be further divided in to oligomers (DP of two to seven) and polymers (DP larger than seven) (Prieur, Rigaud, Cheynier, & Moutounet, 1994). They can be found in grape seeds and wines (Sarneckis et al., 2006), as well as other fruits, such as apples, pears, and berries, nuts, cocoa, and bark of pine trees (Hellström, Törrönen, & Mattila, 2009).

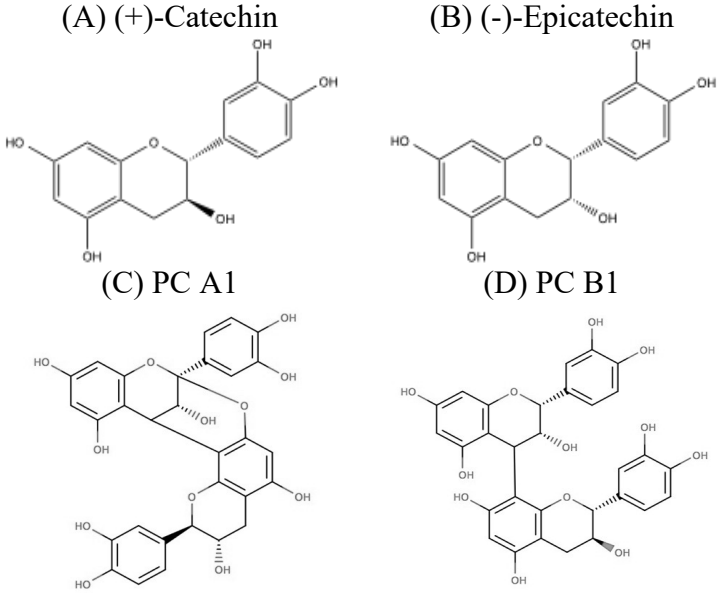


Figure 2. 6. Chemical structures of (A) (+)-catechin, (B) (-)-epicatechin, (C) procyanidin dimer A1 (PC A1), and (D) procyanidin dimer B1 (PC B1)

A-type oligomeric procyanidins [**Figure 2.6 (C)**] in which monomers are linked through C2–*O*–C7 or C2–*O*–C5 bonds, have been identified in only a few plant products, such as plums, avocados, cranberries, and peanuts skins. B-type oligomeric procyanidins [**Figure 2.6 (D)**], in which monomers are linked through C4–C6 or C4–C8 bonds, are present in a wider variety of plant products, such as apples, blueberries, pears, nectarines, kiwi, mango, bananas, citrus fruits, and grape seeds (Appeldoorn et al., 2009).

Flavonols are found in fruits and vegetables such as onions, kale and apples. The major flavonol compounds that have been characterized include quercetin [**Figure 2.7 (A)**], kaempferol [**Figure 2.7 (B)**], myricetin [**Figure 2.7 (C)**] and isorhamnetin [**Figure 2.7 (D)**]. Together with flavones (such as luteolin and apigenin), they contribute to the yellow color in plants, such as the skin of onions (Herrmann, 1976). Flavanones are identified mainly in citrus fruits (Tomás-Barberán & Clifford, 2000).

(A) Quercetin (B) Kaempferol (C) Myricetin (D) Isorhamnetin

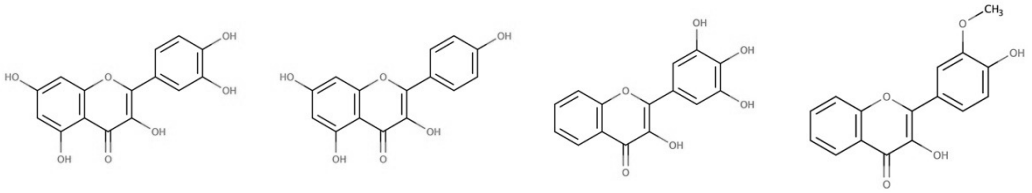


Figure 2. 7. Chemical structures of (A) quercetin, (B) kaempferol, (C) myricetin, and (D) isorhamnetin

Anthocyanins contribute the red, purple and blue color of plants. Due to proton dissociation, the color of anthocyanins depends on the matrix pH; they will appear as red in acidic conditions and blue in basic conditions (Tsao, 2010). They are often used as an indicator for the quality of raw and processed fruits and vegetables (Giusti & Wrolstad, 2001). Major anthocyanins in foods include cyanidin [**Figure 2.8 (A)**], delphinidin [**Figure 2.8 (B)**], pelargonidin [**Figure 2.8 (C)**], and malvidin [**Figure 2.8 (D)**].

(A) Cyanidin (B) Delphinidin (C) Pelargonidin (D) Malvidin

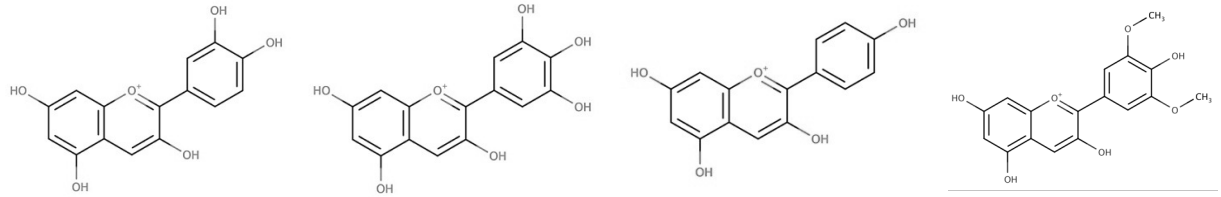


Figure 2. 8. Chemical structures of (A) cyanidin, (B) delphinidin, (C) pelargonidin and (D) malvidin

For the above flavonoids, the B ring is attached to the C2 position of the C ring, however, the B ring can also be attached to other position of the C ring. For isoflavones, the B ring is attached to C3 position of the C ring; for neoflavones, the B ring is attached to C4 position of the C ring [**Figure 2.9 (A)**]; for chalcones, the C ring is open [**Figure 2.9 (B)**]. Soybeans are rich in isoflavones, such as genistin, daidzin, and glycitin, and they have been studied extensively because of the health benefits from soybean-rich diet (Kucuk, 2017; Mahmoud, Yang, & Bosland, 2014; Sacks et al., 2006). Neoflavones, such as dalbergin, are not commonly present in foods, although they have been reported in herbs used in traditional medicine (Chan, Chang, & Kuo, 1997; Koreca, Senschb, & Zoukasb, 2000; Wu et al., 2011). Chalcones are found in tomatoes, roots, hops, beers, and dihydrochalcones are the characteristic of apples (Tomás-Barberán & Clifford, 2000).

(A) Neoflavones (B) Chalcones

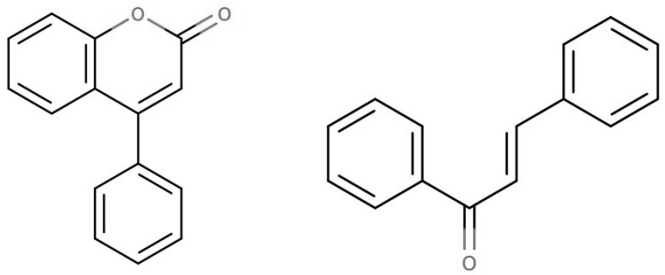


Figure 2. 9. Backbone skeleton of (A) neoflavones and (B) chalcones

Polyphenols with molecular weight larger than 500 are often referred to as tannins (Cheynier, 2005), which were originally discovered as they can "tan" or preserve, leathers (Deshpande, Cheryan, Salunkhe, & Luh, 1986). The word "tannins" is often used in food processing and sensory aspects of foods because of their ability to bind proteins (Ann E Hagerman, 2002). Tannins can be divided into hydrolysable tannins (derivatives of gallic acid) and condensed tannins (procyanidins). The term "tannins" is often used colloquially and with limited precision, although generally refers to larger polyphenols which confer astringency to fruit and fruit products via their ability to bind proteins. However, the ability of current analytical methods to quantify polymeric polyphenols is limited (Neilson, O'Keefe, & Bolling, 2016). A factor contributing to analytical difficulty is the lack of or great expense of analytical standards for these larger compounds. Difficulty in quantifying the polymeric procyanidins has resulted in little available information on their concentrations in foods; the Phenol-Explorer database only includes procyanidins up to trimers (DP = 3). This challenge may have led to these compounds being overlooked in mechanistic or intervention studies on the relationship between dietary polyphenol intake and health benefits. Improved analytical methods for the quantification of polyphenols with larger DP are required in order to gain more understanding of their biological activity.

Besides the complex structure of polyphenols, polyphenols also interact with each other leading to unexpected bioactivities of the mixture. Interaction effect have been found among epicatechin and myricetin on iron uptake (Glahn et al., 2017). Study on the antioxidant capacity of catechin, resveratrol, and quercetin has found that the antioxidant capacity of the mixture of these three polyphenols may be even less than it of the individual polyphenol (inhibitory effect) (Pinelo, Manzocco, Nuñez, & Nicoli, 2004). Synergic antimicrobial effects among flavonoids and the ellagitannins have been found in *Cistus salviifolius* extract (Tomás-Menor et al., 2015). The interaction among polyphenols has been demonstrated on the molecular level. Tannic acid, pyrogallol, and epigallocatechins gallate have been found to interacted with each other via covalent and noncovalent bonds (Sileika, Barrett, Zhang, Lau, & Messersmith, 2013). These interactions influence the results of polyphenol quantification by different absorbance readings resulting due to the positive or negative synergism effects of polyphenol standards in the Folin-Ciocalteu method (Bastola, Guragain, Bhadriraju, & Vadlani, 2017).

In summary, polyphenols exist in nature with a broad diversity of structures due to different linkages and subunits. Thus, it is difficult and challenging to find a reagent only reacting to a specific subunit that all polyphenol compounds share in common while not present in non-polyphenol compounds. Not all polyphenol compounds have a specific structural unit in common in order to be quantified equally by a single analytical method. Also, the target moiety of the polyphenols that reacts to elicit a quantifiable response in each analytical method may also

react with other non-polyphenol (non-target) compounds present in the complex sample matrix. The structural diversity of polyphenols and diversity of fruit juices and fruit beverages containing them can be reasonably expected to influence quantitative analytical results.

2.2 The health benefits of polyphenols

The health benefits of polyphenols are the primary reason why polyphenols are of such great interest in current foods and health research. The accurate quantification of polyphenol content in fruit and fruit products will help us to compare the results of various studies and to gain a better understanding of the roles of polyphenols in human health. These compounds and their purported bioactivities have been extensively studied in recent decades and continue to be an area of current research. One famous study which accelerated broad interest in this topic was the epidemiological study by Renaud and Lorgeril in 1992, colloquially known as "the French Paradox", which pointed out that seemingly paradoxical high dietary intake of saturated fat but low mortality from coronary heart disease in France may be owed to the relatively high consumption of polyphenol-rich wine (Renaud & de Lorgeril, 1992). A polyphenol-rich diet, including tea, wine and cocoa, has been associated with positive health outcomes such as lower incidence of obesity (Farhat, Drummond, & Al-Dujaili, 2017), cancer (Lewandowska, Kalinowska, Lewandowski, Stępkowski, & Brzóska, 2016), cardiovascular disease (Quiñones, Miguel, & Aleixandre, 2013), and Alzheimer's disease (Darvesh, Carroll, Bishayee, Geldenhuys, & Van der Schyf, 2010).

2.2.1 Biological activity and bioavailability

Polyphenols are abundantly present in plants as an antioxidant, an antimicrobial agent, and they can also protect the plant tissue from UV light damage (Michalak, 2006). The antioxidant property allows them to participate in the oxidation-reduction reactions during metabolism, thus affect the activity of enzymes and the cellular signaling (Quiñones et al., 2013). While antioxidant effects were very intensely studied in previous decades, more recent research has focused on other mechanisms potentially underlying the health benefits associated with the intake of dietary polyphenols.

The biological activity and bioavailability of polyphenols varies among different polyphenols; the most abundant polyphenol compound does not necessarily show the highest bioavailability (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). The quantification of "total polyphenols" as a measure in bioactivity studies falls short of ever being able to predict bioactivity (Balentine et al., 2015). The biological activity and bioavailability of polyphenols are highly dependent on the structure of polyphenols, including the glycosylation patterns (Tsao, 2010). Glycosylated polyphenols, which are more common in nature compared to aglycones, have lower bioavailability than aglycones, because aglycones of some polyphenols can be absorbed into the small intestine while glycosylated polyphenols cannot; they need to hydrolyzed by the enzymes present in the enterocyte or made by the microbes present in the intestine (Estrela et al., 2017). The aglycones may also be degraded during hydrolysis. In addition, food processing and the food matrix also impact the bio-efficacy of polyphenols (Neilson & Ferruzzi, 2011). While extensive research has been done on polyphenol bioavailability, current findings show that bioavailability is not required for bioactivity, thus the larger, non-bioavailable dietary polyphenols are of renewed interest, and need to be quantified in this area of research (Neilson et al., 2016).

Many studies have shown high correlations of analytical methods for the quantification of polyphenols with antioxidant activity (Du, Li, Ma, & Liang, 2009; Vinson, Su, Zubik, & Pratima, 2001). However, these correlations may result from other antioxidants present in the

samples (not polyphenols) due to the nonspecific nature of the methods that neither the methods for polyphenol quantification nor antioxidant activity were able to distinguish polyphenols from other antioxidants in the sample. Thus, some existing results of polyphenol quantification using common analytical methods may have been significantly impacted by interference by antioxidants in the sample matrix, and cannot be reliably used to predict the bioactivity or the health benefits of polyphenols, especially via mechanisms other than antioxidant activity.

The results of *in vitro* studies of the proposed mechanisms underlying health benefits associated with dietary polyphenol intake have not always agreed with those of studies performed under *in vivo* conditions, possibly due to the low bioavailability of polyphenols. Thus, researchers have proposed many methods to improve the bioavailability of polyphenols and deliver them at the specific concentrations to the target tissues, including the modification on polyphenol structures, pharmaceutical formulations with nano-scale nutraceuticals, and the development of efficient delivery systems (Estrela et al., 2017). Another proposed explanation for these inconsistencies is that polyphenol bioactivity may be mediated by gut microbiota and/or exerted within the intestinal lumen.

2.2.2 Interaction between polyphenols and intestinal microbiota

A study in rats investigating the metabolic fate of polyphenols showed that the polyphenols detected in the blood and urine were the degradation products of intestinal bacteria (Kohri et al., 2001). The interaction between the polyphenols and intestinal microbiota is very complex (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; Duda-Chodak, Tarko, Satora, & Sroka, 2015). The intestinal bacteria convert the polyphenols into bioavailable metabolites, such as urolithins, and gut microbial ecology affects the metabolism of polyphenols (Tomás-Barberán, Selma, & Espín, 2016). Polyphenols also maintain the balance and health of intestinal microbiota by promoting the growth of the beneficial bacteria (i.e., lactobacilli, A. *muciniphila*, *Akkermansia* spp., and bifidobacteria) and inhibiting pathogenic bacteria (Dueñas et al., 2015). The variability of polyphenol bioavailability of among individuals in clinical trials may be due to the difference of inter-individual gut microbiota (Tomás-Barberán et al., 2016). The relationship among the metabolism of polyphenols, the modulation of intestinal microbiota, and the health status of the host still remain as a popular research topic and can vary among individuals (Tomás-Barberán et al., 2017).

2.2.3 Anti-inflammatory effects

Inflammation is the protective and natural response to injury, disease, and irritations by the immune system (National Cancer Institute, 2018). However, long-term inflammatory stress can lead to tissue damage and failure, and contribute to age-related degenerative disease, such as cardiovascular disease (Gratchev, Sobenin, Orekhov, & Kzhyshkowska, 2012), obesity (Gregor & Hotamisligil, 2011), and Alzheimer's disease (Rosenberg, 2005). Polyphenol-rich products and polyphenol extracts are shown to have anti-inflammatory effects, stabilizing the inflammatory response (Joseph, Edirisinghe, & Burton-Freeman, 2016). Dietary polyphenols from fruits are shown to be effective in relieving the inflammation in both the fasting and postprandial states from clinical trials and human intervention studies (Joseph et al., 2016).

2.2.4 Beneficial effects on cardiovascular disease

Cardiovascular disease is the leading cause of death worldwide, indicated as the cause of an estimated 31% of global deaths in 2015 (World Health Organization, 2017b). Results from a meta-analysis have shown that the high consumption of polyphenol-rich fruits and vegetables lowered the risk of mortality from cardiovascular disease (X. Wang et al., 2014). The beneficial effects of polyphenols on cardiovascular disease come from four aspects: (1) vasodilator effect,

(2) anti-inflammatory effect, (3) anti-atherogenic effect, and (4) anti-thrombotic effect (Quiñones et al., 2013). (1) As antioxidants, polyphenols reacted with the O_2- radicals, which can destroy nitric oxide, an important compound for regulating and maintaining the homeostasis of the blood vessels (Cooke, Dzau, Cooke, & Dzau, 1997). (2) As an inflammatory disease, atherosclerosis is found with the increased concentration of enzymes, such as cyclooxygenase and lipoxygenase (Vila, 2004). Polyphenols have shown to inhibit these two enzymes (Frei & Higdon, 2003). (3) Polyphenols can limit the oxidation of low density lipoproteins (LDL) and protect the endothelial cells from the damage of oxidized LDL (Kurosawa et al., 2005; Steffen, Schewe, & Sies, 2006), thus prevent the accumulation of lipid in the arterial wall and atherosclerosis. (4) Polyphenols also play a role in reducing platelet aggregation (Z. Wang et al., 2002) through the inhibition of enzymes in the synthesis of platelets or the promotion of the formation of platelet aggregation inhibitors (Quiñones et al., 2013).

2.2.5 Anti-cancer effects

Cancer is the second greatest cause of death worldwide, with 1 out of 6 global deaths in 2015 attributed to cancer ("Cancer Fact Sheet," 2017). Cancer refers to a large group of diseases, in which the out-of-control growth of cells can occur in any organ of the human body and then invade to other parts of the body, causing problems and malfunctions of the body (American Cancer Society, 2015).

Herbal medicines have been used in many countries, especially in Asia and Africa as a traditional and alternative cancer treatment valued for perceived lower incidence of side effects compared to chemotherapy (Greenwell & Rahman, 2015). Polyphenols derived from plants, such as curcumin rhizome of turmeric (Wilken, Veena, Wang, & Srivatsan, 2011) and catechins from green tea (Bettuzzi et al., 2006) have shown satisfactory anti-cancer effects through clinical trials in some instances. Several potential mechanisms of polyphenols' anti-cancer effects have been proposed and confirmed using cancer cells, including the inhibition of growth factor and invasion, blocking proliferation, and the increase for apoptosis (Estrela et al., 2017). Although the dosage, toxicity and long-term safety, pharmacokinetics and molecular mechanisms of these compounds need to be determined, they have the potential of being a supplementary therapy in addition to the current cancer treatments (Hosseini & Ghorbani, 2015).

Besides, a positive relationship between the consumption of polyphenol-rich fruits and vegetables and lower cancer incidence in the upper gastrointestinal tract have been observed from many epidemiological studies (Estrela et al., 2017). Although for other common cancers, such as lung cancer, the above relationship was not found (Key, 2011), the American Cancer Society and World Health Organization still make recommendations on incorporating adequate fruits and vegetables into daily diet as a cancer preventative strategy (Kushi et al., 2012; World Health Organization, 2017a).

2.2.6 Polyphenols and obesity

Obesity is caused by the increased size and amount of fat cells in human body (Centers for Disease Control and Prevention, 2018), and more than one third of adults in the US were obese between 2011-2014 (Ogden, Carroll, Fryar, & Flegal, 2015). It is a serious medical condition which is associated with increased risk for cancer (Bhaskaran et al., 2014), hypertension, type-2 diabetes, cardiovascular disease, and other negative health outcomes (Centers for Disease Control and Prevention, 2015).

Polyphenols have shown their potential as an obesity-preventative agent by affecting the lipid and fat metabolism positively (Farhat et al., 2017). The *in vitro* and animal model studies (mice and Wistar rats) have indicated the effectiveness of polyphenols in preventing obesity, but

results from human intervention studies did not show consistent conclusions, and led to an emerging concern for toxic side effects from high doses of polyphenols (Grove & Lambert, 2010; Xu et al., 2015). Several possible mechanisms to explain the role of polyphenols in reducing obesity have been proposed from cellular and animal studies, including their effect on adipocytes, triglycerides and fatty acids, and the modulation of multiple signaling pathways during lipid metabolism, but the underlying mechanisms still remain unclear (Farhat et al., 2017; S. Wang et al., 2014).

Besides the health benefits mentioned above, polyphenols also show potential in reducing the risk of Alzheimer disease (Darvesh et al., 2010), stroke (Hollman, Geelen, & Kromhout, 2010), and osteoporosis (Shen, Wang, Guerrieri, Yeh, & Wang, 2008).

In summary, polyphenols have shown a broad range of bioactivities in cell and animal models and have been associated with many positive health outcomes, but the mechanisms underlying these health benefits remain largely unexplained by clinical intervention studies. The inconclusive results of these studies may be due, in part, to the fact that the analytical methods used for the quantification of polyphenols did not predict the actual polyphenol content or the bioactivity of these compounds. Accurate quantification of polyphenol content in the dietary sources of polyphenols will influence the quality of future work in this area, allowing more accurate quantification of the dietary intake of polyphenols. The challenges to accurate quantification of polyphenols include the large variance of polyphenol content depending on the food type, different mechanism of each analytical method for the quantification of polyphenols, and the complex structure of polyphenols and their unknown interactions with the food matrix. Overcoming these challenges with analytical methods for the quantification of polyphenols will significantly advance our knowledge on how dietary polyphenols exert a positive impact on human health.

2.3 The sensory impact of polyphenols in fruit juice and fruit beverages

Astringency and bitterness are important but different sensory characteristics of foods elicited by polyphenols and contributing to the taste, texture and mouth feel of foods.

The word “astringent” is derived from the Latin words ad, which means to, and stringere, which means bind, thus astringency is referred to as a “binding” reaction (Joslyn & Goldstein, 1964). Astringency has been defined as “the complex of sensations due to shrinking, drawing or puckering of epithelium as result of exposure to substances such as alums or tannins” by the American Society for Testing and Materials (“ASTM E253-17 Standard Terminology Relating to Sensory Evaluation of Materials and Products,” 2017). This tactile sensation is evoked by nonspecific and semi-irreversible hydrogen bonds between o-diphenolic groups and proteins in the mouth, resulting in the distinctive drying and puckering astringency sensation experienced uniformly across the tongue (Joslyn & Goldstein, 1964). Pure astringency can be perceived from certain unripe fruits such as sloes (*Prunus spinosa*), quinces (*Cydonia oblonga*), and perry pears (*Pyrus communis*) (Lea & Arnold, 1978). Consumers generally prefer wine that exhibits a balanced astringency; too much astringency may detract from the value of other components in the wine, while wine with little astringency lacks the expected mouthfeel (Gawel, 1998). The mechanism underlying the sensation of astringency is very complex and several hypotheses have been proposed and tested. One common hypothesis that astringency arises with the loss of salivary lubrication has been challenged by more recent findings that astringency is not always elicited upon decreases in saliva lubricating proteins (Lee, 2010; Lee & Vickers, 2012; Rossetti, Bongaerts, Wantling, Stokes, & Williamson, 2009). Another common hypothesis proposed that the sensation of astringency is caused by direct interaction of polyphenols and the oral

epithelium (C. Payne, Bowyer, Herderich, & Bastian, 2009), and that binding of proline-rich salivary proteins in the oral cavity with polyphenols is protective and can prevent the development of astringency (Horne, Hayes, & Lawless, 2002). However, others have shown that the concentration of non-binding polyphenols in the saliva related closer to astringency than did the concentration of polyphenols bound by the salivary proteins, thus the relationship between the precipitation of polyphenols and salivary proteins and the development of astringency remains inconclusive (Schwarz & Hofmann, 2008). It was proposed recently that the removal by polyphenols of the oral lubricating films that coat the inside of the mouth may contribute to the sensation of astringency (Lee, 2010). Overall, astringency is a complex sensation, the mechanisms of which remain poorly understood and a topic of current research, especially regarding the effect of the structure of the polyphenols on the interaction with salivary proteins (García-Estévez, Ramos-Pineda, & Escribano-Bailón, 2018).

Bitterness is a taste, defined as "pertaining to the taste produced by substances such as quinine or caffeine when in solution" by American Society for Testing and Materials ("ASTM E253-17 Standard Terminology Relating to Sensory Evaluation of Materials and Products," 2017). Bitterness is perceived mostly at the back and sides of the tongue (Reed, Tanaka, & McDaniel, 2006). Pure bitterness is the taste of certain plant-based alkaloids such as caffeine and quinine (Lea & Arnold, 1978). Unlike astringency, the mechanism of bitterness development is well understood. The perception of bitterness is caused by the activation of bitter taste receptors, TAS2Rs, by polyphenols passing into the taste papillae membrane (Soares, Brandão, Mateus, & de Freitas, 2017). There are about 25 TAS2Rs and they are located in the taste buds, which are embedded in the epithelium of the gustatory papillae on the tongue. Different TAS2Rs are able to identify and are activated by bitter compounds, with diverse chemical structures, including polyphenols (Soares et al., 2013).

Although astringency and bitterness are both evoked by polyphenols, the balance of bitterness and astringency can differ in wines and ciders with the same total polyphenol content estimated by absorbance at 280 nm (Brossaud, Cheynier, & Noble, 2001; Lea & Arnold, 1978). The DP of procyanidins affects the balance of bitterness to astringency; oligomeric procyanidins (DP 2 to 5) contributes to bitterness, while more polymerized structures (DP 6 to 10) provide astringency (Robichaud & Noble, 1990).

Analytical methods that quantify procyanidins of both lower and higher DPs and cannot distinguish procyanidins of lower DP from those of higher DP and will not predict the balance of bitterness and astringency of the given sample. Methods that only quantify oligomeric procyanidins will give more information about the bitterness while methods that only quantify polymerized procyanidins will give more information about the astringency. These factors should be taken into account when selecting the most appropriate analytical method for a given purpose.

Unlike in wine and cider, the levels of bitterness and astringency caused by endogenous polyphenols in some products are not always favored in fruit juices and beverages. Consumer acceptability may actually decrease as a result of high polyphenol concentration or the addition of polyphenols as functional ingredients (Tuorila & Cardello, 2002). In juices made from citrus fruits, the bitterness can be reduced by cleaving the moiety attached to the flavanone glycoside naringin and sweetness can be increased by chemically opening the ring to form dihydrochalcone (Lea, 1992). Analytical methods for the quantification of polyphenols will fail for the prediction of bitterness if they cannot distinguish flavanone glycoside naringin, algcone naringin, and dihydrochalcone. This is one example of the potential shortcomings of analytical techniques to

predict sensory characteristics of astringency and bitterness. In summary, because of the important sensory characteristics, both positive and negative, imparted by polyphenols in fruits and fruit products, it is important to quantify polyphenols in these products using an appropriate method to enable targeted adjustment of the polyphenol concentrations to achieve the desired balance of bitterness and astringency.

2.4 Factors influencing the concentration of polyphenols in fruit juices and fruit beverages fruit and fruit products

Polyphenols are stress metabolites in plants synthesized through the shikimate pathway (R. Knaggs, 1999). Many factors have been proven to affect the concentration of polyphenols in plants and plant-based foods, including plant genotype (the type of fruit or vegetable), the environmental conditions under which the plant grows, maturity of the plant or fruit at harvest, and food processing and storage conditions (Pandey & Rizvi, 2009).

Plant genetics is believed to be the most important determining factor of polyphenol content in foods (Tsao, Khanizadeh, & Dale, 2006). Studies on different plants have shown that even among different cultivars of the same species of plants, polyphenol content differs (Crozier, Lean, McDonald, & Black, 1997; Fratianni, Tucci, Palma, Pepe, & Nazzaro, 2007; Neilson & Ferruzzi, 2011; Pereira et al., 2007; Romani, Mulinacci, Pinelli, Vincieri, & Cimato, 1999; Thompson-Witrick et al., 2014). Because of this variation within one food type, such as apples, grapes, etc., compositional data on individual polyphenols has been reported on numerous commonly consumed foods known to be good sources of dietary polyphenols (Hammerstone, Lazarus, & Schmitz, 2000; Rothwell JA, Pérez-Jiménez J, Neveu V, Medina-Ramon A, M'Hiri N, Garcia Lobato P, Manach C, Knox K, Eisner R, Wishart D, 2013). The maturity stage of the fruits also affects the polyphenol content. Some researchers have observed decreases in flavan-3-ol monomer content in grape seed extract using HPLC-UV and total polyphenol content in apples determined using phenol reagent during ripening (Kennedy, Matthews, & Waterhouse, 2000; Murata, Tsurutani, Tomita, Homma, & Kaneko, 1995). However, the reverse trend has been found in mulberry fruits using the Folin–Ciocalteau method and in black currants using HPLC-DAD where polyphenol content increased during ripening (Mahmood et al., 2017; Vuorinen, Määttä, & Törrönen, 2000). There are many factors influencing the observed polyphenol content in fruits during ripening, but one overlooked factor external to the sample itself could be the choice of analytical method used to quantify the polyphenols. The methods and the interference from the samples matrix could contribute more than previously realized to the differences in these results, and in the outcomes of studies based on polyphenol content.

The polyphenol content of plants from the same cultivar also differs if the plants grow in different geographical regions, or under different environmental conditions (Carrillo, Londoño-Londoño, & Gil, 2014; Dragovic-Uzelac, Levaj, Mrkic, Bursac, & Boras, 2007; Häkkinen & Törrönen, 2000; Makris, Kallithraka, & Mamalos, 2006; McGhie, Hunt, & Barnett, 2005). The distinct features of a certain area, such as altitude, climate, soil composition, duration of the day light exposure, etc., all affect plant physiology. The flavonol content in red wines made from the same variety of grapes grown in different regions of the world varies significantly (McDonald et al., 1998). Leafy vegetables (Hertog, Hollman, & Katan, 1992) and tomatoes (Crozier et al., 1997) growing in different seasons differ in polyphenol content. Tomatoes grown in the summer had more polyphenols than tomatoes grown during the spring (Toor, Savage, & Lister, 2006). With the stimulation of UV light (Strack, 1997), plants metabolize more polyphenols to protect themselves against DNA damage from the radiation because polyphenols can absorb UV light.

Higher polyphenol content has been found in tomatoes grown in regions receiving greater sunlight exposure than other regions (Stewart et al., 2000).

Agricultural practices also play a role in the content of polyphenols in plants. Agricultural management practices will affect the polyphenol content in plants, although they have a stronger impact on the polyphenols content in vegetables than in the fruits (Heimler, Romani, & Ieri, 2017). Some studies have shown that organically grown fruits and vegetables contain similar or slightly more polyphenols than conventionally grown ones (Faller & Fialho, 2010), although inconsistent reports exist on this topic (Anttonen & Karjalainen, 2006; Valavanidis, Vlachogianni, Psomas, Zovoili, & Siatis, 2009). When higher rates of nitrogen-containing fertilizers were applied to soil, lower polyphenol content in the plants were generally observed (Heimler et al., 2017). This can be explained by the nitrogen/carbon pathway, whereby in a nutrient-rich environment, resources are distributed to the synthesis of primary metabolites such as proteins with priority over secondary metabolites, such as polyphenols (Nguyen & Niemeyer, 2008). In addition to environmental factors, agricultural practices can also impact the sun exposure to the plants, for example, grape sun exposure is managed through leaf removal, hedging, etc. (practices by which vineyard managers remove foliage and allow the grapes to be exposed to sun), thus impacting the polyphenol concentration in grapes. Greater sun exposure of grape clusters increases the quercetin glycoside concentration in grape skin and the wine made from the highly sun-exposed grapes contains more quercetin glycoside than the wine made from shaded grapes (S. F. Price, Breen, Valladao, & Watson, 1995). Tomatoes and lettuce grown in greenhouses contain less polyphenols than those grown in the fields with open air because they were less exposed to the sunlight from the shielding and filtering of glass or plastic (Romani et al., 2002; Stewart et al., 2000).

Post-harvest factors such as the temperature, humidity, and time of the storage, as well as the presence of metal ions and oxygen, and the pH conditions also affect the polyphenol concentrations in plant-based foods (Aizpurua-Olaizola et al., 2016). The pattern of how polyphenol content in foods changes over storage is not consistent (Aherne & O'Brien, 2002). Loss of polyphenols has been found during the storage of apple juice and juice concentrates (Spanos & Wrolstad, 1992), blueberry products (Brownmiller, Howard, & Prior, 2009), and orange juices (Klimczak, Małecka, Szlachta, & Gliszczyńska-Świgło, 2007) under non-refrigerated temperatures. However, the polyphenol content in black currant and blackberry was unchanged during frozen storage (Bakowska-Barczak & Kolodziejczyk, 2011; Hager, Howard, & Prior, 2010). Monomers and dimers were retained better during storage than polymers possibly due to degradation of polymers into smaller molecules (Howard, Prior, Liyanage, & Lay, 2012).

It is generally believed that food processing steps will lower the polyphenol content as polyphenols are sensitive to light and heat, and they are easily oxidized or degraded. Thermal processing, such as pasteurization, frying, roasting, and microwaving, will cause polyphenol loss in foods (Neilson & Ferruzzi, 2011). Boiling reduces the polyphenol content also because water-soluble polyphenols move from plant tissues into the heat transfer medium, in this case, water. Furthermore, polyphenols are not evenly distributed across plant tissues. Polyphenols usually accumulate in the external tissues of plants, such as skins and leaves (Faller & Fialho, 2010). In kelps, most polyphenols are present in the outer shell which is believed to protect these plants from grazers (Tugwell & Branch, 1989). In grapes and apples, most polyphenols are present in the skins and seeds (Thompson-Witrick et al., 2014; Xia, Deng, Guo, & Li, 2010). The middle layer of the onion bulbs contains more polyphenols than the inner and outer layer (Beesk et al.,

2010). Thus, different processing steps applied to raw materials to remove different plant tissues will result in different polyphenol content in the resulting processed foods (Ewald, Fjelkner-Modig, Johansson, Sjöholm, & Åkesson, 1999). In fruit juice processing, the removal of skins and seeds causes the greatest loss of polyphenols (Howard et al., 2012). Physical processing, such as pressing and crushing, can activate polyphenol oxidase which is a naturally-occurring enzyme present in many plants that reduces polyphenol concentration through oxidation (Spanos & Wrolstad, 1992). Enzymatic clarification of fruit juices can cause polyphenol loss. The extent of polyphenol loss in fruit juices during clarification depends on which clarification method or agent was used (Spanos, Wrolstad, & Heatherbell, 1990). Other processing unit operations, such as fermentation, can also change the polyphenol content in food. The epicatechin content in fermented of cocoa beans for the production chocolate was much lower than in freshly harvested cocoa beans (Kim & Keeney, 1984). The transformation of green tea to black tea via fermentation generally reduces the catechin content because of oxidation (Neilson & Ferruzzi, 2011). Not only does the total polyphenol content change, but the polyphenol profiles also change after processing, as the polymers are more susceptible to processing than the monomers and dimers (Brownmiller et al., 2009). Furthermore, as most prior studies did not quantify polyphenols with larger DP, little is known about how processing and fermentation affect the concentrations of higher DP polyphenols in foods, or the effect of these compounds on human health.

In summary, the polyphenol content in foods can vary tremendously depending on the food type and processing history. Due to the unstable nature of polyphenols, many factors contribute to high variability in polyphenol profile and concentration in fruits and fruit products. Thus, it is important to quantify polyphenol content in fruit juices and fruit beverages accurately and in the most appropriate way for a given research question. Previous research has focused on the relationship between common analytical methods used to quantify polyphenols and their total antioxidant activity, such as g 2,2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) 2,2- diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and the oxygen radical absorption capacity (ORAC) (Soong & Barlow, 2004; Velioglu, Mazza, Gao, & Oomah, 1998). In recent years, the understanding of the health benefits of polyphenols has extended far beyond antioxidant function alone. Current themes of interest in polyphenols and health research include interactions between polyphenols and gut microbes, and recent findings show that the highest antioxidant ability in a given sample was not associated with the highest bioactivity or health benefits, therefore the common methods for polyphenol quantification for antioxidant capacity may not be the best methods in terms of predicting bioactivity (Chang, Alasalvar, & Shahidi, 2018; Tomás-Barberán et al., 2017). With the complex sample matrix, and the discovered possible interference to these methods, much information on how individual polyphenols respond to these methods and to what extent the interfering compounds influence the results should be gained.

2.5 Analytical methods for the quantification of polyphenols

2.5.1 Reading absorbance at 280 nm

Ultraviolet absorbance read at 280 nm is the simplest means for the estimation of total polyphenol content. Most phenol compounds absorb at 280 nm because of the benzene rings (Lorrain, Ky, Pechamat, & Teissedre, 2013). However, the wavelengths at which each individual polyphenol achieves its maximum absorbance are very different (Scalbert, 1992). The maximum absorbance of cinnamic acids and chalcones is not 280 nm. Besides, other non-polyphenol compounds, such as amino acids, which have the benzene rings in their structures which absorb

at 280 nm, will interfere the results by elevating the absorbance reading at 280 nm (Scalbert, 1992). Although low cost and ease of use make this method very convenient, these common sources of error make this method most suitable only as a rough estimation of total polyphenol content. Absorbance read at 230 nm on wine samples was also recently proposed for the fast estimation of astringency intensity (Boulet et al., 2016).

2.5.2 Folin-Ciocalteu method

The Folin-Ciocalteu method is commonly used to determine the total polyphenol concentration in various plant products (Somers & Ziemelis, 1980). Despite the demonstrated interference from compounds naturally existing in the plant products (Everette et al., 2010); this method continues to be very widely applied in food science, nutrition science and horticulture like due to its ease of use and low cost.

2.5.2.1 Development of Folin-Ciocalteu method

This method was first proposed by Folin and Denis for the measurement of tyrosine and the prediction of protein content in 1912. The reaction of a heteropoly phosphotungstatemolybdate reagent and tyrosine generates a blue color thus confirm the presence of tyrosine in the tested sample (Folin & Denis, 1912). Later in 1927, Folin and Ciocalteu modified the method for the determination of tyrosine and tryptophan in proteins (Folin & Ciocalteu, 1927). Lithium sulfate was added to the Folin-Ciocalteu reagent comparing to the Folin-Denis reagent to prevent the random formation of white precipitation, which interferes with the reading from the spectrometer. Also, the amount of molybdate used in the method was increased. Both modifications improved the sensitivity and reproducibility of the method for tyrosine quantification. Lower concentration of tyrosine could then be measured without the isolation of tyrosine from the samples. In 1965, Singleton and Rossi modified the method and adapted it to the measurement of total polyphenols in grape and wine samples (Singleton & Rossi, 1965). The reaction temperature and time, and the concentration of Na_2CO_3 were optimized to obtain the maximum absorbance on wine samples.

2.5.2.2 Mechanism of The Folin-Ciocalteu method

The Folin-Ciocalteu method is commonly used for the measurement of total polyphenols in food and plant extracts. In the Folin-Ciocalteu method, the Folin-Ciocalteu reagent is reduced to a blue color by the reducing compounds in the sample, including but not limited to polyphenols, under base conditions. The intensity of the blue color is proportional to the concentration of the reducing compounds present in the sample. The Folin-Ciocalteu reagent contains the colorless isopolyphosphotungstates with Tungsten in the fully oxidized stage (6+) and yellow isopolyphosphomolybdates, forming heteropolyphosphotungstates-molybdates complexes (Sigma-Aldrich Co. LLC, 2015). In acid solution, the phosphate is in core surrounded by the metal oxides as the hydrated octahedral complexes. When one or two electrons is/are supplied from the polyphenols in the samples, the complexes turn blue in color, such as in the form of $(PMoW_{11}O_{40})^{4-}$ from the yellow-greenish color. But the exact molecular and electronic structure of the ending blue complex remains unknown because of their complex nature (Pope, 2007). Overall it is a method based on redox reactions, thus not only polyphenols but other reducing compounds were also being measured as total reducing capacity of the samples. This has been confirmed by the strong-correlation among the measurements on total polyphenols in wine samples by Folin-Ciocalteu method, the potentiometric titration method (Piljac, Martinez, Valek, Stipcevic, & Ganic, 2005), and the DPPH and FRAP method for antioxidant ability (Šeruga, Novak, & Jakobek, 2011).

Gallic acid was chosen as the standard in this method for its low cost, good solubility in water, ease of purification, and stability in the powder form (Singleton, Orthofer, & Lamuela-Raventós, 1999). Besides, gallic acid was well recovered when spiked in wine samples with different concentrations and measured by the Folin-Ciocalteu method. A wavelength of 760 nm is chosen because other components in the plant extract samples do not exhibit absorbance at this wavelength. Furthermore, the absorbance maximum was observed on both wine samples and gallic acid standard at 760 nm by Folin-Ciocalteu method (Blainski, Lopes, & de Mello, 2013; Singleton & Rossi, 1965). Research has shown that individual polyphenols (caffeic acid, catechin, and vanillic acid) did not react with each other during the measurement by Folin-Ciocalteu method, that the results of the addition of each individual polyphenol, such as gallic acid added into wine, was equal to the results by the measurement on the mixture of the polyphenols (Singleton, 1974).

2.5.2.3 Interferences of matrix constituents with the Folin-Ciocalteu method

However, other reducing compounds in the samples, such as ascorbic acid, sulfite, and aromatic amines, amino phenols, tryptophan, other purines, Cuprous and ferrous ions, cysteine, and hydrogen sulfite can also supply electron to the Folin-Ciocalteu reagent, forming the same blue complex and thus elevating the quantitative result of the assay (Singleton et al., 1999). A thorough study on nitrogen compounds showed that 8.6 mg/L of certain hydroxyamino or hydrazino derivatives can generate the absorbance of 1 absorbance unit (AU) with in the measurement (Ikawa, Schaper, Dollard, & Sasner, 2003). Although the extent of reaction of primary aliphatic amino acids with the Folin-Ciocalteu reagent was negligible, the concentration of 4.6 mg/L of aromatic amino acids contributes to the absorbance of 1 AU. These compounds generate different absorbance with the amount of one mole (molar color yield) upon reaction with the Folin-Ciocalteu reagent. Guanine, xanthine, and uric acid yield the same molar color yield as monophenols (Myers & Singleton, 1979). The molar color of Cysteine was less than the monophenols (Singleton et al., 1999). While the molar color generated by adenine and other purines, and the common pyrimidines was very light (about $1/50^{th}$ of the monophenols) (Ikawa, Dollard, & Schaper, 1988). Free SO_2, ascorbic acid and other compounds contribute 2.5% of the final results when they were separated in white wine samples by gel column chromatography (Myers & Singleton, 1979).

Sulfur dioxide (SO_2) has been widely used in the fruit juice industry and wine and cider making (Boulton, Singleton, Bisson, & Kunkee, 1999). SO_2 can prevent the growth of undesirable microorganisms, especially bacteria, and prevent oxidation by inhibiting the activity of polyphenol oxidase (Boulton et al., 1999). SO_2 can be harmful to humans with asthma at the concentration as low as 5 ppm (Sheppard, Wong, Uehara, Nadel, & Boushey, 1980). The Emergency Exposure Limit was 5 ppm for 24 hours and the Continuous Exposure Limit for 90 days was 1 ppm in workspaces (National Research Council, 1984). Handling SO_2 in gas form and concentrated aqueous form requires extra care. So, the addition of SO_2 is often made by dissolving non-volatile bisulfite salts, such as potassium metabisulfites (57.6% of SO_2 by weight) into acidic solutions, releasing SO_2 in that solution. Three forms exist in wine or fruit beverages treated with SO_2: molecular SO_2, bisulfite HSO_3^-, and sulfite SO_3^{2-}, and the compound is converted among these forms depending on the pH of the solution while maintaining an equilibrium through dissociation (diagram shown in **Figure 2.10**). Together these three forms of sulfites are present in fruit juice and beverages exist in two states: free and bound. They can bind with phenols, acetaldehydes, and sugars. The antioxidant and antimicrobial ability of sulfites comes from the free states, while the bound states are inactive. At the pH for most fruit juices

and beverages (between 3 and 4) (Goldmann, 1949), sulfites are in the form of mostly bisulfite and little of the molecular SO_2. The molecular SO_2 has stronger antimicrobial ability and less antioxidant ability comparing to bisulfite (Carrascon, Ontañón, Bueno, & Ferreira, 2017).

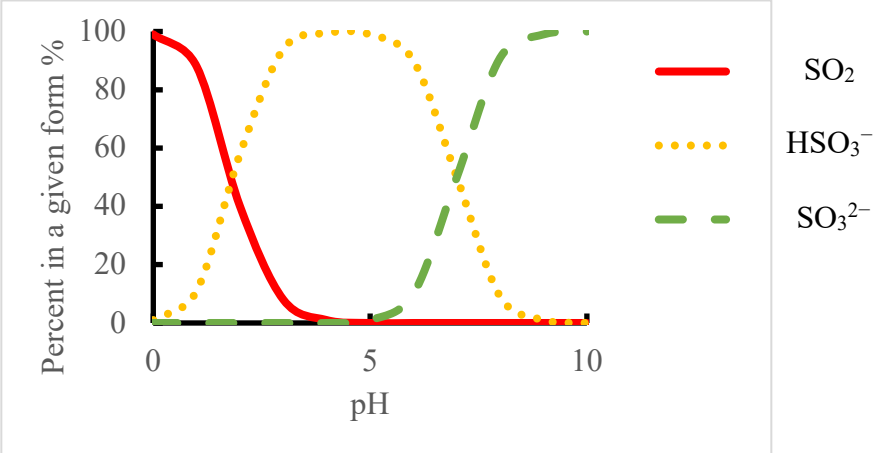


Figure 2. 10. Equilibrium of different form of SO_2 depending on the pH of the solution [figure remade from Henderson, 2019 (Henderson, 2009)]

Ascorbic acid (Vitamin C, or ascorbate) also interferes with the Folin-Ciocalteu method. It is a water-soluble vitamin naturally present in fruits and vegetables. Its chemical structure is shown in **Figure 2.11**.

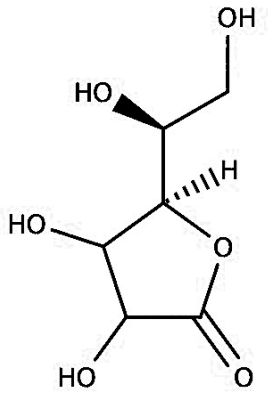


Figure 2. 11. Chemical structure of ascorbic acid

As an antioxidant and reducing agent, ascorbic acid protects tissues against damage by reactive oxygen species and other free radicals that are produced in the normal human metabolism. Fruits, especially Acerola, citrus fruits, kiwifruits, tomatoes, apples, and vegetables, such as peppers, parsley, kales, broccoli, Brussels sprouts, are rich in ascorbic acids, with as high as 1677.60 mg of ascorbic acid in 100 g of raw acerola ("USDA National Nutrient Database for Standard Reference, Release 28.," 2016). Ascorbic acid fortified fruit-flavored drinks are also important source of ascorbic acid in human diet, with as high as 2400 mg of ascorbic acid in 100 g of orange-flavored, breakfast type, low calorie, powder beverages ("USDA National Nutrient Database for Standard Reference, Release 28.," 2016). With 1 mg/L of ascorbic acid contributing to 0.662 mg/L of gallic acid equivalent from the Folin-Ciocalteu method (Everette et al., 2010), the total polyphenols quantified by the Folin-Ciocalteu method will be much higher than the actual polyphenol content in samples with abundant amount of ascorbic acid, contributing to the

confusion in polyphenols and sensory and/or health research. The results of polyphenol quantification in ascorbic acid-rich samples should be interpreted with caution.

Little research has quantified the interference of these compounds contained in the complex matrix of fruit juice and beverage samples, or how the various forms present may influence any interference. Some researchers believe that the amounts of interfering compounds in fruit and fruit beverages, such as wine, are too small to cause a practically significant difference in the results (Singleton et al., 1999). In addition, the degree of interference was also influenced by the concentration of the interfering compounds, for example, small amount of sugar (glucose) cannot react with the Folin-Ciocalteu reagent thus did not interfere with the results, but the reaction can significantly impact quantitative results if the sugar concentration is high (>2.5%) (Slinkard & Singleton, 1977). With advanced analytical methods, the concentrations of these compounds have been precisely measured. But since the matrix is very complex for fruit and fruit beverage samples, no research quantified the extent to which non-target compounds in the sample matrix interfere with results of the Folin-Ciocalteu assay. The mechanism of how non-polyphenol compounds react with the Folin-Ciocalteu reagent thus interfering with the results has not been studied thoroughly and remains unclear. Some of these compounds have strong reducing capacity and can oxidize Folin-Ciocalteu reagent directly. Others can react with the polyphenol compounds and regenerate hydroquinone to further oxidize the Folin-Ciocalteu reagent.

2.5.2.4 Efforts to eliminate interference in the Folin-Ciocalteu method

Studies have been done to identify and eliminate sources of interference in the Folin-Ciocalteu method. Many of these studies targeted ascorbic acid, which is an abundant compound in many fruit-derived samples, although it is possible that the ascorbic acid is oxidized already before the addition of Folin-Ciocalteu reagent, thus retaining little reductive capacity. Using ascorbate oxidase to remove the ascorbic acid has been attempted, but did not entirely eliminate the interference of ascorbic acid for the Folin-Ciocalteu method (Ford, Bai, & Baldwin, 2010), because of finishing product dehydroascorbic acid from the reaction of ascorbic acid and ascorbate oxidase still reacting with the Folin-Ciocalteu reagent and generating color. Removal of ascorbic acid by heating has also been proposed and the content of ascorbic acid in fruit products and beverages obtained by the difference of the results of Folin-Ciocalteu method on the original sample and the sample after the removal of ascorbic acid by heating were similar to the ascorbic acid values quantified by HPLC via direct injection (Georgé, Brat, Alter, & Amiot, 2005). Other methods aimed to first determine the ascorbic acid concentration in the samples, and subtract its contribution to the Folin-Ciocalteu results to get the adjusted final results (Singleton, 1974). Instead of using other methods to measure the ascorbic acid concentration, a method was developed which takes advantage of the fact that ascorbic acid can react with the Folin-Ciocalteu reagent under acidic or neutral pH conditions (without addition of Na_2CO_3 to achieve basic conditions), while the polyphenols and other less strong reducing compounds cannot (Sanchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velazquez, 2013). Polyphenols require basic conditions to be protonated to react with the Folin-Ciocalteu reagent. The contribution of color formed by reaction of ascorbic acid with the Folin-Ciocalteu reagent can be determined under acidic or neutral conditions, with the reading taken immediately after the Folin-Ciocalteu reagent is added. In order to correct for the interference by ascorbic acid, this value can then be subtracted from the final reading after addition of Na_2CO_3 and sufficient reaction time for the polyphenols to react with the Folin-Ciocalteu reagent (Singleton et al., 1999). Recovery on lemon juice and other biological samples with 200 μg/mL of ascorbic acid

showed that this method was efficient on the quantification of ascorbic acid (Jagota & Dani, 1982). However, the method has not been systematically evaluated or tested on a wide range of fruit juice/beverage samples. Thus, it is necessary to validate this method according to the analytical method validation procedures.

Even when ascorbic acid was oxidized already before the addition of Folin-Ciocalteu reagent, it can still interfere with the results because of the first oxidation product dehydroascorbic acid still reacting with the Folin-Ciocalteu reagent (Singleton et al., 1999). Under heated flow automatic analysis, the measurement on dehydroascorbic acid at the concentration of 100 ml/L was 45 mg GAE/L by the Folin-Ciocalteu method (Singleton et al., 1999). However, normal methods to measure ascorbic acid do not include the detection of dehydroascorbic acid and thus could not be used to predict this interfering effect.

2.5.2.5 Analysis of ascorbic acid

The absorbance from ascorbic acid in fruit juice and beverages in the quantification of polyphenols by Folin-Ciocalteu method is not negligible. Besides, in the reducing form, ascorbic acid is not stable; exposure to light, heat, and oxygen during food processing and storage will cause it to degrade (Nielsen, 2017). Thus, it is important to measure ascorbic acid in fruit juice and beverages precisely and accurately for accurate quantification of polyphenols by the Folin-Ciocalteu method.

Many methods have been developed to quantify ascorbic acid in fruits and vegetables. A titration method using 2, 6-dichloroindophenol is the official method for quantification of ascorbic acid in juices, AOAC Method 967.21 Ascorbic Acid in Vitamin Preparations and Juices ("Ascorbic Acid in Vitamin Preparations and Juices, AOAC Method 967.21," 2017). The indicator dye, 2,6-dichloroindophenol, is standardized before usage by standard ascorbic acid solution for tis exact concentration. During the titration, the indicator dye becomes colorless due to the reduction by ascorbic acid. A proper acidic condition is needed for this oxidation-reduction reaction and it can also prevent the auto-oxidation of ascorbic acid under high pH condition. The end point is determined at the color of rose pink by excess of unreduced indicator dye under acidic condition.

Spectrophotometric methods have also been developed for the quantification of ascorbic acid in a variety of samples and they have been approved to be more sensitive, precise and less affected by interference than the above titrimetric method (da Silva, Aguiar-Oliveira, Mazalli, Kamimura, & Maldonado, 2017). Indirect measurement of ascorbic acid by spectrometry is based on reaction of ascorbic acid with the reagent and the increase of generated compounds will be measured because it is stable, can be easily measured by the spectrometer, and follows the Beer-Lambert Law. One reagent is iron (III), and it can be reduced to iron (II) by ascorbic acid and the increase of generated complex of iron (II) with 4-(2-pyridylazo) resorcinol is measured by reading the absorbance 710 nm (Arya, Mahajan, & Jain, 2001). Reagent of 2,4-dinitrophenylhydrazine is also used in the spectrophotometric quantification of ascorbic acid, where ascorbic acid is oxidized into dehydroascorbic, and the increase of the resulting complex formed by the addition of 2,4-dinitrophenylhydrazine and sulfuric acid is measured by reading the absorbance 540 nm (Ball, 2006). Ascorbic acid can be measured directly by spectrometry as well, because of its absorption maximum in the range of 244–265 nm depending on the pH (Gómez Ruiz, Roux, Courtois, & Bonazzi, 2016).

With the development of liquid chromatography, it has been applied for the quantification of ascorbic acid in fruits and beverages (Boonpangrak, Lalitmanat, Suwanwong, Prachayasittikul, & Prachayasittikul, 2016; De Ancos, Cilla, Barberá, Sánchez-Moreno, & Cano,

2017; Rose & Nahrwold, 1981; Wimalasiri & Wills, 1983). The identification and quantification of ascorbic acid by liquid chromatography is more specific and accurate than the spectrophotometric methods (Najwa & Azrina, 2017).

In summary, the Folin-Ciocalteu method is still a common method used for the quantification of total polyphenols in food science and horticulture research despite its limitations. The results from this method should be interpreted with caution because it may not accurately quantify the total polyphenols but rather the total reducing capacity of the samples.

2.5.3 Lowenthal Permanganate Titration

Lowenthal Permanganate Titration has a long history in cider analysis, and has also been applied occasionally in pear, peach, tea and coffee (Barua & Roberts, 1940; Smit, Joslyn, & Lukton, 1955), but has not emerged as a preferred method for products other than apples and ciders. This was the standard method used at the Long Ashton Research Station in the UK, a leading global center for agriculture and horticulture research, especially research on cider and cider apple, from 1903 when it was established as the National Fruit and Cider Institute for the quality improvement of cider production until the Cider Section's closure in the 1980's (Burroughs & Whiting, 1960). In this titration method, polyphenols are oxidized by potassium permanganate solution and indigo carmine is used as a oxidation-reduction indicator for the determination of the end point (Lowenthal, 1877). The results are expressed as tannic acid equivalents. The potassium permanganate solution is sensitive to light and oxidation and should be prepared freshly on the day of use.

There are several limitations of the permanganate titration method. First is the difficulty of standardizing the potassium permanganate solution used in the titration due to its nature of being easily oxidized. Also, the results in tannic acid equivalents for the same amount of different pure polyphenol compounds vary. For example, the quantification result by Lowenthal Permanganate Titration on one gram of quercetin was 1306 mg tannic acid equivalents, on one gram of catechin was 1054 mg tannic acid equivalents, and on one gram of chlorogenic acid was 950 mg tannic acid equivalents (Smit et al., 1955). It is also difficult to determine the end point of the titration by visual observation of the color. The interference from reducing compounds, such as sugars, is believed to be greater for the Lowenthal Permanganate titration than that of the Folin–Ciocalteu method because of potassium permanganate is a stronger oxidizing agent compared to the Folin-Ciocalteu reagent (Singleton et al., 1999). Compared to the Lowenthal Permanganate Assay, it is generally believed that the Folin–Ciocalteu method is preferable because of greater accuracy and less interference. Since this hypothesis has not been tested experimentally, comparison of these two methods by analytical method validation procedures is needed.

2.5.4 Iron salt

Iron salt also has been used in the quantification of total polyphenols, especially in dark beers, because of the lower incidence of interference with this assay by dextrins, melanoidins, and proteins, compared to the Folin-Ciocalteu assay (Singleton et al., 1999). However, it is not preferred over the Folin–Ciocalteu method for the measurement of total polyphenols in samples other than beer. Monophenols cannot be detected by this method and vicinal diphenols and vicinal triphenols generate different colors reacting with the ferrous ions (Singleton et al., 1999).

2.5.5 Prussian Blue assay

The Prussian Blue assay is a spectrophotometric method based on the formation of a metallic complex (Prussian Blue, $Fe_4[Fe(CN)_6]_3$) under low pH conditions by reducing Fe^{3+} to Fe^{2+} (M. L. Price & Butler, 1977). This method has been validated using gallic acid as the

standard (Pueyo & Calvo, 2009). However, the response factors for other pure polyphenol standards, such as catechin and chlorogenic acid, are not equal to one on a molar to molar basis (Slimestad, Vangdal, & Brede, 2009). The type of polyphenols and the relative concentration of polyphenols to each other will greatly impact the results on samples containing a mixture of polyphenols, such as fruit juice and fruit beverages. Significant measurement error using this method can be expected on samples such as fruit juice and beverages which contain different polyphenol compounds and in which gallic acid is not the major polyphenol constituent. For example, the polyphenol content in a sample containing chlorogenic acid, naringin, and ferullic acid will be greatly under estimated by this method because the Molar Response Factors (absorbance per mole of sample divided by the absorbance per mole of gallic acid) for chlorogenic acid, naringin, and ferullic acid were 0.43, 0.20, and 0.61, respectively. It is also possible that the results on a mixture of polyphenols is close to the actual polyphenol content, for example, a sample contain the same amount of catechin and sinapic acid, because the Molar Response Factors for catechin and sinapic acid were 1.19 and 0.63, respectively (Slimestad et al., 2009).

This method has been applied to the determination of total polyphenol concentrations in various samples, such as plant extract (Graham, 1992; Pueyo & Calvo, 2009), tea and grape juice (Margraf, Karnopp, Rosso, & Granato, 2015), strawberries (Budini, Tonelli, & Girotti, 1980), mushrooms (Reis, Martins, Barros, & Ferreira, 2012), and rice (Finocchiaro, Ferrari, & Gianinetti, 2010). The limitations of this method are that samples with high polyphenol concentration need to be diluted and the absorbance should be read shortly after the reaction otherwise precipitate will be formed; the timing for absorbance reading is critical since the color intensity is not stable over time (Graham, 1992); and common interferences from the sample matrix when the reaction is based on redox, similar to those of the Folin-Ciocalteu and the Lowenthal Permanganate assays. Research has shown that H_3PO_4 can stop the reaction and prevent the formation of precipitate. The addition of gum acacia can stabilize the color development of Prussian Blue (Graham, 1992). Pueyo and Calvo improved the method by adapting it into using 96-well micro plates (Pueyo & Calvo, 2009), thus fewer reagents were used in the method and the results of more samples can be read at the same time. However, interference has been found from cysteine, homocysteine, tryptophan, pyridoxine and ascorbic acid (Budini et al., 1980; Graham, 1992). The interference by ascorbic acid (spiked in grape juice at the concentration of 212 mg/L) to the Prussian Blue method is 10% higher than its interference with the Folin-Ciocalteu method (Margraf et al., 2015). The interference caused by ascorbic acid can be corrected for by subtracting the contribution of ascorbic acid from the final results (Budini et al., 1980), but this method requires further validation. Due to its better reproducibility and repeatability over Folin-Ciocalteu Assay on tea and grape juice samples (Margraf et al., 2015), the Prussian Blue assay is very promising for the screening of total polyphenols in apple and hard cider samples.

2.5.6 Bate-Smith assay

The Bate-Smith assay (acid butanol assay) was developed in 1975 by Bate-Smith for the determination of total proanthocyanidins in leaves (Bate-Smith, 1975). Under acidic and heating conditions, proanthocyanidins were depolymerized and converted into mainly cyanidin and delphinidin. The final products have an absorbance at 550 nm and the concentration of proanthocyanidins was quantified (Gessner & Steiner, 2005; Lorrain et al., 2013). The principle of this method could limit its application on sampels rich in cyanidin and delphinidin, where the results will be more than the actual proanthocyanidins. This method is not as simple as other

assays because it requires extraction and purification of the target compounds from the initial samples, for example using relatively pure proanthocyanidins extracted from apples to quantify the proanthocyanidin concentrations in apple samples (C. Li, Trombley, Schmidt, & Hagerman, 2010). Polyphenol extract from quebracho and sorghum have been used as standards in this method, but the color intensity developed by these two standards were not representative for all proanthocyanidins present in samples (Hemingway, 1989). With validation by solid-state 13 C nuclear magnetic resonance (NMR) spectroscopy, proanthocyanidins were poorly extracted and recovered by the acid butanol solution (Harinder P S Makkar, Gamble, & Becker, 1999). The interpretation of results from this method should be critically evaluated due to these limitations.

2.5.7 Vanillin test

The Vanillin test has been developed for the estimation of flavanols in grain Sorghum (Burns, 1971). The principle of this method is that during the reaction between the vanillin reagent and target compounds, a weak electrophilic radical is generated from the vanillin reagent by protonation under acidic conditions. An intermediate compound is formed by this radical reacting with the flavonoid ring at the 6 or 8 position and is then dehydrated to a red colored compound (Ribéreau-Gayon, 1972). The absorbance of the final product is then read at 500 nm.

A single bond (rather than a double bond) between C-2 and C-3 is an essential requirement for a positive reaction in the Vanillin test (Sarkar & Howarth, 1976). This method is specific for the concentration of flavanols, in which there is no carbonyl group at the C-4 position (Swain & Hillis, 1959). The carbonyl group at the C-4 position deactivates the reaction. However, research has shown that non-flavanol compounds, such as phloretin (a dihydrochalcone), phloridzin (a dihydrochalcone glycoside), naringenin, hesperetin, and dihydroquercetin contribute great, intermediate and weak intensity in color development, respectively (Sarkar & Howarth, 1976). Besides, part of the absorbed wavelength (500 nm) of the final product in Vanillin test was also the wavelength at which the natural anthocyanins or converted leucoanthocyanidins exhibit absorbance (McMurrough & McDowell, 1978). Non-flavonoid and other flavonoids cannot be detected by this method because the phloro-glucinol A-rings cannot react with the Vanillin reagent (Kramling & Singleton, 1969). Catechin was often chosen as the standard in this method, but will result in an overestimation of the true flavanols content in samples and the reacting temperature is essential to the color development (M. L. Price, Van Scoyoc, & Butler, 1978). It is best recommended that for samples containing a mixture of catechin and proanthocyanidins, the polyphenols should be separated and quantified using catechin and proanthocyanidins extracts as the reference standards, respectively (Sun, Ricardo-da-Silva, & Spranger, 1998). However, this suggestion can be very time-consuming. The considerable drawbacks of the Vanillin method make it difficult to apply this method as a routine analytical procedure for the quantification of polyphenols in fruit juice and beverages.

2.5.8 The 4-dimethylaminocinnamaldehyde method

The 4-dimethylaminocinnamaldehyde (DMAC) method is another colorimetric method for the quantification of flavanols. Compared to Vanillin, the DMAC method is preferred because it is more sensitive and specific (only flavanols and their gallates are detected) (Rohr, Meier, & Sticher, 2000). The absorbance is read at the wavelength of 640 nm, eliminating the interference from anthocyanins. This method was developed in 1971 (Thies & Fischer, 1971) and first employed on the measurement of flavanols in hops and barleys (McMurrough & McDowell, 1978), then it was modified and applied on beer (Delcour & Varebeke, 1985), red wines (Arnous, Makris, & Kefalas, 2001; Nagel & Glories, 1991), cranberry (Y. Wang et al., 2016), legumes (Y.-G. Li, Tanner, & Larkin, 1996), and cocoa products (M. J. Payne et al.,

2010). This method gained popularity because it was believed that the DMAC reagent only reacted with the C8 carbon at the terminal units on an A-ring proanthocyanidins (Wallace & Giusti, 2010), thus the color intensity produced by different procyanidin compounds would be the same on a molar basis. However, later research showed that monomers gave a higher response than oligomers (the absorbance of epicatechin can be as twice as it of procyanidin B1 with the same concentration and same length of the light pass) (Delcour & Varebeke, 1985; Y.-G. Li et al., 1996; M. J. Payne et al., 2010; Y. Wang et al., 2016). Research on cocoa and cranberry samples also showed that the DP impacted the absorbance reading on a molar basis (Y. Wang et al., 2016). It is essential to choose the representative compounds of interest for a given research question as the standard in this method. Using the wrong standard would result in underestimating or overestimating the amount of flavanols in the samples. Besides, different solvents (ethanol or methanol) used in the DMAC measurement will generate different color intensity when the same compound at the same concentration was measured (Y. Wang et al., 2016).

2.5.9 Bovine serum albumin precipitation method

The concentration of total polyphenols also can be assessed by the precipitation of polyphenols and proteins (in this case bovine serum albumin, BSA). This method was initially reported by Hagerman and Butler in 1978 for the determination of polyphenols in grains (Hagerman-Butler method) (Ann E Hagerman & Butler, 1978). The method is based on the assumption that the precipitation of polyphenols and protein is proportional to the concentration of polyphenols in a given sample. In this method, samples are mixed with excess standard BSA protein solution, allowing for precipitation. Only the precipitate is retained and re-dissolved in sodium dodecyl sulfate-triethanolamine solution. Ferric chloride reagent is then added and reacted with the re-dissolved polyphenols for the generation of color. The absorbance is read at 510 nm, the λ_{max} of the complex formed by polyphenols and protein in alkaline solution. The background absorbance was corrected for by subtracting the absorbance of sodium dodecyl sulfate-triethanolamine solution and ferric chloride reagent from the sample absorbance. In reality, the binding affinity of proteins and polyphenols depends on both the structures of the protein and the polyphenols. Thus, the results cannot be directly compared among polyphenols from different sources. BSA was chosen because it is commonly used as a model protein to study the interaction between polyphenols and proteins (Carvalho, Póvoas, Mateus, & de Freitas, 2006). Other proteins that were evaluated for binding polyphenols in this assay, especially proline-rich proteins, have higher binding affinity with polyphenols comparing with BSA in precipitating polyphenols (A E Hagerman & Butler, 1981). Besides, the DP of polyphenols may affect the results of this method (Ann E Hagerman & Butler, 1978). BSA was able to bind with procyanindin monomers and dimers but not able to form precipitates; instead precipitates were formed between BSA and procyanidins oligomers with higher molecular weights (de Freitas & Mateus, 2001; Siebert & Lynn, 1998). The binding affinity with the BSA becomes weaker as the polymers of polyphenols get bigger (A E Hagerman & Butler, 1981). The pH of the solution during precipitation also plays a role as the polyphenols can bind with more protein at pH near the isoelectric point of the BSA or other proteins used as the binding agent to polyphenols (A E Hagerman & Butler, 1981). The formation of BSA-polyphenol complexes was also affected by the solvents used to extract polyphenols if applied during sample preparation, as trace amounts of acetone have been found to inhibit the formation of the complex (Makkar, 1989).

The Hagerman-Butler method has been modified specifically for the determination of polyphenols in wine samples and the adaptive method was reported in 1999 (Adams &

Harbertson, 1999). This method was able to estimate the total amount of polyphenols that contributed to astringency, which is of particular interest to enologists. The method was based on the principle that the amount of alkaline phosphatase that can bind with polyphenols to form a precipitate is proportional to the amount of total polyphenols in the samples. The tannin-protein complex is formed by adding a mixture of alkaline phosphatase and BSA into given grape or wine sample and the precipitate is re-dissolved in diethanolamine buffer solution at pH of 9.4. The amount of alkaline phosphatase activity in the re-dissolved pellet is measured by adding *p*-nitrophenylphosphate substrate. Compared to the methods which measured how much of the BSA bind with the polyphenols, the measured polyphenol concentration in a given sample in this method positively correlated with the measured alkaline phosphatase activity rather than negatively correlated with the leftover BSA concentration (Ratnavathi & Sashidhar, 1998).

Later, the Hagerman-Butler method was further adapted and applied to the routine determination of total polyphenols in grape and wine samples because of its simple operation, for example the total polyphenols in the skins and seeds of red *Vitis vinifera* wine grapes using catechin as the standard (Adams-Harbertson method, BSA precipitation method) (Harbertson, Kennedy, & Adams, 2002). This adaptation, known as the Adams-Harbertson assay, measured the polyphenols which bind with BSA directly using ferric chloride without the use of alkaline phosphatase. But the method only quantified the polyphenols with four or more subunits, thus indicating more information regarding to the degree of astringency of the samples, than the concentration of total polyphenols, per se. However, poor precision and recovery have been reported with use of the Adams-Harbertson to quantify total procyanidins in bottled wine samples by winery labs and a commercial testing lab (Brooks, McCloskey, Mckesson, & Sylvan, 2008). This may be due to poor training of laboratory personnel, however, rather than inherent flaws in the assay. Regardless, assays used in production environments must be appropriate for the expected skill level of laboratory employees. Despite these extensive attempts at improvement on the BSA method, the results from the original BSA precipitation method still have the strongest correlation with perceived astringency among other methods on red wine samples (Kennedy, Ferrier, Harbertson, & des Gachons, 2006), and closer correlation with perceived astringency than colorimetric methods on other wine samples (Cáceres-Mella et al., 2013). Thus, the BSA precipitation method has the best potential of known analytical methods to predict the intensity of astringency in wine (Boulet et al., 2016) but the expansion of the application to other fruit juices and beverages requires more research and method validation. Research targeting improvement of the BSA precipitation method, such as decreasing the background absorbance, is still on-going (Harbertson, Mireles, Yu, Boulton, & Harbertson, 2015). Overall, despite the complex structure-based reaction between polyphenols and proteins and the limitation of the BSA to precipitate polyphenols of small molecular weights, BSA precipitation method has good potential for a wide application of predicting the astringency in fruit juices and beverages.

2.5.10 Methyl cellulose precipitation method

The methyl cellulose precipitation method is increasingly used to measure polyphenols that can be precipitated with methyl cellulose in red grape juice and red wine – essentially the total tannins excluding monomeric catechins and anthocyanins (Mercurio, Dambergs, Cozzolino, Herderich, & Smith, 2010; Sarneckis et al., 2006). Methyl cellulose is one type of polysaccharide polymer which does not absorb light at 280 nm. By subtracting the absorbance of the original sample and the sample treated with methyl cellulose, the concentration of polyphenols precipitable by methyl cellulose can be determined (Sarneckis et al., 2006).

The application of this simple and robust method on red grape extract and red wine samples is promising because ethanol and relatively low pH, matrix conditions inherent to red wine, had minimal effect on the quantification results and good correlation has been found between this method and the reference HPLC method for the quantification of total tannins excluding monomeric catechins and anthocyanins (Sarneckis et al., 2006), the polyphenols expected to contribute astringency and mouthfeel to red wines.

However, this method has not been validated for samples other than red grape and wine samples. Validation with other sample types, for example white wine or cider, would be required prior to application of this method in samples expected to have substantially different polyphenol composition compared to red grapes or red wine. The importance of validation with new sample types was also emphasized in this study, where polyphenols in commercial tannins with varying polyphenol composition were quantified and compared to the reference values determined by HPLC. Significantly different responses were found depending on polyphenol composition (Sarneckis et al., 2006). The DP pf polyphenol could affect the quantification results, as procyanidin dimer could not precipitate with methyl cellulose but polyphenol extracts from grape seeds with mean DP > 3 was precipitated (Vidal et al., 2003).

The inability to quantify polyphenols in rose and white wine samples using methyl cellulose precipitation has also been reported. This is attributed to the fact that a precipitate was not formed (thus not quantified) due to the low polyphenol concentration (for polyphenols other than monomeric catechins and anthocyanins) in these samples (Cáceres-Mella et al., 2013).

A strong correlation between values obtained through the methyl cellulose method and the BSA precipitation method has been demonstrated on red wine and grape extracts, although the numerical results of the methyl cellulose method are up to 3-fold higher than the numerical values obtained through BSA precipitation (Mercurio & Smith, 2008). These differences are likely attributable to the different mechanisms underlying these two methods, but more work needs to be done to conclusively explain the observed difference (Harbertson & Downey, 2009). The methyl cellulose precipitation method has good potential to predict astringency of red wines because of its high correlation between the quantification results and the perceived astringency on red wine samples (Cáceres-Mella et al., 2013). This method has been adapted into a high throughput analysis format using the 96 well plate (Meagan D. Mercurio, Robert G. Dambergs, Markus J. Herderich, & Smith, 2007), and the application of the methyl cellulose method on other fruit extract, juice, and beverages warrants further investigation.

2.5.11 HPLC-MS

Compared to the colorimetric methods for the determination of total polyphenols, the separation and quantification of individual polyphenols by High Performance Liquid Chromatography coupled with Mass Spectrometer (HPLC-MS) take a different route and provide more precise results for quantifying known target compounds. HPLC-MS methods for individual polyphenol analysis have been well developed and applied on various samples (Alakolanga et al., 2014; Jaiswal & Kuhnert, 2014; Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2015; Thompson-Witrick et al., 2014). However, these methods cannot characterize the structures of polyphenol compounds for sure (only with the application of nuclear magnetic resonance spectroscopy can the chemical structure be confirmed) (Balentine et al., 2015). Lack of commercially available polyphenol standards and the high cost of these standards also limit application of HPLC-MS methods, as it cannot quantify all polyphenols existing in fruit juice and beverages. It is exceedingly impractical if not impossible to obtain authentic analytical standards for all polyphenol compounds present in the fruit juices and

beverages. Summing the individual polyphenol compounds quantified by HPLC-MS may underestimate the total polyphenol concentration since not all polyphenols in the sample are included in the list of target compounds. The use of a single standard to quantify multiple compounds which the authentic standard for a target compound is not available, or to lower the experimental cost is often reported, for example using procyanidin dimer B2 as the standard to quantify other unknown dimers in the samples (Thompson-Witrick et al., 2014). Besides, similar to analysis with biosensors (Portaccio et al., 2006), analysis by HPLC-MS is relatively expensive, requiring advanced instruments which many labs cannot afford or are not equipped with. The lab practices, including the operation and maintenance of the equipment, require technicians with high expertise and specialty in these analyses, a major obstacle to moving from spectrophotometric to HPLC-MS assays. The organic solvents used in elution and separation can be hazardous to personnel and the environment, and require careful handling. Also, clean liquid samples must be introduced to an HPLC-MS system. After the required sample preparation process to obtain a clean liquid from plant tissue, such as extraction and filtration, the polyphenols in the actual analyzed sample will not be exactly the same as the polyphenols present in the samples. Although extraction of the sample is often required, a consistent extraction protocol has not been developed. Different extraction efficiency by different extraction procedures, such as different extraction solvents, time, and temperature, will result in the variability of polyphenol quantification in the samples (Ćujić et al., 2016). Research on olive oil samples showed that the correlation of the polyphenols content measured by Folin-Ciocalteu method and HPLC method did not correlate well ($r = 0.64$) (Andjelkovic et al., 2008). The low correlation may be caused by the drawbacks of HPLC-MS mentioned above. Thus, colorimetric methods with simple sample preparation steps and precise results for the determination of total polyphenols are needed for the lab routine analysis both in academic and in the quality control labs in the industry.

In summary, each analytical method has different advantages and disadvantages, including the lack of specificity towards polyphenols, interferences present in the sample matrix and different response of individual polyphenol compounds to the overall results. To date, no one has systematically evaluated how these factors influence the outcomes of these methods or made recommendations for the best analytical methods to address the specific research questions relating to fruit juices and fruit beverages. The total polyphenol quantification obtained from different analytical methods should be compared and evaluated critically with careful consideration of the limitations and potential interferences with each method, since the results obtained from the same sample by different methods may not be consistent. The inappropriate application (or at least lack of context in terms of sources of error) of analytical methods for the quantification of polyphenols limits the progress on polyphenol and health research, leading to that fact that 1) no conclusive recommendation has been made on the type and amount of dietary polyphenols intake for consumption on the purpose of promoting health while extensive research has shown the health benefits of polyphenols (Balentine et al., 2015), and 2) no conclusive recommendation has been made on adjusting polyphenols (type and amount) in the fruit juice and beverage to achieve the desired sensory characteristic of the products for better consumer acceptance and preference. It is not only important to accurately quantify total polyphenols, but to be able to choose the analytical method that quantifies the compounds of importance to a given research question. Accurate quantitative information on the polyphenol content in the fruit juice and beverages should be obtained before the start of any clinical studies on the health benefits of polyphenols in these samples and more information on the correlation of the

quantification results of polyphenols and the sensory quality of the fruit juice and beverages should be gained.

2.6 Analytical method validation

An analytical method is a procedure by which target compounds in certain samples can be analyzed. Analytical method validation is the systematic process by which to determine whether the analytical method can achieve the intended purpose or not (Swartz & Krull, 2012).

Analytical method validation was not yet employed in the 1940's, as review articles from that decade on quantitative analysis in chemistry did not mention the comparison of the same or similar analytical methods for precision or accuracy (Strong, 1947). With rapid growth in the number of reported analytical methods in the 1970s, a series of papers were published which brought attention to the importance of comparing the advantages and disadvantages of analytical methods (Wilson, 1970a, 1970b, 1973, 1974). To evaluate the performance characteristics of the reported quantitative analytical methods, general problems or aspects of analytical methods were identified and their importance were assessed (Wilson, 1970a). Several consistent definitions, criteria and parameters of the methods were developed and suggested for reporting the methods, such as standard deviation of analytical results being used for reporting the precision of the methods (Wilson, 1970b, 1973), definitions of range, and the power of detection (limit of detection) were proposed (Wilson, 1973). Beginning with this attention in the literature, it is widely agreed upon by current researchers that the validation of analytical methods not only contributes to the quality, accuracy, reliability and consistency of the measurements in research labs, and it is also often required for regulatory and quality control labs (Araujo, 2009) . Validation of the analytical methods should be conducted before the initial use of the method for routine testing in a lab, or when the method is being transferred to a different lab, or in the situation where one or multiple of the conditions or parameters of the analysis procedure of the previous validated method changes/change and the change is beyond the parameters or conditions that has been studied for precision and robustness during the previous method validation (Huber, 2010). When analyses are conducted appropriately with a validated method, the achieved measurements should be accurate, precise, reliable, and truly representative of the analyte in the samples (Araujo, 2009).

Guidelines on analytical method validation are provided by several well-known international organizations (Araujo, 2009), among which the references from the United States Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) are primarily used in the Food Science field.

Eight typical characteristics of the analytical method under consideration will be evaluated during the process according to the Analytical Procedures and Methods Validation for Drugs and Biologics published by U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), and Center for Biologics Evaluation and Research (CBER) in 2015 (U.S. Food and Drug Administration, 2015). This document was adjusted to meet the specific requirements when working with drugs and biologics from Validation of Analytical Procedures: Text and Methodology (Q2(R1)) published by International Conference on Harmonization (ICH) in 2005 (International Conference on Harmonization (ICH), 2005).

Eight typical characteristics of analytical methods are: accuracy, limits of detection (LOD), limits of quantitation (LOQ), linearity, precision (repeatability, intermediate precision, and reproducibility), range, robustness and specificity. Appropriate statistical analysis should be used for the interpretation of results.

2.6.1 Accuracy

The accuracy (trueness) of an analytical method is the nearness of the results to the true value. It can be assessed by the percent of recovery, which measures different known concentrations of the analyte spiked into the same background sample. A minimum of three concentration levels should be spiked and measured in three analytical replicates (a total of nine measurements). All the concentrations selected for testing should be within certain ranges and the accuracy of a method is often reported with a range of concentrations of the analyte. The difference between the mean and the confidence intervals of the measurements and the know concentrations of the spiked standards should be reported (Gustavo González & Ángeles Herrador, 2007). The closer the measured results are to the truth, the higher accuracy the method is considered to achieve.

2.6.2 Precision

The precision of an analytical method is how close or scattered the results are when the same sample is measured by the same method under the same conditions. There are three levels of precision: repeatability, intermediate precision and reproducibility. The measured sample should be an authentic standard with known concentration, or a prepared sample with the concentration validated. A minimum of six measurements on the chosen sample should be tested. The variance, standard deviation, relative standard deviation (RSD, coefficient of variation) and the confidence intervals of a certain group of measurements are the parameters to be reported and considered when evaluating the precision of an analytical method. The closer or less scattered the measurements are, the more precise the method is.

Repeatability (intra-day precision) can be measured by evaluating the same sample multiple times with a small time interval between measurements on the same day, using the same method and conditions, and the same personnel in the same lab. This characteristic is expected to achieve the smallest variance among all measurements.

Intermediate precision (inter-day precision) can be measured by evaluating the same sample multiple times on different days with the same method and conditions and the same personnel in the same lab. Days are chosen because it is typical in routine lab analysis that the measurements will be done on different days regarding to time limits on a single day. This characteristic is expected to achieve a larger variance among all measurements than the repeatability (intra-day precision). With the above two characteristics, an analytical method can be validated in a single lab.

Reproducibility (between-lab precision) can be measured by evaluating the same sample multiple times by different labs. It is the standardization of the method itself and not required for the validation of analytical method within a single lab.

2.6.3 Linearity

Linearity of an analytical method is the parameter indicating whether the measurements (readings of color intensity or signals from the equipment) are proportional to the concentration of the target compounds in the samples. When conducting linear regression with X being the known concentration of standards, and Y being the response or signal of the measurements, the R (correlation coefficient) and R^2 (coefficient of determination) of the analytical curve are expected to be as close to a value of one as possible, for good linearity of the method. In some case, when the direct data of the measurements are not linear for the tested concentrations, the direct data can be transformed mathematically prior to regression analysis. For most methods, linearity can be achieved within a certain range of concentrations of the analytes in the samples. At least five known concentrations of the standard should be used in building the analytical

curve. When using linear regression to calculate the concentration of analytes in the samples, it is important to make sure that the concentration of the analytes is within the linear range of the method, and not to extrapolate beyond that range.

2.6.4 Range

The Range of an analytical method is the span between the lower and higher concentration of the analyte in the sample where the measurements of the analyte with these concentrations are precise, accurate and linear. It is necessary to make sure that the concentrations of the analyte in the sample are within the range of the method. The range is often achieved by the determination of linearity. A range including values equal to 80% to 120% of the estimated concentration of the analyte in the samples should be considered for testing.

2.6.5 Limits of detection (LOD)

The limit of detection (LOD) of an analytical method is the lowest concentration at which the target compounds can be detected, but accurate quantification is not required. It is an important characteristic of the method especially when the method is used to test if the target compound is present in the samples or not. There are several ways to determine the LOD, the choice of which should depend on the specific method being evaluated.

The first approach is by visual evaluation. A series of samples with known concentrations of the analyte can be evaluated visually to determine the minimum concentration of the analyte at which it can be reliably detected visually.

For methods where baseline noise is an issue, a signal-to-noise ratio of 3 or 2 is generally acceptable for the estimation of LOD. A signal response can be obtained by the measurement of a sample with known low concentration of the analyte. The lowest concentration should be found which the concentration can be reliably detected. A noise response can be obtained by the measurement on a blank sample. Then the signal-to-noise ratio can be calculated.

The LOD also can be determined by the standard deviation of the measurements and the slope of the analytical curve from the linear regression analysis. LOD can be calculated by the following equations:

$$LOD = \frac{3.3s}{b}$$

Where s is the standard deviation of the measurements and b is the slope of the analytical curve (Shrivastava & Gupta, 2011). The measurements can be done on a series of samples with known concentrations and the standard deviation of the measurements can be calculated on the intercept of the ordinate axis obtained from linear regression analysis.

2.6.6 Limits of quantitation (LOQ)

Limit of quantitation (LOQ) of an analytical method is the lowest concentration at which the target compounds can be not only detected but also quantified accurately and precisely by the method. There are several ways to determine the LOQ and the choice of which should depend on the method being evaluated.

The first approach is by visual evaluation. A series of samples with known concentrations of the analyte can be evaluated to determine the minimum concentration of the analyte at which the analyte can be quantified visually with sufficient accuracy and precision.

For methods in which baseline noise is an issue, a signal-to-noise ratio of 10 is generally acceptable for the estimation of LOQ. A signal response can be obtained by the measurement on a sample with known low concentration of the analyte. The lowest concentration at which the concentration can be determined with sufficient accuracy and precision should be determined. A

noise response can be obtained by the measurement on a blank sample. Then the signal-to-noise ratio can be calculated.

The LOQ also can be determined by the standard deviation of the measurements and the slope of the analytical curve from linear regression analysis. LOQ can be calculated by the following equations:

$$LOQ = \frac{10s}{b}$$

Where s is the standard deviation of the measurements and b is the slope of the analytical curve (Shrivastava & Gupta, 2011). The measurements can be done on a series of blank samples or the standard deviation can be calculated on the intercept of the ordinate axis obtained from the linear regression analysis.

2.6.7 Specificity

The specificity of an analytical method is its capability to identify and measure the target analyte without interference from other compounds naturally present in the sample. The complexity and variability of the chemical composition of the sample matrix for fruit juice and beverages contributes to the considerable significance of specificity in fruit and fruit beverage analysis. Poor specificity of a method can result in underestimation or overestimation of the target compounds. High specificity means the method only measures exactly the compounds that it targets to measure accurately. An identification test can be performed to distinguish the analyte and the compounds that have similar chemical structures compared to the analyte. The choice of compounds with similar chemical structures compared to the analyte should be based on their potential for interference, as demonstrated by structural similarity and evidence from previous studies. A negative result should be obtained on the samples with the compounds that have similar chemical structures compared to the analyte but without the analyte present in the sample. A comparison of measurements on samples containing the analyte and not containing the analyte should be conducted. High specificity is demonstrated when a positive result is obtained for the samples containing the analyte and a negative result is obtained for the samples in which the analyte is not present.

2.6.8 Robustness

The robustness of an analytical method is its capacity to maintain the consistency of measurements under noticeable variance of the method conditions, such as light, pH of the reaction system, and the stability of the reagents under normal lab conditions. The higher robustness of a method is, the more reliable it is whenever being conducted under lab conditions. The robustness of an analytical method towards certain parameters of the method can be quantified by the measurements on the same sample with variations in that particular method parameter (for example light exposure during the reaction). If the measurements do not vary, we can conclude that this method is robust with light exposure during the reaction.

In summary, using the systematic approach prescribed by the analytical method validation procedures outlined in this section for evaluation of analytical methods for the quantification of polyphenols will provide guidance for selecting the best analytical method to fit a specific research objective in fruit and fruit beverage analysis.

2.7 Conclusion

From the above literature review, the complex structure polyphenols contributed to the complicate yet distinct polyphenol composition among fruit juice and beverages. However, the concentration of polyphenols could vary greatly. The quantification results were very different using different analytical methods on the same sample and compounds in sample matrix were

found to interfere the quantification results. Each method possess its own reaction principles, advantages and disadvantages. Because of the health benefits and sensory attributes of polyphenols to these food products, it is important to exam and compare common analytical methods for the quantification of polyphenols in fruit juice and beverages systematically using analytical method validation parameters. The study described in Chapter 3 was designed to compare the Folin−Ciocalteu method and Lowenthal permanganate titration method for the quantification of total polyphenols and to compare DMAC and BSA precipitation methods on the quantification of total procyanidins. The study in Chapter 4 was further developed to exam the interaction effect of individual polyphenol compounds and potential interfering compounds to the quantification results of analytical methods using factorial design. Finally, The purpose of study in Chapter 5 was to characterize the amino acid composition in apples with potential use in cider making, and to assess the extent to which amino acid concentration and composition vary among juice samples, and Chapter 6 is aimed to evaluate the impact of using a cell phone-based personal response system on academic performance and students' perceptions of learning in an upper-level undergraduate Food Science course.

2.8 References

Adams, D. O., & Harbertson, J. F. (1999). Use of alkaline phosphatase for the analysis of tannins in grapes and red wines. American Journal of Enology and Viticulture, 50(3), 247. Retrieved from http://www.ajevonline.org/content/50/3/247.abstract

Aherne, S. A., & O'Brien, N. M. (2002). Dietary flavonols: chemistry, food content, and metabolism. Nutrition, 18(1), 75–81. https://doi.org/http://dx.doi.org/10.1016/S0899-9007(01)00695-5

Aizpurua-Olaizola, O., Navarro, P., Vallejo, A., Olivares, M., Etxebarria, N., & Usobiaga, A. (2016). Microencapsulation and storage stability of polyphenols from Vitis vinifera grape wastes. Food Chemistry, 190, 614–621. https://doi.org/https://doi.org/10.1016/j.foodchem.2015.05.117

Alakolanga, A. G. A. W., Siriwardane, A. M. D. A., Savitri Kumar, N., Jayasinghe, L., Jaiswal, R., & Kuhnert, N. (2014). LC-MSn identification and characterization of the phenolic compounds from the fruits of *Flacourtia indica* (Burm. F.) Merr. and *Flacourtia inermis* Roxb. Food Research International, 62, 388–396. https://doi.org/http://dx.doi.org/10.1016/j.foodres.2014.03.036

American Cancer Society. (2015). What is cancer? Retrieved February 18, 2019, from https://www.cancer.org/cancer/cancer-basics/what-is-cancer.html

Andjelkovic, M., Van Camp, J., Pedra, M., Renders, K., Socaciu, C., & Verhé, R. (2008). Correlations of the phenolic compounds and the phenolic content in some Spanish and French olive oils. Journal of Agricultural and Food Chemistry, 56(13), 5181–5187. https://doi.org/10.1021/jf0724419

Anttonen, M. J., & Karjalainen, R. O. (2006). High-performance liquid chromatography analysis of black currant (*Ribes nigrum* L.) fruit phenolics grown either conventionally or organically. Journal of Agricultural and Food Chemistry, 54(20), 7530–7538. https://doi.org/10.1021/JF0615350

Appeldoorn, M. M., Sanders, M., Vincken, J.-P., Cheynier, V., Le Guernevé, C., Hollman, P. C. H., & Gruppen, H. (2009). Efficient isolation of major procyanidin A-type dimers from peanut skins and B-type dimers from grape seeds. Food Chemistry, 117(4), 713–720. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2009.04.047

Araujo, P. (2009). Key aspects of analytical method validation and linearity evaluation. Journal of Chromatography B, 877(23), 2224–2234. https://doi.org/http://dx.doi.org/10.1016/j.jchromb.2008.09.030

Arnous, A., Makris, D. P., & Kefalas, P. (2001). Effect of principal polyphenolic components in relation to antioxidant characteristics of aged red wines. Journal of Agricultural and Food Chemistry, 49(12), 5736–5742. https://doi.org/10.1021/jf010827s

Arya, S. P., Mahajan, M., & Jain, P. (2001). Spectrophotometric determination of vitamin C with iron(II)-4-(2-pyridylazo)resorcinol complex. Analytica Chimica Acta, 427(2), 245–251. https://doi.org/http://dx.doi.org/10.1016/S0003-2670(00)01092-8

Ascorbic Acid in Vitamin Preparations and Juices, AOAC Method 967.21. (2017). Association of Official Analytical Chemists (AOAC) International.

ASTM E253-17 Standard terminology relating to sensory evaluation of materials and products. (2017). In Annual Book of ASTM Standards. West Conshohocken, PA: American Society for Testing and Materials International. Retrieved from http://www.astm.org

Bakowska-Barczak, A. M., & Kolodziejczyk, P. P. (2011). Black currant polyphenols: Their storage stability and microencapsulation. Industrial Crops and Products, 34(2), 1301–1309. https://doi.org/http://dx.doi.org/10.1016/j.indcrop.2010.10.002

Balentine, D. A., Dwyer, J. T., Erdman, J. J. W., Ferruzzi, M. G., Gaine, P. C., Harnly, J. M., & Kwik-Uribe, C. L. (2015). Recommendations on reporting requirements for flavonoids in research. The American Journal of Clinical Nutrition, 101(6), 1113–1125. https://doi.org/10.3945/ajcn.113.071274

Ball, G. F. M. (2006). Vitamins in foods : analysis, bioavailability, and stability (pp: 156). Boca Raton, FL: Taylor & Francis.

Barua, D. N., & Roberts, E. A. (1940). Methods for the volumetric estimation of tea tannin in green-leaf and black tea. A new alkaline permanganate method. The Biochemical Journal, 34(12), 1524–1531. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16747284

Bastola, K. P., Guragain, Y. N., Bhadriraju, V., & Vadlani, P. V. (2017). Evaluation of standards and interfering compounds in the determination of phenolics by Folin-Ciocalteu assay method for effective bioprocessing of biomass. American Journal of Analytical Chemistry, 08(06), 416–431. https://doi.org/10.4236/ajac.2017.86032

Bate-Smith, E. C. (1975). Phytochemistry of proanthocyanidins. Phytochemistry, 14(4), 1107–1113. https://doi.org/http://dx.doi.org/10.1016/0031-9422(75)85197-1

Beesk, N., Perner, H., Schwarz, D., George, E., Kroh, L. W., & Rohn, S. (2010). Distribution of quercetin-3,4’-O-diglucoside, quercetin-4’-O-monoglucoside, and quercetin in different parts of the onion bulb (*Allium cepa* L.) influenced by genotype. Food Chemistry, 122(3), 566–571. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2010.03.011

Bettuzzi, S., Brausi, M., Rizzi, F., Castagnetti, G., Peracchia, G., & Corti, A. (2006). Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: A preliminary report from a one-year proof-of-principle study. Cancer Research, 66(2), 1234. Retrieved from http://cancerres.aacrjournals.org/content/66/2/1234.abstract

Bhaskaran, K., Douglas, I., Forbes, H., dos-Santos-Silva, I., Leon, D. A., & Smeeth, L. (2014). Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5.24 million UK adults. Lancet (London, England), 384(9945), 755–765. https://doi.org/10.1016/S0140-6736(14)60892-8

Blainski, A., Lopes, G., & de Mello, J. (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium Brasiliense* L. Molecules, 18(6), 6852–6865. https://doi.org/10.3390/molecules18066852

Boonpangrak, S., Lalitmanat, S., Suwanwong, Y., Prachayasittikul, S., & Prachayasittikul, V. (2016). Analysis of ascorbic acid and isoascorbic acid in orange and guava fruit juices distributed in Thailand by LC-IT-MS/MS. Food Analytical Methods, 9(6), 1616–1626. https://doi.org/10.1007/s12161-015-0337-x

Boulet, J.-C., Trarieux, C., Souquet, J.-M., Ducasse, M.-A., Caillé, S., Samson, A., … Cheynier, V. (2016). Models based on ultraviolet spectroscopy, polyphenols, oligosaccharides and polysaccharides for prediction of wine astringency. Food Chemistry, 190, 357–363. https://doi.org/https://doi.org/10.1016/j.foodchem.2015.05.062

Boulton, R. B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (1999). Yeast and biochemistry of ethanol fermentation. In Principles and Practices of Winemaking (pp. 102–192). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4757-6255-6_4

Brooks, L., McCloskey, L., Mckesson, D., & Sylvan, M. (2008). Adams-Harbertson protein precipitation-based wine tannin method found invalid. Journal of AOAC International, 91(5), 1090–1094.

Brossaud, F., Cheynier, V., & Noble, A. C. (2001). Bitterness and astringency of grape and wine polyphenols. Australian Journal of Grape and Wine Research, 7(1), 33–39. https://doi.org/10.1111/j.1755-0238.2001.tb00191.x

Brownmiller, C., Howard, L. R., & Prior, R. L. (2009). Processing and storage effects on procyanidin composition and concentration of processed blueberry products. Journal of Agricultural and Food Chemistry, 57(5), 1896–1902. https://doi.org/10.1021/jf803015s

Budini, R., Tonelli, D., & Girotti, S. (1980). Analysis of total phenols using the Prussian Blue method. Journal of Agricultural and Food Chemistry, 28(6), 1236–1238. https://doi.org/10.1021/jf60232a056

Burns, R. E. (1971). Method for estimation of tannin in grain sorghum. Agronomy Journal, 63(3), 511–512. https://doi.org/10.2134/agronj1971.00021962006300030050x

Burroughs, L. F., & Whiting, G. C. (1960). Annual report Long Ashton Research Station for 1960.

Cáceres-Mella, A., Peña-Neira, Á., Narváez-Bastias, J., Jara-Campos, C., López-Solís, R., & Canals, J. M. (2013). Comparison of analytical methods for measuring proanthocyanidins in wines and their relationship with perceived astringency. International Journal of Food Science & Technology, 48(12), 2588–2594. https://doi.org/10.1111/ijfs.12253

Cancer Fact Sheet. (2017). Retrieved from http://www.who.int/mediacentre/factsheets/fs297/en/

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F. J., & Queipo-Ortuño, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. The Journal of Nutritional Biochemistry, 24(8), 1415–1422. https://doi.org/http://dx.doi.org/10.1016/j.jnutbio.2013.05.001

Carrascon, V., Ontañón, I., Bueno, M., & Ferreira, V. (2017). Gas chromatography-mass spectrometry strategies for the accurate and sensitive speciation of sulfur dioxide in wine. Journal of Chromatography A, 1504, 27–34. https://doi.org/10.1016/J.CHROMA.2017.05.012

Carrillo, L. C., Londoño-Londoño, J., & Gil, A. (2014). Comparison of polyphenol, methylxanthines and antioxidant activity in Theobroma cacao beans from different cocoa-

growing areas in Colombia. Food Research International, 60, 273–280. https://doi.org/http://dx.doi.org/10.1016/j.foodres.2013.06.019

Carvalho, E., Póvoas, M. J., Mateus, N., & de Freitas, V. (2006). Application of flow nephelometry to the analysis of the influence of carbohydrates on protein–tannin interactions. Journal of the Science of Food and Agriculture, 86(6), 891–896. https://doi.org/10.1002/jsfa.2430

Centers for Disease Control and Prevention. (2015). The health effects of overweight and obesity. Retrieved from https://www.cdc.gov/healthyweight/effects/index.html

Centers for Disease Control and Prevention. (2018, September). Adult Obesity Facts. https://doi.org/10.1377/hlthaff.28.5.w822

Chan, S.-C., Chang, Y.-S., & Kuo, S.-C. (1997). Neoflavonoids from *Dalbergia odorifera*. Phytochemistry, 46(5), 947–949. https://doi.org/http://dx.doi.org/10.1016/S0031-9422(97)00365-8

Chang, S. K., Alasalvar, C., & Shahidi, F. (2018). Superfruits: Phytochemicals, antioxidant efficacies, and health effects – A comprehensive review. Critical Reviews in Food Science and Nutrition, 1–25. https://doi.org/10.1080/10408398.2017.1422111

Cheynier, V. (2005). Polyphenols in foods are more complex than often thought. The American Journal of Clinical Nutrition, 81(1), 223S–229S. Retrieved from http://ajcn.nutrition.org/content/81/1/223S.abstract

Cooke, M. D. J. P., Dzau, M. D. V. J., Cooke, J. P., & Dzau, V. J. (1997). Nitric oxide synthase: Role in the genesis of vascular disease. Annual Review of Medicine, 48(1), 489–509. https://doi.org/10.1146/annurev.med.48.1.489

Cooper, K. A., Campos-Giménez, E., Jiménez Alvarez, D., Nagy, K., Donovan, J. L., & Williamson, G. (2007). Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate. Journal of Agricultural and Food Chemistry, 55(8), 2841–2847. https://doi.org/10.1021/jf063277c

Crozier, A., Lean, M. E. J., McDonald, M. S., & Black, C. (1997). Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. Journal of Agricultural and Food Chemistry, 45(3), 590–595. https://doi.org/10.1021/jf960339y

Ćujić, N., Šavikin, K., Janković, T., Pljevljakušić, D., Zdunić, G., & Ibrić, S. (2016). Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. Food Chemistry, 194, 135–142. https://doi.org/10.1016/J.FOODCHEM.2015.08.008

da Silva, T. L., Aguiar-Oliveira, E., Mazalli, M. R., Kamimura, E. S., & Maldonado, R. R. (2017). Comparison between titrimetric and spectrophotometric methods for quantification of vitamin C. Food Chemistry, 224, 92–96. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2016.12.052

Darvesh, A. S., Carroll, R. T., Bishayee, A., Geldenhuys, W. J., & Van der Schyf, C. J. (2010). Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents. Expert Review of Neurotherapeutics, 10(5), 729–745. https://doi.org/10.1586/ern.10.42

De Ancos, B., Cilla, A., Barberá, R., Sánchez-Moreno, C., & Cano, M. P. (2017). Influence of orange cultivar and mandarin postharvest storage on polyphenols, ascorbic acid and antioxidant activity during gastrointestinal digestion. Food Chemistry, 225, 114–124. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2016.12.098

de Freitas, V., & Mateus, N. (2001). Structural Features of Procyanidin Interactions with Salivary Proteins. Journal of Agricultural and Food Chemistry, 49(2), 940–945. https://doi.org/10.1021/jf000981z

Delcour, J. A., & Varebeke, D. J. de. (1985). A new colorimetric assay for flavanoids in Pilsner beers. Journal of the Institute of Brewing, 91(1), 37–40. https://doi.org/10.1002/j.2050-0416.1985.tb04303.x

Deshpande, S. S., Cheryan, M., Salunkhe, D. K., & Luh, B. S. (1986). Tannin analysis of food products. C R C Critical Reviews in Food Science and Nutrition, 24(4), 401–449. https://doi.org/10.1080/10408398609527441

Dragovic-Uzelac, V., Levaj, B., Mrkic, V., Bursac, D., & Boras, M. (2007). The content of polyphenols and carotenoids in three apricot cultivars depending on stage of maturity and geographical region. Food Chemistry, 102(3), 966–975. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2006.04.001

Du, G., Li, M., Ma, F., & Liang, D. (2009). Antioxidant capacity and the relationship with polyphenol and Vitamin C in Actinidia fruits. Food Chemistry, 113(2), 557–562. https://doi.org/10.1016/J.FOODCHEM.2008.08.025

Duda-Chodak, A., Tarko, T., Satora, P., & Sroka, P. (2015). Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. European Journal of Nutrition, 54(3), 325–341. https://doi.org/10.1007/s00394-015-0852-y

Dueñas, M., Muñoz-González, I., Cueva, C., Jiménez-Girón, A., Sánchez-Patán, F., Santos-Buelga, C., … Bartolomé, B. (2015). A survey of modulation of gut microbiota by dietary polyphenols. BioMed Research International, 2015, 15. https://doi.org/10.1155/2015/850902

Estrela, J. M., Mena, S., Obrador, E., Benlloch, M., Castellano, G., Salvador, R., & Dellinger, R. W. (2017). Polyphenolic phytochemicals in cancer prevention and therapy: Bioavailability versus bioefficacy. Journal of Medicinal Chemistry. https://doi.org/10.1021/acs.jmedchem.6b01026

Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). Thorough study of reactivity of various compound classes toward the Folin−Ciocalteu reagent. Journal of Agricultural and Food Chemistry, 58(14), 8139–8144. https://doi.org/10.1021/jf1005935

Ewald, C., Fjelkner-Modig, S., Johansson, K., Sjöholm, I., & Åkesson, B. (1999). Effect of processing on major flavonoids in processed onions, green beans, and peas. Food Chemistry, 64(2), 231–235. https://doi.org/http://dx.doi.org/10.1016/S0308-8146(98)00136-8

Faller, A. L. K., & Fialho, E. (2010). Polyphenol content and antioxidant capacity in organic and conventional plant foods. Journal of Food Composition and Analysis, 23(6), 561–568. https://doi.org/http://dx.doi.org/10.1016/j.jfca.2010.01.003

Farhat, G., Drummond, S., & Al-Dujaili, E. A. S. (2017). Polyphenols and their role in obesity management: A systematic review of randomized clinical trials. Phytotherapy Research, 31(7), 1005–1018. https://doi.org/10.1002/ptr.5830

Finocchiaro, F., Ferrari, B., & Gianinetti, A. (2010). A study of biodiversity of flavonoid content in the rice caryopsis evidencing simultaneous accumulation of anthocyanins and proanthocyanidins in a black-grained genotype. Journal of Cereal Science, 51(1), 28–34. https://doi.org/http://dx.doi.org/10.1016/j.jcs.2009.09.003

Folin, O., & Ciocalteu, V. (1927). On tryosine and tryptophane determinations in proteins. Journal of Biological Chemistry, 73(2), 627–650. Retrieved from http://www.jbc.org/content/73/2/627.short

Folin, O., & Denis, W. (1912). On phosphotungstic-phosphomolybdic compounds as color reagents. Journal of Biological Chemistry, 12(2), 239–243. Retrieved from http://www.jbc.org/content/12/2/239.short

Ford, B., Bai, J., & Baldwin, E. (2010). Improved removal of ascorbate interference in the Folin-Ciocalteu assay of "total phenolic" content. Proceedings of Florida State Horticulture Society, 123, 220–222.

Fratianni, F., Tucci, M., Palma, M. De, Pepe, R., & Nazzaro, F. (2007). Polyphenolic composition in different parts of some cultivars of globe artichoke (*Cynara cardunculus* L. *var. scolymus* (L.) Fiori). Food Chemistry, 104(3), 1282–1286. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2007.01.044

Frei, B., & Higdon, J. V. (2003). Antioxidant activity of tea polyphenols *in vivo*: Evidence from animal studies. The Journal of Nutrition, 133(10), 3275S–3284S. Retrieved from http://jn.nutrition.org/content/133/10/3275S.abstract

García-Estévez, I., Ramos-Pineda, A. M., & Escribano-Bailón, M. T. (2018). Interactions between wine phenolic compounds and human saliva in astringency perception. Food & Function, 9(3), 1294–1309. https://doi.org/10.1039/C7FO02030A

Gawel, R. (1998). Red wine astringency: a review. Australian Journal of Grape and Wine Research, 4(2), 74–95. https://doi.org/10.1111/j.1755-0238.1998.tb00137.x

Georgé, S., Brat, P., Alter, P., & Amiot, M. J. (2005). Rapid determination of polyphenols and vitamin C in plant-derived products. Journal of Agricultural and Food Chemistry, 53(5), 1370–1373. https://doi.org/10.1021/jf048396b

Gessner, M. O., & Steiner, D. (2005). Acid butanol assay for proanthocyanidins (condensed tannins). In M. A. S. Graça, F. Bärlocher, & M. O. Gessner (Eds.), Methods to Study Litter Decomposition: A Practical Guide (pp. 107–114). Dordrecht: Springer Netherlands. https://doi.org/10.1007/1-4020-3466-0_16

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. In Current Protocols in Food Analytical Chemistry. John Wiley & Sons, Inc. https://doi.org/10.1002/0471142913.faf0102s00

Glahn, R., Tako, E., Hart, J., Haas, J., Lung'aho, M., & Beebe, S. (2017). Iron bioavailability studies of the first generation of iron-biofortified beans released in Rwanda. Nutrients, 9(7). https://doi.org/10.3390/nu9070787

Goldmann, M. E. (1949). The pH of fruit juices. Journal of Food Science, 14(4), 275–277. https://doi.org/10.1111/j.1365-2621.1949.tb16233.x

Gómez Ruiz, B., Roux, S., Courtois, F., & Bonazzi, C. (2016). Spectrophotometric method for fast quantification of ascorbic acid and dehydroascorbic acid in simple matrix for kinetics measurements. Food Chemistry, 211, 583–589. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2016.05.107

Graham, H. D. (1992). Stabilization of the Prussian blue color in the determination of polyphenols. Journal of Agricultural and Food Chemistry, 40(5), 801–805. https://doi.org/10.1021/jf00017a018

Gratchev, A., Sobenin, I., Orekhov, A., & Kzhyshkowska, J. (2012). Monocytes as a diagnostic marker of cardiovascular diseases. Immunobiology, 217(5), 476–482. https://doi.org/http://dx.doi.org/10.1016/j.imbio.2012.01.008

Greenwell, M., & Rahman, P. (2015). Medicinal plants: Their use in anticancer treatment. International Journal of Pharmaceutical Sciences and Research, 6(10), 4103–4112. https://doi.org/10.13040/IJPSR.0975-8232.6(10).4103-12

Gregor, M. F., & Hotamisligil, G. S. (2011). Inflammatory mechanisms in obesity. Annual Review of Immunology, 29(1), 415–445. https://doi.org/10.1146/annurev-immunol-031210-101322

Grove, K. A., & Lambert, J. D. (2010). Laboratory, epidemiological, and human intervention studies show that tea (*Camellia sinensis*) may be useful in the prevention of Obesity. The Journal of Nutrition, 140(3), 446–453. https://doi.org/10.3945/jn.109.115972

Gustavo González, A., & Ángeles Herrador, M. (2007). A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. TrAC Trends in Analytical Chemistry, 26(3), 227–238. https://doi.org/http://dx.doi.org/10.1016/j.trac.2007.01.009

Hager, T. J., Howard, L. R., & Prior, R. L. (2010). Processing and storage effects on the ellagitannin composition of processed blackberry products. Journal of Agricultural and Food Chemistry, 58(22), 11749–11754. https://doi.org/10.1021/jf102964b

Hagerman, A. E. (2002). Hydrolyzable tannin structural chemistry. Tannin Handbook (Http://Www. Users. Muohio. Edu/Hagermae/Tannin. Pdf).

Hagerman, A. E., & Butler, L. G. (1978). Protein precipitation method for the quantitative determination of tannins. Journal of Agricultural and Food Chemistry, 26(4), 809–812. https://doi.org/10.1021/jf60218a027

Hagerman, A. E., & Butler, L. G. (1981). The specificity of proanthocyanidin-protein interactions. Journal of Biological Chemistry, 256(9), 4494–4497. Retrieved from http://www.jbc.org/content/256/9/4494.abstract

Häkkinen, S. H., & Törrönen, A. R. (2000). Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: influence of cultivar, cultivation site and technique. Food Research International, 33(6), 517–524. https://doi.org/http://dx.doi.org/10.1016/S0963-9969(00)00086-7

Hammerstone, J. F., Lazarus, S. A., & Schmitz, H. H. (2000). Procyanidin content and variation in some commonly consumed foods. The Journal of Nutrition, 130(8), 2086S–2092S. Retrieved from http://jn.nutrition.org/content/130/8/2086S.abstract

Harbertson, J. F., & Downey, M. O. (2009). Investigating differences in tannin levels determined by methylcellulose and protein precipitation. American Journal of Enology and Viticulture, 60, 246–249. Retrieved from http://www.ajevonline.org/content/60/2/246

Harbertson, J. F., Kennedy, J. A., & Adams, D. O. (2002). Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. American Journal of Enology and Viticulture, 53(1), 54. Retrieved from http://www.ajevonline.org/content/53/1/54.abstract

Harbertson, J. F., Mireles, M., Yu, Y., Boulton, R., & Harbertson, J. F. (2015). Improvement of BSA tannin precipitation assay by reformulation of resuspension buffer. American Journal of Enology and Viticulture, 66(1), 95–99. https://doi.org/10.5344/ajev.2014.14082

Harborne, J. B., & Williams, C. A. (1982). Flavone and flavonol glycosides. In J. B. Harborne & T. J. Mabry (Eds.), The Flavonoids: Advances in Research (pp. 261–311). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4899-2915-0_5

Heimler, D., Romani, A., & Ieri, F. (2017). Plant polyphenol content, soil fertilization and agricultural management: a review. European Food Research and Technology, 243(7), 1107–1115. https://doi.org/10.1007/s00217-016-2826-6

Hellström, J. K., Törrönen, A. R., & Mattila, P. H. (2009). Proanthocyanidins in common food products of plant origin. Journal of Agricultural and Food Chemistry, 57(17), 7899–7906. https://doi.org/10.1021/jf901434d

Hemingway, R. W. (1989). Structural variations in proanthocyanidins and their derivatives. In R. W. Hemingway, J. J. Karchesy, & S. J. Branham (Eds.), Chemistry and Significance of Condensed Tannins (pp. 83–107). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4684-7511-1_5

Henderson, P. (2009). Sulfur dioxide: Science behind this anti-microbial, anti-oxidant wine additive. Practical Winery & Vineyard Journal, 1–6. Retrieved from http://www.gencowinemakers.com/docs/Sulfur Dioxide-Science Behind this Anti-microbial, Anti-oxidant Wine Additive.pdf

Henning, S. M., Niu, Y., Lee, N. H., Thames, G. D., Minutti, R. R., Wang, H., … Heber, D. (2004). Bioavailability and antioxidant activity of tea flavanols after consumption of green tea, black tea, or a green tea extract supplement. The American Journal of Clinical Nutrition, 80(6), 1558–1564. Retrieved from http://ajcn.nutrition.org/content/80/6/1558.abstract

Herrmann, K. (1976). Flavonols and flavones in food plants: a review†. International Journal of Food Science & Technology, 11(5), 433–448. https://doi.org/10.1111/j.1365-2621.1976.tb00743.x

Hertog, M. G. L., Hollman, P. C. H., & Katan, M. B. (1992). Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. Journal of Agricultural and Food Chemistry, 40(12), 2379–2383. https://doi.org/10.1021/jf00024a011

Hollman, P. C. H., & Arts, I. C. W. (2000). Flavonols, flavones and flavanols – nature, occurrence and dietary burden. Journal of the Science of Food and Agriculture, 80(7), 1081–1093. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<1081::AID-JSFA566>3.0.CO;2-G

Hollman, P. C. H., Geelen, A., & Kromhout, D. (2010). Dietary flavonol intake may lower stroke risk in men and women. The Journal of Nutrition, 140(3), 600–604. https://doi.org/10.3945/jn.109.116632

Horne, J., Hayes, J., & Lawless, H. T. (2002). Turbidity as a measure of salivary protein reactions with astringent substances. Chemical Senses, 27(7), 653–659. https://doi.org/10.1093/chemse/27.7.653

Hosseini, A., & Ghorbani, A. (2015). Cancer therapy with phytochemicals: evidence from clinical studies. Avicenna Journal of Phytomedicine, 5(2), 84–97. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4418057/

Howard, L. R., Prior, R. L., Liyanage, R., & Lay, J. O. (2012). Processing and storage effect on berry polyphenols: Challenges and implications for bioactive properties. Journal of Agricultural and Food Chemistry, 60(27), 6678–6693. https://doi.org/10.1021/jf2046575

Huber, L. (2010). Validation of Analytical Methods. Agilent Technologies.

Ikawa, M., Dollard, C. A., & Schaper, T. D. (1988). Reaction of Folin-Ciocalteau phenol reagent with purines, pyrimidines, and pteridines and its relationship to structure. Journal of Agricultural and Food Chemistry, 36(2), 309–311. https://doi.org/10.1021/jf00080a017

Ikawa, M., Schaper, T. D., Dollard, C. A., & Sasner, J. J. (2003). Utilization of Folin−Ciocalteu phenol reagent for the detection of certain nitrogen compounds. https://doi.org/10.1021/JF021099R

International Conference on Harmonization (ICH). (2005). Validation of Analytical Procedures: Text and Methodology (Q2(R1)).

Jagota, S. K., & Dani, H. M. (1982). A new colorimetric technique for the estimation of vitamin C using Folin phenol reagent. Analytical Biochemistry, 127(1), 178–182. https://doi.org/http://dx.doi.org/10.1016/0003-2697(82)90162-2

Jaiswal, R., & Kuhnert, N. (2014). Identification and characterization of the phenolic glycosides of *Lagenaria siceraria* Stand. (Bottle Gourd) fruit by liquid chromatography–tandem mass spectrometry. Journal of Agricultural and Food Chemistry, 62(6), 1261–1271. https://doi.org/10.1021/jf4053989

Joseph, S. V, Edirisinghe, I., & Burton-Freeman, B. M. (2016). Fruit polyphenols: A review of anti-inflammatory effects in humans. Critical Reviews in Food Science and Nutrition, 56(3), 419–444. https://doi.org/10.1080/10408398.2013.767221

Joslyn, M. A., & Goldstein, J. L. (1964). Astringency of fruits and fruit products in relation to phenolic content. Advances in Food Research, 13, 179–217. https://doi.org/http://dx.doi.org/10.1016/S0065-2628(08)60101-9

Kennedy, J. A., Ferrier, J., Harbertson, J. F., & des Gachons, C. P. (2006). Analysis of tannins in red wine using multiple methods: Correlation with perceived astringency. American Journal of Enology and Viticulture, 57(4), 481–485.

Kennedy, J. A., Matthews, M. A., & Waterhouse, A. L. (2000). Changes in grape seed polyphenols during fruit ripening. Phytochemistry, 55(1), 77–85. https://doi.org/http://dx.doi.org/10.1016/S0031-9422(00)00196-5

Key, T. J. (2011). Fruit and vegetables and cancer risk. British Journal of Cancer, 104(1), 6–11. https://doi.org/10.1038/sj.bjc.6606032

Kim, H., & Keeney, P. G. (1984). (-)-Epicatechin content in fermented and unfermented cocoa beans. Journal of Food Science, 49(4), 1090–1092. https://doi.org/10.1111/j.1365-2621.1984.tb10400.x

Klimczak, I., Małecka, M., Szlachta, M., & Gliszczyńska-Świgło, A. (2007). Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. Journal of Food Composition and Analysis, 20(3), 313–322. https://doi.org/http://dx.doi.org/10.1016/j.jfca.2006.02.012

Kohri, T., Matsumoto, N., Yamakawa, M., Suzuki, M., Nanjo, F., Hara, Y., & Oku, N. (2001). Metabolic fate of (−)-[4-3H] epigallocatechin gallate in rats after oral administration. Journal of Agricultural and Food Chemistry, 49(8), 4102–4112.

Koreca, R., Senschb, K. H., & Zoukasb, T. (2000). Effects of the neoflavonoid coutareagenin, one of the antidiabetic active substances of Hintonia latiflora, on streptozotocin-induced diabetes mellitus in rats. Arzneimittelforschung, 50(02), 122–128.

Kramling, T. E., & Singleton, V. L. (1969). An estimate of the nonflavonoid phenols in wines. American Journal of Enology and Viticulture, 20(2), 86–92.

Kucuk, O. (2017). Soy foods, isoflavones, and breast cancer. Cancer, 123(11), 1901–1903. https://doi.org/10.1002/cncr.30614

Kurosawa, T., Itoh, F., Nozaki, A., Nakano, Y., Katsuda, S., Osakabe, N., … Itakura, H. (2005). Suppressive effect of cocoa powder on atherosclerosis in Kurosawa and Kusanagi-

hypercholesterolemic rabbits. Journal of Atherosclerosis and Thrombosis, 12(1), 20–28. https://doi.org/10.5551/jat.12.20

Kushi, L. H., Doyle, C., McCullough, M., Rock, C. L., Demark-Wahnefried, W., Bandera, E. V, … Physical Activity Guidelines Advisory, C. (2012). American Cancer Society guidelines on nutrition and physical activity for cancer prevention. CA: A Cancer Journal for Clinicians, 62(1), 30–67. https://doi.org/10.3322/caac.20140

Lea, A. G. H. (1992). Flavor, color, and stability in fruit products: The effect of polyphenols. In Plant Polyphenols (pp. 827–847). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4615-3476-1_49

Lea, A. G. H., & Arnold, G. M. (1978). The phenolics of ciders: Bitterness and astringency. Journal of the Science of Food and Agriculture, 29(5), 478–483. https://doi.org/10.1002/jsfa.2740290512

Lee, C. A. (2010). Sensory aspects of astringency and changes of the oral environment in the mechanism of astringency. Department of Food Science and Nutrition. University of Minnesota, Ann Arbor, MI.

Lee, C. A., & Vickers, Z. M. (2012). Astringency of foods may not be directly related to salivary lubricity. Journal of Food Science, 77(9), S302–S306. https://doi.org/10.1111/j.1750-3841.2012.02860.x

Lewandowska, H., Kalinowska, M., Lewandowski, W., Stępkowski, T. M., & Brzóska, K. (2016). The role of natural polyphenols in cell signaling and cytoprotection against cancer development. The Journal of Nutritional Biochemistry, 32, 1–19. https://doi.org/http://dx.doi.org/10.1016/j.jnutbio.2015.11.006

Li, C., Trombley, J. D., Schmidt, M. A., & Hagerman, A. E. (2010). Preparation of an acid butanol standard from fresh apples. Journal of Chemical Ecology, 36(5), 453–460. https://doi.org/10.1007/s10886-010-9784-4

Li, Y.-G., Tanner, G., & Larkin, P. (1996). The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. Journal of the Science of Food and Agriculture, 70(1), 89–101. https://doi.org/10.1002/(SICI)1097-0010(199601)70:1<89::AID-JSFA470>3.0.CO;2-N

Lorrain, B., Ky, I., Pechamat, L., & Teissedre, P.-L. (2013). Evolution of analysis of polyhenols from grapes, wines, and extracts. Molecules, 18(1), 1076. Retrieved from http://www.mdpi.com/1420-3049/18/1/1076

Lowenthal. (1877). Uber die Bestimmung des Gerbstoffs. Analytical and Bioanalytical Chemistry (Previous as Zeitschrift Für Analytische Chemie), 16, 33–48.

Mahmood, T., Anwar, F., Afzal, N., Kausar, R., Ilyas, S., & Shoaib, M. (2017). Influence of ripening stages and drying methods on polyphenolic content and antioxidant activities of mulberry fruits. Journal of Food Measurement and Characterization. https://doi.org/10.1007/s11694-017-9602-6

Mahmoud, A. M., Yang, W., & Bosland, M. C. (2014). Soy isoflavones and prostate cancer: A review of molecular mechanisms. The Journal of Steroid Biochemistry and Molecular Biology, 140, 116–132. https://doi.org/http://dx.doi.org/10.1016/j.jsbmb.2013.12.010

Makkar, H. P. S. (1989). Protein precipitation methods for quantitation of tannins: a review. Journal of Agricultural and Food Chemistry, 37(4), 1197–1202. https://doi.org/10.1021/jf00088a083

Makkar, H. P. S., Gamble, G., & Becker, K. (1999). Limitation of the butanol–hydrochloric acid–iron assay for bound condensed tannins. Food Chemistry, 66(1), 129–133. https://doi.org/http://dx.doi.org/10.1016/S0308-8146(99)00043-6

Makris, D. P., Kallithraka, S., & Mamalos, A. (2006). Differentiation of young red wines based on cultivar and geographical origin with application of chemometrics of principal polyphenolic constituents. Talanta, 70(5), 1143–1152. https://doi.org/http://dx.doi.org/10.1016/j.talanta.2006.03.024

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. The American Journal of Clinical Nutrition, 79(5), 727–747. Retrieved from http://ajcn.nutrition.org/content/79/5/727.abstract

Margraf, T., Karnopp, A. R., Rosso, N. D., & Granato, D. (2015). Comparison between Folin-Ciocalteu and Prussian Blue assays to estimate the total phenolic content of juices and teas using 96-well microplates. Journal of Food Science, 80(11), C2397–C2403. https://doi.org/10.1111/1750-3841.13077

McDonald, M. S., Hughes, M., Burns, J., Lean, M. E. J., Matthews, D., & Crozier, A. (1998). Survey of the free and conjugated myricetin and quercetin content of red wines of different geographical origins. Journal of Agricultural and Food Chemistry, 46(2), 368–375. https://doi.org/10.1021/jf970677e

McGhie, T. K., Hunt, M., & Barnett, L. E. (2005). Cultivar and Growing Region Determine the Antioxidant Polyphenolic Concentration and Composition of Apples Grown in New Zealand. Journal of Agricultural and Food Chemistry, 53(8), 3065–3070. https://doi.org/10.1021/jf047832r

McMurrough, I., & McDowell, J. (1978). Chromatographic separation and automated analysis of flavanols. Analytical Biochemistry, 91(1), 92–100. https://doi.org/http://dx.doi.org/10.1016/0003-2697(78)90819-9

Meagan D. Mercurio, Robert G. Dambergs, Markus J. Herderich, and, & Smith, P. A. (2007). High throughput analysis of red wine and grape phenolics adaptation and validation of methyl cellulose precipitable tannin assay and modified Somers color assay to a rapid 96 well plate format. https://doi.org/10.1021/JF063674N

Mercurio, M. D., Dambergs, R. G., Cozzolino, D., Herderich, M. J., & Smith, P. A. (2010). Relationship between red wine grades and phenolics. 1. Tannin and total phenolics concentrations. Journal of Agricultural and Food Chemistry, 58(23), 12313–12319. https://doi.org/10.1021/jf103230b

Mercurio, M. D., & Smith, P. A. (2008). Tannin quantification in red grapes and wine: Comparison of polysaccharide- and protein-based tannin precipitation techniques and their ability to model wine astringency. Journal of Agricultural and Food Chemistry, 56(14), 5528–5537. https://doi.org/10.1021/jf8008266

Michalak, A. (2006). Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Environmental Studies, 15(4), 523–530. Retrieved from http://www.pjoes.com/Phenolic-Compounds-and-Their-Antioxidant-Activity-in-Plants-Growing-under-Heavy-Metal,87899,0,2.html

Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F., & Veberic, R. (2015). A comparison of fruit quality parameters of wild bilberry (*Vaccinium myrtillus* L.) growing at different locations. Journal of the Science of Food and Agriculture, 95(4), 776–785. https://doi.org/10.1002/jsfa.6897

Murata, M., Tsurutani, M., Tomita, M., Homma, S., & Kaneko, K. (1995). Relationship between apple ripening and browning: changes in polyphenol content and polyphenol oxidase. Journal of Agricultural and Food Chemistry, 43(5), 1115–1121.

Myers, T. E., & Singleton, V. L. (1979). The nonflavonoid phenolic fraction of wine and its analysis. American Journal of Enology and Viticulture, 30(2), 98–102.

Nagel, C. W., & Glories, Y. (1991). Use of a modified dimethylaminocinnamaldehyde reagent for analysis of flavanols. American Journal of Enology and Viticulture, 42(4), 364–366.

Najwa, R. F., & Azrina, A. (2017). Comparison of vitamin C content in citrus fruits by titration and high performance liquid chromatography (HPLC) methods. International Food Research Journal, 24(2).

National Cancer Institute. (2018). Definition of inflammation - NCI Dictionary of Cancer Terms - National Cancer Institute. Retrieved February 18, 2019, from https://www.cancer.gov/publications/dictionaries/cancer-terms/def/inflammation

National Research Council. (1984). Emergency and continuous exposure limits for selected airborne contaminants. Vol. 2. National Academies Press.

Neilson, A. P., & Ferruzzi, M. G. (2011). Influence of formulation and processing on absorption and metabolism of flavan-3-Ols from tea and cocoa. Annual Review of Food Science and Technology, 2(1), 125–151. https://doi.org/10.1146/annurev-food-022510-133725

Neilson, A. P., O'Keefe, S. F., & Bolling, B. W. (2016). High-molecular-weight proanthocyanidins in foods: Overcoming analytical challenges in pursuit of novel dietary bioactive components. Annual Review of Food Science and Technology, 7(1), 43–64. https://doi.org/10.1146/annurev-food-022814-015604

Nguyen, P. M., & Niemeyer, E. D. (2008). Effects of nitrogen fertilization on the phenolic composition and antioxidant properties of Basil (*Ocimum basilicum* L.). Journal of Agricultural and Food Chemistry, 56(18), 8685–8691. https://doi.org/10.1021/jf801485u

Nielsen, S. S. (2017). Vitamin C determination by indophenol method. In Food Analysis Laboratory Manual (pp. 143–146). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-44127-6_15

Ogden, C. L., Carroll, M. D., Fryar, C. D., & Flegal, K. M. (2015). Prevalence of obesity among adults and youth: United States, 2011–2014. (N. C. for H. Statistics, Ed.). Hyattsville, MD: National Center for Health Statistics.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity, 2(5), 270–278. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2835915/

Payne, C., Bowyer, P. K., Herderich, M., & Bastian, S. E. P. (2009). Interaction of astringent grape seed procyanidins with oral epithelial cells. Food Chemistry, 115(2), 551–557. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2008.12.061

Payne, M. J., Hurst, W. J., Stuart, D. A., Ou, B., Fan, E., Ji, H., & Kou, Y. (2010). Determination of total procyanidins in selected chocolate and confectionery products using DMAC. Journal of AOAC International, 93(1), 89–96.

Pereira, J. A., Oliveira, I., Sousa, A., Valentão, P., Andrade, P. B., Ferreira, I. C. F. R., … Estevinho, L. (2007). Walnut (*Juglans regia* L.) leaves: Phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. Food and Chemical Toxicology, 45(11), 2287–2295. https://doi.org/http://dx.doi.org/10.1016/j.fct.2007.06.004

Piljac, J., Martinez, S., Valek, L., Stipcevic, T., & Ganic, K. K. (2005). A Comparison of methods used to define the phenolic content and antioxidant activity of Croatian wines. Food Technology and Biotechnology, 43(3), 271–276.

Pinelo, M., Manzocco, L., Nuñez, M. J., & Nicoli, M. C. (2004). Interaction among phenols in food fortification: Negative Synergism on Antioxidant Capacity. https://doi.org/10.1021/JF0350515

Pope, M. T. (2007). Molybdenum oxygen chemistry: Oxides, oxo Complexes, and polyoxoanions. In Progress in Inorganic Chemistry (pp. 181–257). John Wiley & Sons, Inc. https://doi.org/10.1002/9780470166406.ch4

Portaccio, M., Di Martino, S., Maiuri, P., Durante, D., De Luca, P., Lepore, M., … Mita, D. G. (2006). Biosensors for phenolic compounds: The catechol as a substrate model. Journal of Molecular Catalysis B: Enzymatic, 41(3–4), 97–102. https://doi.org/http://dx.doi.org/10.1016/j.molcatb.2006.05.002

Price, M. L., & Butler, L. G. (1977). Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. Journal of Agricultural and Food Chemistry, 25(6), 1268–1273. https://doi.org/10.1021/jf60214a034

Price, M. L., Van Scoyoc, S., & Butler, L. G. (1978). A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. Journal of Agricultural and Food Chemistry, 26(5), 1214–1218. https://doi.org/10.1021/jf60219a031

Price, S. F., Breen, P. J., Valladao, M., & Watson, B. T. (1995). Cluster sun exposure and quercetin in pinot-noir grapes and vine. American Journal of Enology and Viticulture, 46(2), 187–194. Retrieved from http://vt.summon.serialssolutions.com/link/0/eLvHCXMwY2BQSEszSzVLNk0F1k2WJkYm5hZpyYbAVoGxQWpqmqWRaSLKjnek0txNlEHbzTXE2UMXuQqIL4AcuxAPOqDVNMjFyNICXMgaijHwJoJWgeeVgHeLpfC9qMx-rZrgpzb7b4lwgGyrLQBg-Sbm

Prieur, C., Rigaud, J., Cheynier, V., & Moutounet, M. (1994). Oligomeric and polymeric procyanidins from grape seeds. Phytochemistry, 36(3), 781–784. https://doi.org/http://dx.doi.org/10.1016/S0031-9422(00)89817-9

Pueyo, I. U., & Calvo, M. I. (2009). Assay conditions and validation of a new UV spectrophotometric method using microplates for the determination of polyphenol content. Fitoterapia, 80(8), 465–467. https://doi.org/http://dx.doi.org/10.1016/j.fitote.2009.06.008

Quiñones, M., Miguel, M., & Aleixandre, A. (2013). Beneficial effects of polyphenols on cardiovascular disease. Pharmacological Research, 68(1), 125–131. https://doi.org/http://dx.doi.org/10.1016/j.phrs.2012.10.018

R. Knaggs, A. (1999). The biosynthesis of shikimate metabolites. Natural Product Reports, 16(4), 525–560. https://doi.org/10.1039/A707501D

Ratnavathi, C. ., & Sashidhar, R. . (1998). Microassay for the quantitation of protein precipitable polyphenols: use of bovine serum albumin–benzidine conjugate as a protein probe. Food Chemistry, 61(3), 373–380. https://doi.org/10.1016/S0308-8146(97)00056-3

Reed, D. R., Tanaka, T., & McDaniel, A. H. (2006). Diverse tastes: Genetics of sweet and bitter perception. Physiology & Behavior, 88(3), 215–226. https://doi.org/10.1016/J.PHYSBEH.2006.05.033

Reis, F. S., Martins, A., Barros, L., & Ferreira, I. C. F. R. (2012). Antioxidant properties and phenolic profile of the most widely appreciated cultivated mushrooms: A comparative study between in vivo and in vitro samples. Food and Chemical Toxicology, 50(5), 1201–1207. https://doi.org/http://dx.doi.org/10.1016/j.fct.2012.02.013

Renaud, S., & de Lorgeril, M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. The Lancet, 339(8808), 1523–1526. https://doi.org/10.1016/0140-6736(92)91277-F

Ribéreau-Gayon, P. (1972). Plant Phenolics. Oliver and Boyd. Retrieved from https://books.google.com/books?id=EHzwAAAAMAAJ

Robichaud, J. L., & Noble, A. C. (1990). Astringency and bitterness of selected phenolics in wine. Journal of the Science of Food and Agriculture, 53(3), 343–353. https://doi.org/10.1002/jsfa.2740530307

Rohr, G. E., Meier, B., & Sticher, O. (2000). Analysis of procyanidins. Studies in Natural Products Chemistry, 21, 497–570. https://doi.org/10.1016/S1572-5995(00)80013-7

Romani, A., Mulinacci, N., Pinelli, P., Vincieri, F. F., & Cimato, A. (1999). Polyphenolic content in five Tuscany cultivars of *Olea europaea* L. Journal of Agricultural and Food Chemistry, 47(3), 964–967. https://doi.org/10.1021/jf980264t

Romani, A., Pinelli, P., Galardi, C., Sani, G., Cimato, A., & Heimler, D. (2002). Polyphenols in greenhouse and open-air-grown lettuce. Food Chemistry, 79(3), 337–342. https://doi.org/http://dx.doi.org/10.1016/S0308-8146(02)00170-X

Rose, R. C., & Nahrwold, D. L. (1981). Quantitative analysis of ascorbic acid and dehydroascorbic acid by high-performance liquid chromatography. Analytical Biochemistry, 114(1), 140–145. https://doi.org/http://dx.doi.org/10.1016/0003-2697(81)90464-4

Rosenberg, P. B. (2005). Clinical aspects of inflammation in Alzheimer's disease. International Review of Psychiatry, 17(6), 503–514. https://doi.org/10.1080/02646830500382037

Rossetti, D., Bongaerts, J. H. H., Wantling, E., Stokes, J. R., & Williamson, A. M. (2009). Astringency of tea catechins: more than an oral lubrication tactile percept. Food Hydrocolloids, 23(7), 1984–1992. https://doi.org/10.1016/j.foodhyd.2009.03.001

Rothwell JA, Pérez-Jiménez J, Neveu V, Medina-Ramon A, M'Hiri N, Garcia Lobato P, Manach C, Knox K, Eisner R, Wishart D, S. A. (2013). Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Retrieved September 20, 2002, from http://phenol-explorer.eu/

Sacks, F. M., Lichtenstein, A., Van Horn, L., Harris, W., Kris-Etherton, P., & Winston, M. (2006). Soy protein, isoflavones, and cardiovascular health. An American Heart Association Science Advisory for Professionals From the Nutrition Committee, 113(7), 1034–1044. https://doi.org/10.1161/circulationaha.106.171052

Sanchez-Rangel, J. C., Benavides, J., Heredia, J. B., Cisneros-Zevallos, L., & Jacobo-Velazquez, D. A. (2013). The Folin-Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. Analytical Methods, 5(21), 5990–5999. https://doi.org/10.1039/C3AY41125G

Sarkar, S. K., & Howarth, R. E. (1976). Specificity of the vanillin test for flavanols. Journal of Agricultural and Food Chemistry, 24(2), 317–320. https://doi.org/10.1021/jf60204a041

Sarneckis, C. J., Dambergs, R. G., Jones, P., Mercurio, M., Herderich, M. J., & Smith, P. A. (2006). Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. Australian Journal of Grape and Wine Research, 12(1), 39–49. https://doi.org/10.1111/j.1755-0238.2006.tb00042.x

Scalbert, A. (1992). Quantitative methods for the estimation of tannins in plant tissues. In Plant Polyphenols (pp. 259–280). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4615-3476-1_15

Schwarz, B., & Hofmann, T. (2008). Is there a direct relationship between oral astringency and human salivary protein binding? European Food Research and Technology, 227(6), 1693. https://doi.org/10.1007/s00217-008-0895-x

Šeruga, M., Novak, I., & Jakobek, L. (2011). Determination of polyphenols content and antioxidant activity of some red wines by differential pulse voltammetry, HPLC and spectrophotometric methods. Food Chemistry, 124(3), 1208–1216. https://doi.org/http://doi.org/10.1016/j.foodchem.2010.07.047

Shen, C. L., Wang, P., Guerrieri, J., Yeh, J. K., & Wang, J. S. (2008). Protective effect of green tea polyphenols on bone loss in middle-aged female rats. Osteoporosis International, 19(7), 979–990. https://doi.org/10.1007/s00198-007-0527-5

Sheppard, D., Wong, W. S., Uehara, C. F., Nadel, J. A., & Boushey, H. A. (1980). Lower threshold and greater bronchomotor responsiveness of asthmatic subjects to sulfur dioxide. American Review of Respiratory Disease, 122(6), 873–878. https://doi.org/10.1164/ARRD.1980.122.6.873

Shrivastava, A., & Gupta, V. (2011). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles of Young Scientists, 2(1), 21–25. https://doi.org/10.4103/2229-5186.79345

Siebert, K. J., & Lynn, P. Y. (1998). Comparison of polyphenol interactions with iolyvinylpolypyrrolidone and haze-active protein. Journal of American Society of Brewing Chemists, 56(1), 24–31.

Sigma-Aldrich Co. LLC. (2015). Product information_47641 Folin-Ciocalteu´s phenol reagent. Retrieved from http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Datasheet/6/47641dat.pdf

Sileika, T. S., Barrett, D. G., Zhang, R., Lau, K. H. A., & Messersmith, P. B. (2013). Colorless multifunctional coatings inspired by polyphenols found in tea, chocolate, and wine. Angewandte Chemie, 125(41), 10966–10970. https://doi.org/10.1002/ange.201304922

Singleton, V. L. (1974). Analytical fractionation of the phenolic substances of grapes and wine and some practical uses of such analyses. In Chemistry of Winemaking (Vol. 137, pp. 184–211). AMERICAN CHEMICAL SOCIETY. https://doi.org/doi:10.1021/ba-1974-0137.ch00910.1021/ba-1974-0137.ch009

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In Methods in Enzymology (Vol. Volume 299, pp. 152–178). Academic Press. https://doi.org/http://dx.doi.org/10.1016/S0076-6879(99)99017-1

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16(3), 144–158.

Slimestad, R., Vangdal, E., & Brede, C. (2009). Analysis of phenolic compounds in six Norwegian plum cultivars (*Prunus domestica* L.). Journal of Agricultural and Food Chemistry, 57(23), 11370–11375. https://doi.org/10.1021/jf902054x

Slinkard, K., & Singleton, V. L. (1977). Total phenol analysis: Automation and comparison with manual methods. American Journal of Enology and Viticulture, 28(1), 49. Retrieved from http://www.ajevonline.org/content/28/1/49.abstract

Smit, C. J. B., Joslyn, M. A., & Lukton, A. (1955). Determination of tannins and related polyphenols in foods. Analytical Chemistry, 27(7), 1159–1162.

Soares, S., Brandão, E., Mateus, N., & de Freitas, V. (2017). Sensorial properties of red wine polyphenols: Astringency and bitterness. Critical Reviews in Food Science and Nutrition, 57(5), 937–948. https://doi.org/10.1080/10408398.2014.946468

Soares, S., Kohl, S., Thalmann, S., Mateus, N., Meyerhof, W., & De Freitas, V. (2013). Different phenolic compounds activate distinct human bitter taste receptors. Journal of Agricultural and Food Chemistry, 61(7), 1525–1533. https://doi.org/10.1021/jf304198k

Somers, T. C., & Ziemelis, G. (1980). Gross interference by sulphur dioxide in standard determinations of wine phenolics. Journal of the Science of Food and Agriculture, 31, 600–610.
Soong, Y.-Y., & Barlow, P. J. (2004). Antioxidant activity and phenolic content of selected fruit seeds. Food Chemistry, 88(3), 411–417. https://doi.org/10.1016/j.foodchem.2004.02.003

Spanos, G. A., & Wrolstad, R. E. (1992). Phenolics of apple, pear, and white grape juices and their changes with processing and storage. A review. Journal of Agricultural and Food Chemistry, 40(9), 1478–1487. https://doi.org/10.1021/jf00021a002
Spanos, G. A., Wrolstad, R. E., & Heatherbell, D. A. (1990). Influence of processing and storage on the phenolic composition of apple juice. Journal of Agricultural and Food Chemistry, 38(7), 1572–1579. https://doi.org/10.1021/jf00097a031

Steffen, Y., Schewe, T., & Sies, H. (2006). Myeloperoxidase-mediated LDL oxidation and endothelial cell toxicity of oxidized LDL: attenuation by (−)-epicatechin. Free Radical Research, 40(10), 1076–1085. https://doi.org/10.1080/10715760600883247

Stewart, A. J., Bozonnet, S., Mullen, W., Jenkins, G. I., Lean, M. E. J., & Crozier, A. (2000). Occurrence of flavonols in tomatoes and tomato-based products. Journal of Agricultural and Food Chemistry, 48(7), 2663–2669. https://doi.org/10.1021/jf000070p

Strack, D. (1997). Phenolic metabolism. In P. M. Dey & J. B. Harborne (Eds.), Plant Biochemistry. London: Academic Press.

Strong, F. C. (1947). Trends in quantitative analysis. Analytical Chemistry, 19(12), 968–971. https://doi.org/10.1021/ac60012a008

Sun, B., Ricardo-da-Silva, J. M., & Spranger, I. (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. Journal of Agricultural and Food Chemistry, 46(10), 4267–4274. https://doi.org/10.1021/jf980366j

Swain, T., & Hillis, W. E. (1959). The phenolic constituents of *Prunus domestica.* I.—The quantitative analysis of phenolic constituents. Journal of the Science of Food and Agriculture, 10(1), 63–68. https://doi.org/10.1002/jsfa.2740100110

Swartz, M. E., & Krull, I. S. (2012). Handbook of Analytical Validation. Taylor & Francis. Retrieved from https://books.google.com/books?id=wK6yRXe_QvUC

Thies, M., & Fischer, R. (1971). Über eine neue Farbreaktion zum mikrochemischen Nachweis und zur quantitativen Bestimmung von Catechinen. Microchimica Acta, 59(1), 9–13. https://doi.org/10.1007/BF01216876

Thompson-Witrick, K. A., Goodrich, K. M., Neilson, A. P., Hurley, E. K., Peck, G. M., & Stewart, A. C. (2014). Characterization of the polyphenol composition of 20 cultivars of cider, processing, and dessert apples (Malus × domestica Borkh.) grown in Virginia. Journal of Agricultural and Food Chemistry, 62(41), 10181–10191. https://doi.org/10.1021/jf503379t

Tomás-Barberán, F. A., & Clifford, M. N. (2000). Flavanones, chalcones and dihydrochalcones – nature, occurrence and dietary burden. Journal of the Science of Food and

Agriculture, 80(7), 1073–1080. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<1073::AID-JSFA568>3.0.CO;2-B

Tomás-Barberán, F. A., González-Sarrías, A., García-Villalba, R., Núñez-Sánchez, M. A., Selma, M. V, García-Conesa, M. T., & Espín, J. C. (2017). Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. Molecular Nutrition & Food Research, 61(1), 1500901–n/a. https://doi.org/10.1002/mnfr.201500901

Tomás-Barberán, F. A., Selma, M. V, & Espín, J. C. (2016). Interactions of gut microbiota with dietary polyphenols and consequences to human health. Current Opinion in Clinical Nutrition & Metabolic Care, 19(6), 471–476. https://doi.org/10.1097/mco.0000000000000314

Tomás-Menor, L., Barrajón-Catalán, E., Segura-Carretero, A., Martí, N., Saura, D., Menéndez, J. A., … Micol, V. (2015). The Promiscuous and synergic molecular interaction of polyphenols in bactericidal activity: An opportunity to improve the performance of antibiotics? Phytotherapy Research, 29(3), 466–473. https://doi.org/10.1002/ptr.5296

Toor, R. K., Savage, G. P., & Lister, C. E. (2006). Seasonal variations in the antioxidant composition of greenhouse grown tomatoes. Journal of Food Composition and Analysis, 19(1), 1–10. https://doi.org/http://dx.doi.org/10.1016/j.jfca.2004.11.008

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. Nutrients, 2(12), 1231–1246. https://doi.org/10.3390/nu2121231

Tsao, R., Khanizadeh, S., & Dale, A. (2006). Designer fruits and vegetables with enriched phytochemicals for human health. Canadian Journal of Plant Science, 86(3), 773–786. https://doi.org/10.4141/P05-138

Tugwell, S., & Branch, G. M. (1989). Differential polyphenolic distribution among tissues in the kelps Ecklonia maxima, Laminaria pallida and Macrocystis angustifolia in relation to plant-defence theory. Journal of Experimental Marine Biology and Ecology, 129(3), 219–230. https://doi.org/http://dx.doi.org/10.1016/0022-0981(89)90104-4

Tuorila, H., & Cardello, A. V. (2002). Consumer responses to an off-flavor in juice in the presence of specific health claims. Food Quality and Preference, 13(7–8), 561–569. https://doi.org/10.1016/S0950-3293(01)00076-3

U.S. Food and Drug Administration. (2015). Analytical procedures and methods validation for drugs and Biologics. Guidance for Industry.

USDA National Nutrient Database for Standard Reference, Release 28. (2016). US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory. Retrieved from https://ndb.nal.usda.gov/ndb/search/list

Valavanidis, A., Vlachogianni, T., Psomas, A., Zovoili, A., & Siatis, V. (2009). Polyphenolic profile and antioxidant activity of five apple cultivars grown under organic and conventional agricultural practices. International Journal of Food Science & Technology, 44(6), 1167–1175. https://doi.org/10.1111/j.1365-2621.2009.01937.x

Velioglu, Y. S., Mazza, G., Gao, L., & Oomah, B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products, 46(10), 4113–4117. https://doi.org/10.1021/JF9801973

Vidal, S., Francis, L., Guyot, S., Marnet, N., Kwiatkowski, M., Gawel, R., … Waters, E. J. (2003). The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. Journal of the Science of Food and Agriculture, 83(6), 564–573. https://doi.org/10.1002/jsfa.1394

Vila, L. (2004). Cyclooxygenase and 5-lipoxygenase pathways in the vessel wall: Role in atherosclerosis. Medicinal Research Reviews, 24(4), 399–424. https://doi.org/10.1002/med.10065

Vinson, J., Su, X., Zubik, L., & Pratima, B. (2001). Phenol antioxidant quantity and quality in foods: Fruits. Journal of Agricultural and Food Chemistry, 49(11), 5315–5321. https://doi.org/10.1021/JF0009293

Vuorinen, H., Määttä, K., & Törrönen, R. (2000). Content of the flavonols myricetin, quercetin, and kaempferol in Finnish berry wines. Journal of Agricultural and Food Chemistry, 48(7), 2675–2680. https://doi.org/10.1021/jf991388o

Wallace, T. C., & Giusti, M. M. (2010). Evaluation of parameters that affect the 4-dimethylaminocinnamaldehyde assay for flavanols and proanthocyanidins. Journal of Food Science, 75(7), C619–C625. https://doi.org/10.1111/j.1750-3841.2010.01734.x

Wang, S., Moustaid-Moussa, N., Chen, L., Mo, H., Shastri, A., Su, R., … Shen, C.-L. (2014). Novel insights of dietary polyphenols and obesity. The Journal of Nutritional Biochemistry, 25(1), 1–18. https://doi.org/http://dx.doi.org/10.1016/j.jnutbio.2013.09.001

Wang, X., Ouyang, Y., Liu, J., Zhu, M., Zhao, G., Bao, W., & Hu, F. B. (2014). Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. BMJ : British Medical Journal, 349. https://doi.org/10.1136/bmj.g4490

Wang, Y., Singh, A. P., Hurst, W. J., Glinski, J. A., Koo, H., & Vorsa, N. (2016). Influence of degree-of-polymerization and linkage on the quantification of proanthocyanidins using 4-dimethylaminocinnamaldehyde (DMAC) assay. Journal of Agricultural and Food Chemistry, 64(11), 2190–2199. https://doi.org/10.1021/acs.jafc.5b05408

Wang, Z., Huang, Y., Zou, J., Cao, K., Xu, Y., & Wu, J. M. (2002). Effects of red wine and wine polyphenol resveratrol on platelet aggregation in vivo and in vitro. International Journal of Molecular Medicine, 9(1), 77–79.

Weber, F., Schulze-Kaysers, N., & Schieber, A. (2014). Chapter 15 - Characterization and quantification of polyphenols in fruits - Watson, Ronald Ross. In Polyphenols in Plants (pp. 293–304). San Diego: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-397934-6.00015-2

Wilken, R., Veena, M. S., Wang, M. B., & Srivatsan, E. S. (2011). Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. Molecular Cancer, 10(1), 12. https://doi.org/10.1186/1476-4598-10-12

Wilson, A. L. (1970a). The performance-characteristics of analytical methods—I. Talanta, 17(1), 31–44. https://doi.org/http://dx.doi.org/10.1016/0039-9140(70)80046-7

Wilson, A. L. (1970b). The performance-characteristics of analytical methods—II. Talanta, 17(1), 31–44. https://doi.org/10.1016/0039-9140(70)80047-9

Wilson, A. L. (1973). The performance characteristics of analytical methods—III. Talanta, 20(8), 725–732. https://doi.org/http://dx.doi.org/10.1016/0039-9140(73)80249-8

Wilson, A. L. (1974). Performance characteristics of analytical methods—IV. Talanta, 21(11), 1109–1121. https://doi.org/http://dx.doi.org/10.1016/0039-9140(74)80094-9

Wimalasiri, P., & Wills, R. B. H. (1983). Simultaneous analysis of ascorbic acid and dehydroascorbic acid in fruit and vegetables by high-performance liquid chromatography. Journal of Chromatography A, 256, 368–371. https://doi.org/http://dx.doi.org/10.1016/S0021-9673(01)88253-4

World Health Organization. (2017a). Cancer Fact Sheet. Retrieved February 18, 2019, from http://pinkoak.org/wp-content/uploads/2018/03/WHO-Cancer-Fact-Sheet-2017.pdf

World Health Organization. (2017b). Cardiovascular diseases (CVDs). Retrieved February 18, 2019, from https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)

Wu, S.-F., Chang, F.-R., Wang, S.-Y., Hwang, T.-L., Lee, C.-L., Chen, S.-L., … Wu, Y.-C. (2011). Anti-inflammatory and cytotoxic neoflavonoids and benzofurans from pterocarpus santalinus. Journal of Natural Products, 74(5), 989–996. https://doi.org/10.1021/np100871g

Xia, E.-Q., Deng, G.-F., Guo, Y.-J., & Li, H.-B. (2010). Biological activities of polyphenols from grapes. International Journal of Molecular Sciences, 11(2), 622. Retrieved from http://www.mdpi.com/1422-0067/11/2/622

Xu, Y., Zhang, M., Wu, T., Dai, S., Xu, J., & Zhou, Z. (2015). The anti-obesity effect of green tea polysaccharides, polyphenols and caffeine in rats fed with a high-fat diet. Food & Function, 6(1), 296–303.

CHAPTER 3 Comparison of common analytical methods for the quantification of total polyphenols and flavanols in fruit juices and ciders

Submitted to Journal of Food Science, Food Chemistry Section

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ABSTRACT

Multiple analytical methods are used for quantification of total polyphenols and total flavanols in fruit juice and beverages. Four methods were evaluated in this study: Folin-Ciocalteu (F-C), Lowenthal permanganate (L-P), 4-dimethylaminocinnamaldehyde (DMAC) and the bovine serum albumin (BSA) precipitation method. Method validation parameters including working range, limit of detection (LOD), limit of quantitation (LOQ), precision (repeatability), accuracy, and specificity were assessed and compared. The F-C method was not specific to polyphenols, the L-P method had the widest working range but lacked accuracy. The DMAC method was the most specific to flavanols, and the BSA method was not suitable for quantification of smaller flavanols such as catechin and epicatechin. Quantitative performance was evaluated using commercial fruit juice samples (n = 14), apple juice samples of different cultivars (n = 22), and commercial ciders (n = 17). In general, the L-P titration method and DMAC method resulted in higher quantitative values than the F-C method and BSA precipitation method, respectively. However, ratios of results obtained by the L-P and F-C method ranged from 1 to 28, and ratios of results obtained by the DMAC and BSA precipitation method ranged from <1 to 280. This tremendous variation is likely due to variation in polyphenol composition. This information provides perspective for comparison of results obtained through these different methods, and a basis for choosing the most appropriate analytical method for quantification of polyphenols to address a specific research question when working with commercial fruit juice, apple juice from different apple cultivars and commercial ciders.

PRACTICAL APPLICATION

This study compared results obtained when four common polyphenol quantification methods were applied to a diverse selection of fruit juices and beverages with distinct polyphenol composition. The matrix and polyphenol composition of the samples significantly influenced the results. Our findings can help manufacturers of fruit-based products choose the most appropriate analytical method for polyphenol quantification as part of a quality assurance program or to convey information on dietary polyphenol content to consumers. An assessment of analytical method validation parameters is provided for each of the four methods, which will help users of these methods to understand their limitations

3.1 Introduction

3.1.1 Polyphenols in fruit juice and beverages

Polyphenols are secondary metabolites of plants with highly diverse chemical structures. More than 8000 polyphenols of plant origin have been characterized (Pandey & Rizvi, 2009). All polyphenols contain hydroxylated phenyl moieties, often present as glycosides (Weber, Schulze-Kaysers, & Schieber, 2014). Polyphenols are classified into flavonoids, phenolic acids, and other classes including stilbenes and lignans based on their aglycone structures (Tsao, 2010). Raw fruits and vegetables, such as grapes, apples and cocoa, as well as the products made from them, such as wines, cider (the fermented alcoholic beverage made from apples) and chocolate, are major sources of dietary polyphenols globally (Tsao, 2010). The concentration and composition of polyphenols in fruits vary greatly depending on the plant species (Pandey & Rizvi, 2009). For example, anthocyanins (a subgroup of flavonoids) are the most predominant class of polyphenols in cranberry, red grape, and pomegranate. For citrus fruits including lime, lemon and grapefruit, flavanones (a subgroup of flavonoids) predominate. In American cranberry and prune, the predominant phenolic compounds are benzoic acid and neochlorogenic acid, respectively (both phenolic acids). Chlorogenic acid (a phenolic acid) is prevalent in most apple and blueberry cultivars. Substantial variation in polyphenol composition may also exist within different genotypes of a given species (Anastasiadi et al., 2017; Rothwell et al., 2013).

Flavanols, a subgroup of flavonoids made up of flavanol subunits, impart astringency and bitterness to fruit juices and beverages (Lea & Arnold, 1978). Bitterness and astringency can differ in wines and ciders with the same reported total polyphenol content due to variations in sensory impact among polyphenols (Brossaud, Cheynier, & Noble, 2001; Lea & Arnold, 1978). Several methods for polyphenol quantification have been used as predictors of bitterness and/or astringency, however identifying the most effective method for this purpose remains a topic of current research (Boulet et al., 2016).

Consumption of dietary polyphenols is associated with positive human health outcomes. Information on polyphenol content is thus routinely reported in fruit juice and beverage marketing or point-of-sale materials (Sun-Waterhouse, 2011). Fruit producers use a range of analytical methods to quantify polyphenol content for this purpose, including those evaluated in this study (Aleixandre-Tudo, Buica, Nieuwoudt, Aleixandre, & du Toit, 2017).

3.1.2 Analytical methods for polyphenol quantification

Several analytical methods are routinely used to determine a “total” polyphenol value for fruit juices and beverages. The Folin-Ciocalteu (F-C), Lowenthal permanganate (L-P), 4-dimethylaminocinnamaldehyde (DMAC) and bovine serum albumin (BSA) precipitation methods are historically relevant and represent four of the most widely applied methods used in fruit juice and beverage analysis (Weber et al., 2014). Other relevant methods for polyphenol quantification also exist, including reading absorbance at 280 nm, precipitation with methyl

cellulose, Prussian Blue, Bate-Smith, and Vanillin test (Aleixandre-Tudo et al., 2017). These methods are all generally nonspecific due to the complexity and expense of determining concentrations of individual polyphenol constituents (Neilson, O'Keefe, & Bolling, 2016).

The F-C method is widely used for quantification of total polyphenols in various fruit juices and beverages (Everette et al., 2010). It is based on redox reactions between reducing compounds in the sample, including but not limited to polyphenols, and the F-C reagent. Several compounds, including ascorbic acid, reducing sugars, SO2, tyrosine, have been found to interfere with the results of the F-C method and are inadvertently quantified as polyphenols (Everette et al., 2010). However, this method continues to be very widely applied in food science, nutrition science and horticulture likely due to its ease of use and low cost.

The L-P titration method was commonly applied in apple and cider analysis. While the L-P method has also been applied occasionally in the analysis of pear, peach, tea and coffee (Barua & Roberts, 1940; Smit, Joslyn, & Lukton, 1955), it has not emerged as a preferred method for products beyond apples and ciders, perhaps due to its reported limitations such as difficulty in visually determining the titration endpoint. The method relies on the oxidation of polyphenols by potassium permanganate in the presence of indigo carmine as a 'redox indicator'. Compared to the L-P method, it is generally believed that the F-C method is preferable because of greater accuracy and less interference (Singleton, Orthofer, & Lamuela-Raventós, 1999), however, this hypothesis has not been rigorously tested.

The DMAC method has been widely used for total flavanol quantification in various samples for decades, especially in fruit juice and beverage samples because of the sensory properties imparted by flavanols. The DMAC method relies on the reaction between the DMAC reagent and flavanols, resulting in a spectrophotometrically quantifiable color change. The variation of degree of polymerization (DP) and linkage of flavanols found in fruit juice and beverages has been reported to cause variations in the quantification results (Wang et al., 2016).

The BSA precipitation method has the best potential of known analytical methods to predict the intensity of astringency in wine, and is thus commonly applied in wine analysis. Astringency is a tactile sensation that can be evoked by precipitation of proteins in the mouth by polyphenols (Brossaud et al., 2001). The BSA method is based on the assumption that the precipitation of polyphenols and protein is proportional to the concentration of polyphenols in a given sample. The expansion of the application to predict astringency in other fruit juices and beverages of different polyphenol composition requires more research and method validation.

The objective of this study was to determine analytical method validation parameters and assess quantitative performance of four analytical methods (F-C, L-P, DMAC and BSA) using a broad range of fruit juice and beverage samples to allow assessment of the influence of differences in polyphenol composition among fruit types (e.g. berries, citrus, apples) on quantitative results. We hypothesized that different methods would exhibit different strengths and weaknesses in terms of analytical method validation parameters and that the quantitative results of F-C would differ from L-P, and DMAC would differ from BSA. Furthermore, we expected that the magnitude of the differences would be influenced by polyphenol composition and sample matrix.

3.2 Materials and methods

3.2.1 Chemicals and standards

F-C's phenol reagent, gallic acid (GA), sodium carbonate, procyanidin (PC) B2 (a PC dimer), (+)-catechin, (−)-epicatechin, chlorogenic acid, quercetin, phloretin, indigo carmine, potassium permanganate, DMAC, and sodium oxalate were purchased from Sigma-Aldrich (St.

Louis, MO, USA); J. T. Baker Analyzed HPLC Ultra Gradient acetonitrile and formic acid were purchased from Avantor Performance Materials (Center Valley, PA, USA); Analytical standards PC B1 and B5 (a PC dimer), C1 (a PC trimer), and Cinnamtannin A2 (Cinn A2, a PC tetramer) were purchased from Planta Analytica (Danbury, CT, USA); L-ascorbic acid, methanol, ethyl acetate, hydrochloric acid, sulfuric acid and acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA); BSA Fraction V was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

3.2.2 Analytical method validation

3.2.2.1 Working range

Analytical curves for the F-C, L-P, DMAC, and BSA precipitation methods were built using aqueous solutions of GA (n = 11, concentrations of 0 to 1000 mg/L with 100 mg/L interval), aqueous solutions of GA (n = 13, concentrations of 0 to 12 g/L with 1 g/L interval), PC B2 solutions prepared in methanol (n = 9, concentrations of 0, 1, 5, 10, 20, 30, 40, 50 and 60 mg/L) and aqueous solutions of catechin (n = 9, concentrations of 0, 10, 25, 50, 100, 150, 200, 250, and 300 mg/L), respectively. Each concentration of each standard was prepared in triplicate, and three measurements were made per solution (Nunes, Alvarenga, de Souza Sant'Ana, Santos, & Granato, 2015). Initial concentration ranges evaluated were identified based on prior reports of linear standard curves. The upper limit of the working range was determined by evaluating increasing concentrations until either (1) the curve became nonlinear as determined through linear regression, (2) maximum solubility of the standard was reached, or (3) absorbance readings ≥ 1 were obtained. The lower limit of the working range was determined as LOQ. Linear regression analysis was conducted (Microsoft Excel, Redmond, WA, USA) wherein an equation describing the linear relationship of the data and an R^2 value were obtained for each method. ANOVA of the linear regression was conducted to test the significance of the model, where significance was defined as $p < 0.05$.

3.2.2.2 LOD and LOQ

LOD and LOQ were calculated as:

$$\mathrm{LOD} = \frac{3.3\mathrm{s}}{\mathrm{b}} \quad (1)$$

$$\mathrm{LOQ} = \frac{10\mathrm{s}}{\mathrm{b}} \quad (2)$$

Where s is the standard deviation of the y-intercept and b is the slope of the analytical curve (Shrivastava & Gupta, 2011).

3.2.2.3 Repeatability and accuracy

Repeatability was determined using the relative standard deviation (RSD). Accuracy was determined using the recovery and 95% Confidence Interval (CI). Solutions with known concentrations (concentrations of the standard near the middle of the working range for each method) of 300 mg/L GA, 5 g/L GA, 20 mg/L of PC B2, and one cider sample were measured 10 times each with a 15-min interval on the same day under the same conditions (materials, equipment, temperature) by the same personnel using the L-P, F-C, DMAC, and BSA precipitation methods, respectively. A cider sample expected to contain PCs of DP > 4 was evaluated in the BSA method due to the expense of purified PCs of DP > 4. RSD (n = 10) was calculated as:

$$\mathrm{RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100 \quad (3)$$

Accuracy (trueness) is assessed by percent recovery. Recovery was calculated for the L-P, F-C, and DMAC method using $n = 10$ replicates:

$$\text{Recovery} = \frac{\text{Quantification result}}{\text{Known concentration of the standard}} \times 100 \quad (4)$$

The 95% ($\alpha = 0.05$, $t_{0.025}$) CI of the mean was calculated for $n = 10$ replicates:

$$\text{CI}(95\%\ \text{confidence}) = \text{Mean of the sample concentrations} \pm t_{\alpha/2} \frac{\text{Standard deviation}}{\sqrt{n}} \quad (5)$$

3.2.2.4 Selectivity

Selectivity was assessed by spiking potentially interfering compounds into solutions of polyphenol compounds. Concentrations of potentially interfering compounds ascorbic acid (1 g/L), glucose (100 g/L), and tyrosine (10 mg/L), were identified according to values present in apple juice ("USDA Food Composition Database" 2019). Sodium metabisulfite concentration (0.1 g/L) was representative of cider or wine (Zoecklein, Fugelsang, Gump, & Nury, 1999).

For each of the four methods evaluated, a solution of the standard for that method was prepared in triplicate at a concentration near the middle of the working range for each method, as determined in section 2.2.1. Specifically, to evaluate selectivity of the F-C method, triplicate 300 mg/L aqueous solutions of GA were made and spiked with the four potentially interfering compounds at the aforementioned concentrations. Triplicate 5 g/L aqueous solutions of GA for the L-P, 20 mg/L aqueous solutions of PC B2 for DMAC and cider samples for BSA were spiked in the same manner, respectively. One measurement per replicate was taken for a total of three measurements for each potentially interfering compound using each method. One-way ANOVA followed by Tukey's multiple comparison test (significance where $p < 0.05$) was conducted to determine whether the presence of these compounds interfered with the results.

3.2.3 Evaluation of quantitative performance

3.2.3.1 Sample preparation

A wide range of commercially available fruit juices ($n = 14$) (not from concentrate, no added ascorbic acid) and commercially available ciders ($n = 17$, identified in Supplementary Information Table 3.1) were purchased from local grocery stores. Apples of different cultivars ($n = 22$) obtained from orchards in Virginia, USA, were pressed into juice in our laboratory (Champion Juicer, Lodi, CA, USA). The number of samples in each category was determined based on availability, thus sample number varied by category. All of the samples were centrifuged at 2300 x g for 10 min, and the supernatant was flushed with nitrogen and stored at −80°C until analysis.

3.2.3.2 Quantification of total polyphenol content by F-C and L-P methods

F-C method. Total polyphenol content was determined by the F-C method reported by Spanos and Wrolstad, with minor modification (Spanos & Wrolstad, 1992). A six-point standard curve (0 to 500 mg/L GA in water) was used for quantification of experimental samples. Samples were diluted in water to fall within the working range for the F-C method, determined in section 2.2.1. The F-C reagent was dissolved in water to 0.2 M. A 50 µL aliquot of each sample (diluted in water, if needed) was mixed with 450 µL of water in a cuvette (polystyrene, Fisher Scientific, Fair Lawn, NJ, USA). A 1.25 mL aliquot of 0.2 M F-C reagent was added and mixed. A 1 mL aliquot of 75 g/L Na_2CO_3 solution was then added to the cuvette and mixed. Following incubation (two hours, dark, room temperature), absorbance was read at 765 nm (GENESYS 10S UV-Vis spectrophotometer, Thermo Scientific, Waltham, MA, USA). This analysis, including sample dilution, was conducted in triplicate for each sample. Total

polyphenol concentration was calculated using the standard curve and expressed as GA equivalents in mg/L.

L-P titration method. Total polyphenol content was also determined by the L-P titration method (Lowenthal, 1877). A 0.02 N aqueous $KMnO_4$ solution was standardized against sodium oxalate (AOAC, 1995). Concentration of the standardized titrant was calculated as:

$$\text{Concentration of } KMnO_4 \text{ solution (in N)} = \frac{\text{mass of sodium oxalate (in g)} \times 1000}{\text{volume of } KMnO_4 \text{ (in mL)} \times 66.999} \quad (6)$$

Whereas 66.999 is the factor to convert into normality of the $KMnO_4$ solution. A 1 mL aliquot of the sample, 5 mL of the 0.1% indigo carmine indicator [0.1% (w/v) indigo carmine in 0.92 M sulfuric acid], and 200 mL deionized water were added to a 500 mL flask, and titrated to a light green endpoint while stirring. The volume of titrant used was recorded as X mL. A blank titration using 5 mL of indigo carmine indicator with 200 mL water was also carried out, with volume of titrant recorded as Y mL. Titration was conducted in triplicate for each sample and the blank. Total polyphenol content expressed as 'tannic acid' (TA) equivalents in mg/L was calculated as:

$$\text{Total Polyphenol } \left(\frac{\text{mg}}{\text{L}} \text{ of TA equivalents}\right) = (X - Y) \times 4.157 \times \text{concentration of } KMnO_4 \text{ solution (in N)} \times 10000 \quad (7)$$

Where the 4.147 is the factor to convert from $KMnO_4$ equivalents to TA equivalents and 10000 is the factor to convert from percentage of tannic acid into mg/L of TA equivalents.

2.3.3 Quantification of total flavanol content using DMAC and BSA precipitation methods

DMAC method. Total flavanol content in the samples was determined by the DMAC method (Payne et al., 2010). A six-point standard curve with concentrations from 0 to 50 mg/L PC B2 in methanol was used for quantification of the experimental samples. Samples were diluted in water to within the working range of the DMAC method, determined in section 2.2.1. Prior work has shown that using methanol vs. water as the solvent did not impact color development at the catechin concentrations evaluated in our study (Wallace & Giusti, 2010). A 200 µL aliquot of diluted sample or standard was mixed with 1 mL of the 0.1% DMAC solution {DMAC dissolved in acidified methanol [6 mL concentrated (36%) hydrochloric acid in 54 mL methanol]} in a cuvette. Absorbance at 640 nm was recorded. This analysis, including sample dilution, was conducted in triplicate. Total flavanol concentration expressed as PC B2 equivalents in mg/L was calculated using the standard curve.

BSA precipitation method. Total flavanol content was also quantified using the BSA precipitation method (Harbertson, Kennedy, & Adams, 2002). A six-point standard curve [0 to 150 mg/L (+)-catechin] was used for quantification of the experimental samples. To make the standards, stock solution of 1000 mg/L of (+)-catechin in methanol was added to Buffer 1 [5% triethanolamine (v/v) and 10% sodium dodecyl sulfate (w/v)] in cuvettes to a total volume of 875 µL. Then, 125 µL of $FeCl_3$ reagent (0.01 M $FeCl_3$ in 0.01 N HCl) was added and incubated at room temperature for 10 min. The absorbance was read at 510 nm. The BSA method involves precipitation of flavanols followed by washing, redissolution, and spectrophotometric quantification. Briefly, samples were diluted in Buffer 2 (12% ethanol in water (v/v) containing 5 g/L potassium bitartrate adjusted to pH 3.3 with HCl) to within the working range determined in section 2.2.1. A 1 mL aliquot of the BSA solution [1 mg/mL BSA in Buffer 3 (0.2 M acetic acid and 0.17 M NaCl adjusted to pH 4.9 with NaOH)] was then mixed with 500 µL of diluted sample, incubated at room temperature for 15 min with slow agitation, and centrifuged for 1 min (13,500 x *g*). The supernatant was poured off and the precipitate was dissolved in 250 µL of

Buffer 3. This mixture was centrifuged for 1 min (13,500 x *g*) and the supernatant poured off. The precipitate was dissolved in 875 µL of Buffer 1 and incubated at room temperature for 10 min. Absorbance at 510 nm was recorded as A_1. A 125 µL aliquot of $FeCl_3$ reagent was added and the absorbance at 510 nm after 10 min was recorded as A_2. This analysis, including sample dilution, was conducted in triplicate for each sample. The difference between A_2 and A_1 was used as the Y value to calculate flavanol content, as mg/L (+)-catechin equivalents, from the standard curve for each experimental sample.

3.2.3.4 Quantification of individual polyphenol compounds by UPLC/MS

To understand how polyphenol composition may influence results of the four analytical methods evaluated, individual polyphenol compounds commonly found in apple juice and cider were quantified using the LC-MS method described by Ma *et al*. (Ma et al., 2018). Separation gradient and retention times, molecular weights, and SIR channels are provided in Supplementary Information Table 3.2 and 3.3, respectively. Due to vastly different polyphenol composition expected in commercial fruit juice samples and the expense of the wide range of analytical standards that would be required, analysis of the individual polyphenol profile of these samples was not conducted. Instead, a subset of 12 apple juices and 12 ciders expected to vary in polyphenol composition, based on prior reports of polyphenol composition (Anastasiadi et al., 2017) and on informal evaluation of their sensory attributes, were selected. Standards of PC B1, PC B2, PC B5, PC C1, Cinn A2, (+)-catechin, (−)-epicatechin, chlorogenic acid, quercetin, and phloretin were used to build five-point standard curves for the quantification of these compounds.

3.2.3.5 Statistical analysis

Results are reported as means ± Standard Error of the Mean (SEM) for three replicates. The total individual polyphenol concentration by LC/MS was defined as the sum of all analyzed individual polyphenols for a given sample. Un-paired t-tests were conducted between the quantification results to compare values for total polyphenols obtained through L-P vs. F-C, and for total flavanols obtained through DMAC vs. BSA, respectively, using GraphPad Prism v6.0e (GraphPad Software Inc., La Jolla, CA, USA). To determine whether the relationships between these values are influenced by sample composition, one-way ANOVA with Tukey's multiple comparison test was used to determine whether there were significant differences in the ratios of L-P to F-C values and ratios of DMAC to BSA values. Significant difference was defined as $p < 0.05$.

3.3 Results and discussion

3.3.1 Analytical method validation

3.3.1.1 Working range

Working range for the four methods are summarized in Table 3.1. The values of R^2 and $R^2_{adjusted}$ near one for all the analytical curves (Supplementary Information Figure 3.1) show that each of the linear models explained the majority of the experimental variability. The *p*-values for all analytical curves were significant ($p < 0.0001$), indicating the existence of a strong relationship between responses and concentrations of standards. The upper limit of the working range for F-C found in this study (500 mg/L of GA) is in agreement with a previously suggested maximum concentration (Singleton et al., 1999). For L-P, the upper limit of the working range was 12,000 mg/L of GA. The maximum solubility of GA in water is approximately 12 g/L (Budavari, 1996), representing the highest concentration evaluated in this study.

While a upper limit of the working range of 50 mg/L of PC B2 was found for DMAC in this study, standard curves with wider linear ranges have been reported by others, including 0.1

to 100 mg/L of PC B2 (Payne et al., 2010) and 3.125 to 100 mg/L of PC B2 (Prior et al., 2010). It is likely that those linear ranges reached higher maximum concentrations than we observed because values obtained via absorbance values ≥ 1 were included. For this study, we defined absorbance ≥ 1 as a criterion for the upper limit of the method due to the increased error associated with absorbance readings ≥ 1 (Nielsen, 2010). For BSA, a standard curve linear between 0 to 100 mg/L of tannic acid was previously reported (Hagerman & Butler, 1978), however we observed a wider working range up to 150 mg/L of catechin. While tannic acid was historically used as the standard for BSA precipitation, catechin is preferred as the standard for fruit juice and beverage analysis because it is found in fruit (Harbertson et al., 2002), while tannic acid is not (*Food Chemicals Codex*, 2019).

Table 3. 1. Analytical curves, LOD, and LOQ for the F-C, L-P, DMAC and BSA precipitation methods. Note that the units differ among these values and are listed in the footnotes provided.

Method	Analytical curve[1]	Working range	R^2	$R^2_{adjusted}$	*P* value[2]	LOD	LOQ
F-C[3]	y = 0.0019x + 0.0096	42.9 to 500	0.999	0.999	<0.0001	14.2	42.9
L-P[3]	y = 1256.1x + 48.61	1.47 to 12000	0.995	0.995	<0.0001	0.485	1.47
DMAC[4]	y = 0.017x + 7E05	5.71 to 50	0.998	0.997	<0.0001	1.71	5.71
BSA[5]	y = 0.0067x + 0.0108	13.7 to 150	0.999	0.998	<0.0001	4.51	13.7

[1] Regression analysis was conducted using the concentrations of standard in the x-axis and the measurements in the y-axis.
[2] P-value < 0.05 indicates that the slope of the linear regression is non-zero.
[3] mg/L of GA equivalents
[4] mg/L of PC B2 equivalents
[5] mg/L of catechin equivalents

The limitations of this study merit consideration. The use of varying numbers of standards (n = 11, n = 13, n = 9 and n = 9) for each assay could have biased the results in terms of comparing R^2 values of standard curves within working ranges. Additionally, the low end of the working range was defined as the LOQ in this study. Although samples at the concentration of 0 mg/L (reagent blank) were evaluated for each method, the number of values near 0 mg/L was limited, and 1/x weighting was not applied. This could lend disproportionate weight to the samples at higher concentrations in determination of the linear fit, leading to increased error at the low end of the linear ranges reported in this study. To minimize the introduction of error due to this limitation, a good practice is to concentrate or dilute samples to fit within 20% to 80% of the working range of the method (Nielsen, 2010).

3.3.1.2 LOD and LOQ

LOD and LOQ are summarized in Table 3.1. For F-C, LOD and LOQ were 14.2 mg/L GA and 42.9 mg/L GA, respectively. Others have reported lower LOD and LOQ of 0.25 mg/L GA and 0.82 mg/L GA, respectively (Margraf, Karnopp, Rosso, & Granato, 2015). This difference could be attributable to stronger color intensity imparted by more concentrated F-C reagent, which results in greater slope of the analytical curve, and lower LOD and LOQ. LOD and LOQ for the L-P method have not been previously reported. For the DMAC method, we observed LOD and LOQ of 1.71 mg/L PC B2 and 5.71 mg/L PC B2. A comparable LOD of 1.94 mg/L and LOQ of 6.47 mg/L were reported by others using PC A2 as the standard (Feliciano et al., 2012). Our results indicate that the DMAC method has slightly lower LOD and LOQ compared to the BSA precipitation method (4.51 mg/L catechin, and 13.68 mg/L catechin). No

prior reports of the LOD and LOQ of the BSA precipitation method are available for comparison.

3.3.1.3 Repeatability and accuracy

Repeatability (%RSD) and accuracy (recovery and 95% CI) are listed in Table 3.2. The %RSD for each of the four methods was small, indicating low variation among measurements made under the same operating conditions over short time intervals, i.e. high intra-day precision. Higher %RSD of the F-C method has been reported by others (4.98% and 6.65%), compared to 0.66 % found in this study. For DMAC, we found 2.2% RSD, slightly lower than reported by others [4.0 to 9.5% RSD for intermediate products of chocolate manufacturing (Payne et al., 2010), and 2.3 to 6.1% RSD for commercial cranberry samples (Prior et al., 2010)]. Differences in repeatability with DMAC could be attributable to matrix differences of food samples vs. standard solutions. For the BSA method, a prior report of 1.2%-7.2% RSD for dry red wine samples is in agreement with our finding of 3.6% RSD (Mercurio & Smith, 2008).

Table 3. 2. Repeatability and recovery of F-C, L-P, DMAC and BSA precipitation methods. Data were expressed as mean ± SEM.

Method	Repeatability/(%RSD)	Recovery/%	$CI_{95\%}$
F-C	0.66	102.9 ± 0.21	307.3 to 309.9 mg/L in GA equivalents
L-P	0.70	143.5 ± 0.32	7145 to 7207 mg/L in TA equivalents
DMAC	2.2	104.1 ± 0.72	20.5 to 21.1 mg/L in PC B2 equivalents
BSA	3.6	N/A	N/A

Nearly 100% recovery for F-C and DMAC (Table 3.2) indicates measurements very close to reference values. Similar recovery for F-C, 90% (Blainski, Lopes, & de Mello, 2013) and 98.20% (Margraf et al., 2015) have been reported. L-P recovery was much higher, 143.5 ± 0.32%, a value beyond the accepted range of 80% to 120% (U.S. Food and Drug Administration, 2015). Though accuracy of L-P is poor compared to F-C, L-P is still used for relative comparison of polyphenol content among samples with similar polyphenol compositions, such as apples of a single cultivar, due to its high repeatability, wide linear range, and low cost.

3.3.1.4 Selectivity

Selectivity is reported in Table 3.3. Ascorbic acid, potassium metabisulfite, glucose and tyrosine significantly increased total polyphenol values by F-C (Table 3, $p < 0.001$) by 241%, 13%, 2% and 29%, respectively. Low selectivity of F-C has been observed by others (Everette et al., 2010). Reducing compounds in the sample, including but not limited to polyphenols, reduce the F-C reagent under basic conditions. Accordingly, the presence of other non-polyphenol reducing compounds in the sample matrix could significantly impact results. While interference from glucose may not be of practical significance (< 10%), the other compounds evaluated could significantly interfere with comparisons of total polyphenol values, especially between different types of fruit.

Others hypothesized that interference by sugars in the sample matrix would be greater for L-P than for F-C because potassium permanganate is a stronger oxidizing agent compared to the F-C reagent (Singleton et al., 1999). However, our results were numerically close to the reference values (< 10% difference, Table 3.3). Others found that 8 g/L glucose had no effect on L-P results (Celeste, Tomas, Cladera, Estela, & Cerda, 1993), however we found that the addition of 100 g/L of glucose, the concentration found in apple juice, significantly decreased results. In the L-P method, potassium permanganate does not specifically oxidize polyphenols,

but all other reducing compounds that are oxidized more rapidly by potassium permanganate than indigo carmine (Smit et al., 1955). Under acidic conditions, polyphenols bind with sugars through hydrogen bonds (Bordenave, Hamaker, & Ferruzzi, 2014), decreasing polyphenol redox by making the active site (reactive hydroxyl groups) unavailable (Bors & Michel, 2002), resulting in lower apparent polyphenol concentration in the presence of sugar.

Table 3. 3. Selectivity of F-C, L-P, DMAC and BSA precipitation method. Data were expressed as mean ± SEM. Different lower-case letters after the value indicate significant difference ($p < 0.05$) among treatments for each method (each row, including the control), by one-way ANOVA and Tukey's HSD test.

Methods	Control[1]	Ascorbic acid[2]	Potassium metabisulfite[3]	Glucose[4]	Tyrosine[5]
F-C[6]	308.6 ± 0.64 e	1053 ± 2.4 a	350 ± 0.17 c	316 ± 1.2 d	398 ± 3.3 b
L-P[7]	7176 ± 16 b	7803 ± 43 a	7238 ± 21 b	6745 ± 25 c	7685 ± 104 a
DMAC[8]	20.8 ± 0.14 a	21.5 ± 0.33 a	21.1 ± 0.043 a	20.9 ± 0.14 a	21.4 ± 0.049 a
BSA[9]	81.0 ± 0.92 a	80.2 ± 3.3 a	75.3 ± 2.0 ab	66.3 ± 2.8 b	76.4 ± 2.7 a

[1] 300 mg/L of GA for F-C method, 5 g/L of GA for L-P method, 20 mg/L of PC B2 for DMAC method, and a cider sample for BSA precipitation method for $n = 10$ replicates
[2] 1 g/L ascorbic acid was spiked into the respective control for $n = 6$ replicates
[3] 0.1 g/L potassium metabisulfite was spiked into the respective control for $n = 6$ replicates
[4] 100 g/L glucose was spiked into the respective control for $n = 6$ replicates
[5] 10 mg/L tyrosine was spiked into the respective control for $n = 6$ replicates
[6] in mg/L GA equivalents
[7] in mg/L TA equivalents
[8] in mg/L PC B2 equivalents
[9] in mg/L catechin equivalents

DMAC showed the greatest selectivity of all methods evaluated in this study. None of the compounds evaluated altered results compared to the control (20 mg/L of PC B2, Table 3.3). In the DMAC method, the reagent reacts with the C8 carbon at the terminal unit on A-ring flavanols (Wallace & Giusti, 2010). Because the four potentially interfering compounds evaluated in this study do not share this specific structure required for color development, they did not interfere with the quantitative results of the DMAC method.

For BSA, glucose was the only compound that interfered, resulting in 18% lower values for total flavanols possibly due to glucose interfering in binding between polyphenols and BSA (Bordenave et al., 2014). Other fruit juice or cider sample matrix constituents not evaluated in this project, like proteins or polysaccharides including pectin, could also interfere with the BSA precipitation method and this merits further investigation.

3.3.2 Evaluation of quantitative performance

3.3.2.1 Comparison of total polyphenol quantification by F-C and L-P

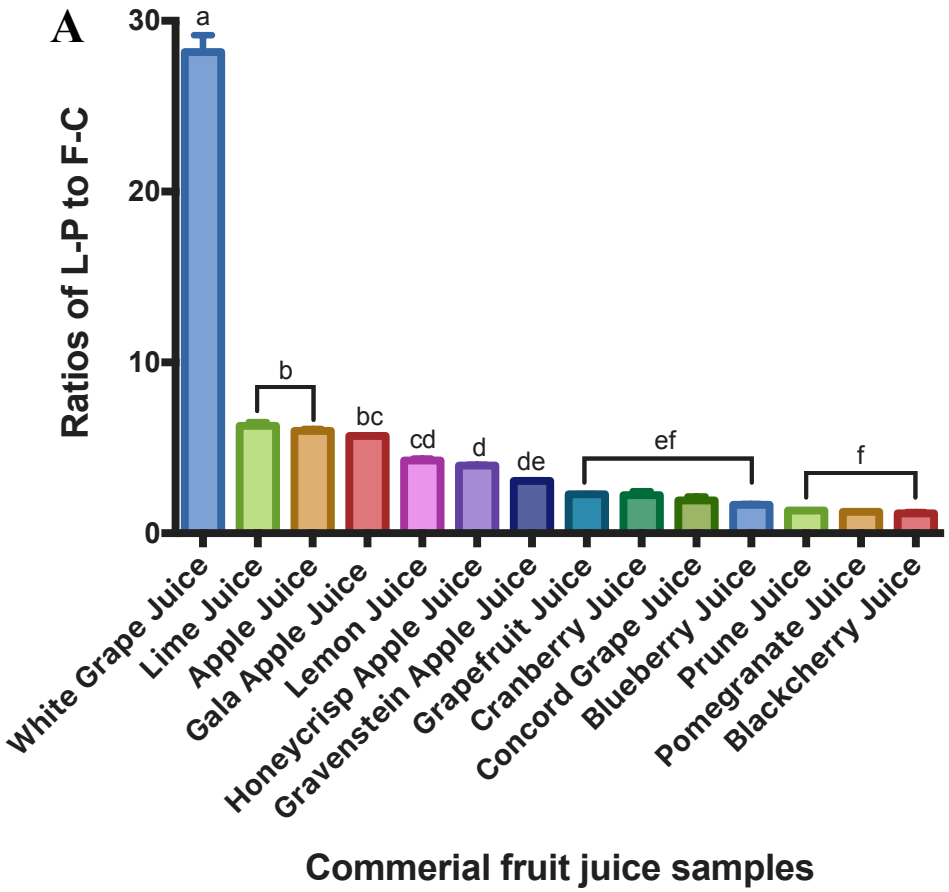
Results obtained using F-C were lower for all samples compared to L-P, with the exception of black cherry juice (Table 3.4). This observation is consistent with prior reports on red and white wine samples (Celeste et al., 1993). The absolute values for F-C and L-P were expected to differ due to different principles of these methods. Perhaps more interestingly, significant differences in in the ratio of L-P to F-C values for a given sample ($p < 0.0001$ were observed for all three sets of samples. These ratios range from 1.2 to 28 (Table 3.4). For fruit juice of lighter color (white grape, lime, apple, lemon), the ratios were higher compared to fruit juice of darker color (blueberry juice, cranberry juice, concord grape juice, grapefruit juice) (Figure 3.1A). The color of these fruits is imparted mainly by anthocyanins (Bridle & Timberlake, 1997), a class of polyphenols that may quantitatively contribute more in F-C than in

L-P. Additionally, within the sample set of apple juices from different cultivars, ratios range from 1.1 to 11 ($p < 0.0001$) (Figure 3.1B), likely due to substantial variation in polyphenol composition among apple cultivars (Anastasiadi et al., 2017) (Table 3.5). The ratios of L-P to F-C values for commercial cider samples fall into a narrower range of 1.3 to 5.8 (Figure 3.1C), with 2 to 3 being the most prevalent ratio. Greater variation in polyphenol composition in apple juices compared to ciders made from those juices has been reported (Ewing, Peck, Ma, Neilson, & Stewart, 2019; Ma et al., 2018), and our findings are in general agreement with this.

Table 3. 4. Total polyphenol content of commercial fruit juice samples (n = 14), apple juice samples of different cultivar (n = 22), and commercially available cider samples (n = 17) quantified by F-C and L-P method. Data were expressed as mean ± SEM for n = 3 replicates. P values were reported from comparisons between the two methods by un-paired t-test.

Samples	F-C (mg/L GA equivalents)	L-P (mg/L TA equivalents)	P values	Ratio L-P/F-C
Commercial fruit juices				
Apple Juice	204.8 ± 0.04	1226 ± 23	<0.001	6.0 ± 0.1
Blueberry Juice	1270 ± 0.6	2108 ± 67	<0.001	1.7 ± 0.06
Blackcherry Juice	1860 ± 0.04	2163 ± 150	0.113	1.2 ± 0.08
Cranberry Juice	705.4 ± 0.3	1573 ± 149	0.004	2.2 ± 0.2
Concord Grape Juice	1161 ± 0.3	2231 ± 264	0.015	1.9 ± 0.2
Gala Apple Juice	209.3 ± 0.2	1193 ± 12	<0.001	5.7 ± 0.04
Grapefruit Juice	599.6 ± 0.2	1365 ± 3.2	<0.001	2.3 ± 0.02
Gravenstein Apple Juice	452.2 ± 0.1	1384 ± 23	<0.001	3.1 ± 0.04
Honeycrisp Apple Juice	325.8 ± 0.06	1290 ± 17	<0.001	4.0 ± 0.06
Lemon Juice	319.6 ± 0.2	1359 ± 32	<0.001	4.3 ± 0.1
Lime Juice	198.1 ± 0.2	1245 ± 41	<0.001	6.3 ± 0.2
Pomegranate Juice	3120 ± 0.5	3897 ± 51	<0.001	1.2 ± 0.01
Prune Juice	1769 ± 0.2	2354 ± 23	<0.001	1.3 ± 0.01
White Grape Juice	38.46 ± 0.05	1083 ± 34	<0.001	28 ± 1
apple juice samples of different cultivar				
Arkansas Black	908.1 ± 0.8	1568 ± 77	<0.001	1.8 ± 0.1
Ashmead's Kernel	692.7 ± 0.2	1441 ± 41	<0.001	2.1 ± 0.05
Black Twig	421.6 ± 0.1	3501 ± 24	<0.001	8.3 ± 0.08
Cameo	277.1 ± 0.2	3045 ± 59	<0.001	11 ± 0.2
Fuji	333.7 ± 0.03	1734 ± 58	<0.001	5.2 ± 0.2
Gold Rush	383.0 ± 0.4	1770 ± 25	<0.001	4.6 ± 0.1
Golden Delicious	344.6 ± 0.5	1695 ± 18	<0.001	4.9 ± 0.1
Golden Russet	572.7 ± 0.8	1767 ± 16	<0.001	3.1 ± 0.04
Granny Smith	442.3 ± 1	1601 ± 17	<0.001	3.6 ± 0.1
Harrison 1	1169 ± 0.1	2108 ± 13	<0.001	1.8 ± 0.01
Harrison 2	1170 ± 0.01	2169 ± 18	<0.001	1.9 ± 0.02
Hewes	2180 ± 0.1	2456 ± 16	<0.001	1.1 ± 0.01
Ida Red	368.1 ± 0.1	1643 ± 190	0.003	4.5 ± 0.5
Jonagold	389.2 ± 0.9	1577 ± 19	<0.001	4.1 ± 0.1
King David	462.5 ± 0.4	1601 ± 22	<0.001	3.5 ± 0.06

Manchurian	201.4 ± 0.3	1474 ± 17	<0.001	7.3 ± 0.1
Pink Lady	360.2 ± 0.6	1625 ± 21	<0.001	4.5 ± 0.1
Red Delicious	644.0 ± 0.2	1474 ± 26	<0.001	2.3 ± 0.05
Rome	585.3 ± 0.4	1571 ± 22	<0.001	2.7 ± 0.05
Snowdrift	6607 ± 0.5	10730 ± 31	<0.001	1.6 ± 0.01
Virginia Gold	362.1 ± 0.2	1550 ± 19	<0.001	4.3 ± 0.06
York	507.6 ± 0.2	1640 ± 19	<0.001	3.2 ± 0.05
Commercial ciders				
1	205.1 ± 0.1	923.4 ± 29	<0.001	4.5 ± 0.1
2	567.0 ± 0.6	1220 ± 3.3	<0.001	2.2 ± 0.04
3	238.6 ± 0.2	966.3 ± 20	<0.001	4.0 ± 0.07
4	2187 ± 0.8	2830 ± 190	0.029	1.3 ± 0.1
5	378.3 ± 0.3	1052 ± 22	<0.001	2.8 ± 0.05
6	677.5 ± 0.6	1375 ± 5.7	<0.001	2.0 ± 0.03
7	465.1 ± 0.4	1121 ± 49	<0.001	2.4 ± 0.08
8	243.6 ± 0.2	996.0 ± 17	<0.001	4.1 ± 0.08
9	179.6 ± 0.1	986.1 ± 6.6	<0.001	5.5 ± 0.05
10	160.3 ± 0.2	926.7 ± 27	<0.001	5.8 ± 0.1
11	396.0 ± 0.9	1000 ± 6.0	<0.001	2.5 ± 0.07
12	456.9 ± 0.1	1078 ± 34	<0.001	2.4 ± 0.07
13	367.1 ± 0.1	1051 ± 33	<0.001	2.9 ± 0.1
14	638.5 ± 0.3	1356 ± 18	<0.001	2.1 ± 0.04
15	385.8 ± 0.2	1036 ± 18	<0.001	2.7 ± 0.06
16	396.9 ± 0.02	1142 ± 14	<0.001	2.9 ± 0.03
17	1318 ± 0.1	2235 ± 12	<0.001	1.7 ± 0.01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Gala Apple Juice
Lemon Juice
Honeycrisp Apple Juice
Gravenstein Apple Juice
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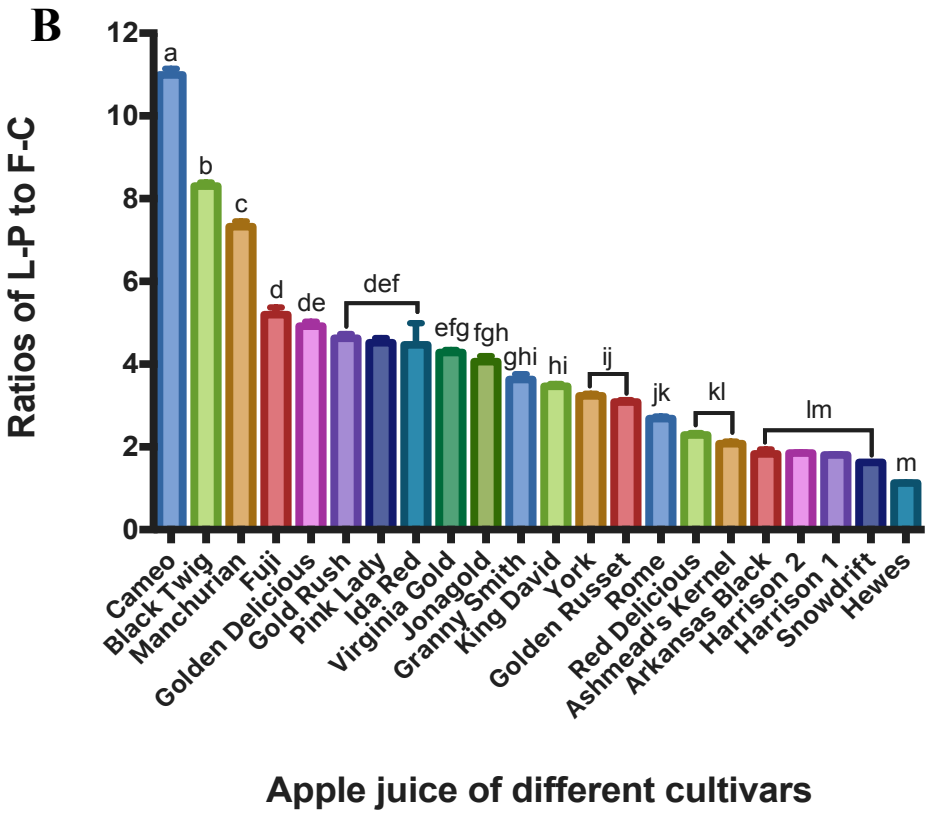
Concord Grape Juice
Blueberry Juice
Prune Juice
Pomegranate Juice
Blackcherry Juice
Commerial fruit juice samples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Ratios of L-P to F-C
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Black Twig
Manchurian
Fuji
Golden Delicious
Gold Rush
Pink Lady
Ida Red
Virginia Gold
Jonagold
Granny Smith
King David
York
Golden Russet
Rome
Red Delicious
Ashmead's Kernel
Arkansas Black
Harrison 2
Harrison 1
Snowdrift
Hewes
Apple juice of different cultivars

C

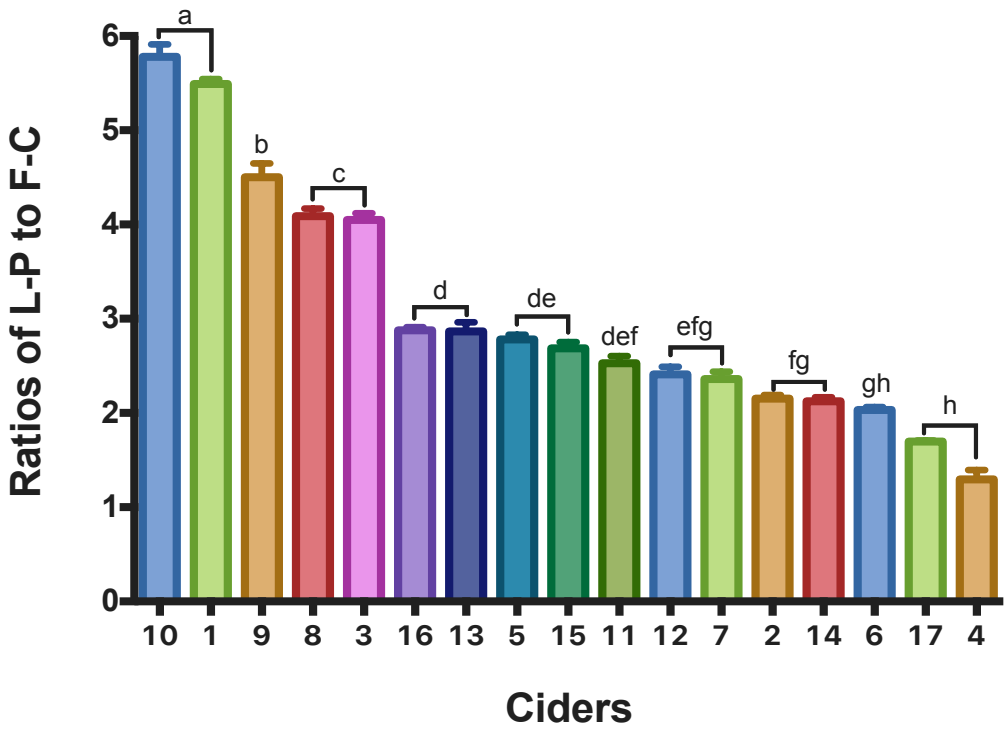


Figure 3. 1. Multiple comparisons of ratios of L-P to F-C results among fruit juice samples (A), apple juice of different cultivars (B), and ciders (C). Bars represent the mean and error bars represent the SEM for $n = 3$ replicates. Lower case letters represent significant differences between the means. Significance was defined as $p < 0.05$.

Table 3. 5. Concentration of individual polyphenol compounds in mg/L of (A) apple juice from different apple cultivars (*n* = 12) and (B) commercial ciders (*n* = 12). Data were expressed as mean ± SEM for n = 3 replicates.

Cultivar	Catechin	Epicatechin	PC B1	PC B2	PC B5
Ashmead's Kernel	3.14 ± 0.15	5.49 ± 0.31	1.77 ± 0.23	3.47 ± 0.45	0.433 ± 0.033
Black Twig	0.229 ± 0.020	0.156 ± 0.014	0.0938 ± 0.012	0.0952 ± 0.012	0.074 ± 0.0072
Cameo	0.0563 ± 0.011	0.0376 ± 0.0071	0.0095 ± 0.0019	0.0100 ± 0.0018	0.0138 ± 0.0032
Fuji	0.122 ± 0.0068	0.0811 ± 0.0050	0.031 ± 0.00036	0.0331 ± 0.0011	0.0206 ± 0.00078
Gold Rush	0.416 ± 0.024	0.277 ± 0.019	1.30 ± 0.10	0.889 ± 0.038	0.476 ± 0.020
Granny Smith	0.497 ± 0.071	0.353 ± 0.043	2.65 ± 0.12	0.891 ± 0.12	0.287 ± 0.035
Harrison 1	0.398 ± 0.015	0.262 ± 0.011	0.58 ± 0.056	0.673 ± 0.056	0.140 ± 0.011
Harrison 2	5.26 ± 0.16	5.98 ± 0.44	1.97 ± 0.11	1.52 ± 0.076	0.387 ± 0.010
Hewes	1.45 ± 0.32	21.0 ± 4.6	1.67 ± 0.47	14.7 ± 3.1	1.790 ± 0.52
Manchurian	0.00447 ± 0.0025	0.132 ± 0.022	0.0023 ± 0.00060	nd	nd
Snowdrift	266 ± 8.3	158 ± 14	475 ± 30	351 ± 36	22.7 ± 1.2
York	0.124 ± 0.014	0.282 ± 0.031	0.046 ± 0.0061	0.140 ± 0.0063	0.0416 ± 0.0043

Table 3.5. (A) Continued

Cultivar	PC C1	Cinn A2	Chlorogenic acid	Phloretin	Quercetin	Total
Ashmead's Kernel	0.523 ± 0.044	0.286 ± 0.035	59.4 ± 3.1	0.0220 ± 0.00224	0.0435 ± 0.0014	74.6 ± 2.0
Black Twig	0.0563 ± 0.0083	0.0531 ± 0.0074	35.3 ± 1.9	0.00688 ± 0.00086	0.0262 ± 0.00051	36.1 ± 2.0
Cameo	0.00777 ± 0.0017	0.0142 ± 0.0023	19.9 ± 0.48	0.00195 ± 0.000050	0.00485 ± 0.00073	20.1 ± 0.48
Fuji	0.0132 ± 0.00054	0.0161 ± 0.0068	25.7 ± 0.93	0.00120 ± 0.00010	0.00593 ± 0.00034	26.0 ± 0.92
Gold Rush	0.649 ± 0.036	0.468 ± 0.0068	43.9 ± 2.1	0.0112 ± 0.00073	0.0260 ± 0.0010	48.4 ± 2.0
Granny Smith	0.591 ± 0.088	0.503 ± 0.044	9.17 ± 0.044	0.00478 ± 0.00065	0.0125 ± 0.0030	15.0 ± 0.25
Harrison 1	0.227 ± 0.024	0.108 ± 0.0096	318 ± 15	0.0084 ± 0.00070	0.00167 ± 0.00030	321 ± 15
Harrison 2	0.581 ± 0.012	0.210 ± 0.012	195 ± 4.4	0.0214 ± 0.0017	0.00738 ± 0.00056	212 ± 5.1
Hewes	0.830 ± 0.14	2.46 ± 0.38	58.5 ± 8.1	0.0022 ± 0.00060	0.0101 ± 0.0019	102 ± 17
Manchurian	nd	nd	2.38 ± 0.39	0.00378 ± 0.00059	0.0226 ± 0.0025	2.55 ± 0.42
Snowdrift	164 ± 11	82.0 ± 9.2	27.53 ± 1.5	0.244 ± 0.0081	0.294 ± 0.021	1550 ± 100
York	0.0694 ± 0.0029	0.0489 ± 0.0047	21.0 ± 1.9	0.00288 ± 0.00028	0.0102 ± 0.0011	21.8 ± 2.0

Table 3.5. (B)

Sample number	Catechin	Epicatechin	PC B1	PC B2	PC B5
2	0.594 ± 0.049	0.611 ± 0.050	0.136 ± 0.013	0.521 ± 0.054	0.0408 ± 0.0031
3	0.0704 ± 0.023	0.338 ± 0.022	0.0118 ± 0.0034	0.0780 ± 0.0074	0.0176 ± 0.0010
4	9.22 ± 0.62	28.7 ± 1.7	3.80 ± 0.10	18.6 ±2.4	2.09 ± 0.030
5	0.374 ± 0.047	1.62 ± 0.24	0.0741 ± 0.011	0.376 ± 0.040	0.105 ± 0.018
6	2.97 ± 0.074	9.32 ± 0.47	2.06 ± 0.22	6.27 ± 1.0	0.708 ± 0.020
8	0.141 ± 0.015	0.515 ± 0.057	0.0254 ± 0.0054	0.611 ± 0.14	0.0608 ± 0.0088
10	0.0769 ± 0.0061	0.153 ± 0.014	0.0117 ± 0.0021	0.0492 ± 0.0073	0.00503 ± 0.00046
12	0.472 ± 0.058	3.69 ± 0.45	0.491 ± 0.058	3.29 ± 0.80	0.351 ± 0.044
13	0.598 ± 0.039	5.23 ± 0.87	0.675 ± 0.11	4.32 ± 0.60	0.462 ± 0.0055
14	0.529 ± 0.026	2.37 ± 0.10	0.202 ± 0.043	0.660 ± 0.060	0.124 ± 0.0038
15	0.159 ± 0.022	0.375 ± 0.048	0.0145 ± 0.0031	0.141 ± 0.033	0.0325 ± 0.0049
17	3.94 ± 0.23	36.3 ± 3.4	3.60 ± 0.30	48.7 ± 5.4	4.44 ± 0.022

Table 3.5. (B) Continued

Sample number	PC C1	Cinn A2	Chlorogenic acid	Phloretin	Quercetin	Total
2	0.0547 ± 0.0029	nd	10.5 ± 0.36	0.0296 ± 0.00047	0.0417 ± 0.00072	12.5 ± 0.54
3	nd	nd	9.72 ± 0.060	0.0249 ± 0.00061	0.0705 ± 0.0017	10.3 ± 0.12
4	0.625 ± 0.029	1.88 ± 0.025	8.13 ± 0.19	0.277 ± 0.00075	0.573 ± 0.0066	73.9 ± 5.2
5	0.0811 ± 0.0074	nd	17.0 ± 1.8	0.766 ± 0.13	0.180 ± 0.0045	20.6 ± 2.3
6	1.03 ± 0.093	0.185 ± 0.010	43.2 ± 1.0	1.05 ± 0.011	0.0380 ± 0.00080	66.8 ± 2.9
8	0.124 ± 0.027	nd	11.1 ± 0.36	0.181 ± 0.0028	0.183 ± 0.0036	12.9 ± 0.62
10	nd	nd	1.07 ± 0.093	0.445 ± 0.0035	0.285 ± 0.0041	2.09 ± 0.13
12	0.737 ± 0.054	0.287 ± 0.020	53.6 ± 1.6	0.267 ± 0.0043	0.00440 ± 0.00022	63.2 ± 3.1
13	0.946 ± 0.097	0.324 ± 0.029	3.46 ± 0.043	0.180 ± 0.035	0.00912 ± 0.0013	16.2 ± 1.8
14	0.108 ± 0.015	nd	133 ± 9.1	0.184 ± 0.0081	0.0151 ± 0.00090	137 ± 9.3
15	nd	nd	15.0 ± 1.1	0.328 ± 0.0058	0.0696 ± 0.0013	16.2 ± 1.2
17	2.19 ± 0.16	nd	70.1 ± 3.2	0.115 ± 0.0032	0.0805 ± 0.00098	170 ± 13

*nd=not detected

Differences in reactivity among polyphenol compounds have been reported for F-C (Supplementary Information Figure 3.2) and L-P (Smit et al., 1955), and could be expected to translate to differences in ratios of L-P to F-C results for samples with very different polyphenol composition, such as the sample set evaluated in this study. For example, for a solution of pure hydroquinone, the result by L-P was 2.4 times higher than the result by F-C (Hyman, Sansome-Smith, Shears, & Wood, 1985). Hydroquinone is the oxidation product of phenols, and is thus present during the reaction for both F-C and L-P. L-P results were higher compared to F-C for chlorogenic acid, catechol, and pyrogallol, while the F-C method yielded higher results for catechin, quercetin, and phenol (Smit et al., 1955). Taken together, these findings illustrate that polyphenol composition may impact both the absolute values obtained using F-C and L-P methods, and also the relationship between values obtained using these two methods.

3.3.2.2 Comparison of total flavanol quantification by DMAC and BSA

For all samples except pomegranate juice, total flavanol results by DMAC were higher than those obtained using BSA (Table 3.6). In fact, many of the samples contained low or non-detectable concentrations of flavanols as determined by BSA. Differences in absolute values obtained using these two different methods were expected due to differences in the mechanisms of the two methods. While DMAC quantifies only flavanols sharing a common structure of a C8 carbon at the terminal unit on the A-ring (Wallace & Giusti, 2010), BSA only quantifies flavanols with DP 3 and larger due to the inability of BSA to form precipitates with flavanols monomers and dimers (Harbertson, Kilmister, Kelm, & Downey, 2014).

Table 3. 6. Total flavanol content of commercial fruit juice samples (*n* = 14), apple juice samples of different cultivar (*n* = 22), and commercially available cider samples (*n* = 17) quantified by DMAC and BSA precipitation method. Data were expressed as mean ± SEM for *n* = 3 replicates. P values were reported from between methods along the rows by un-paired t-test. For these methods, values that were not detected (below the LOD of each method) were treated as 0 when calculating the ratios.

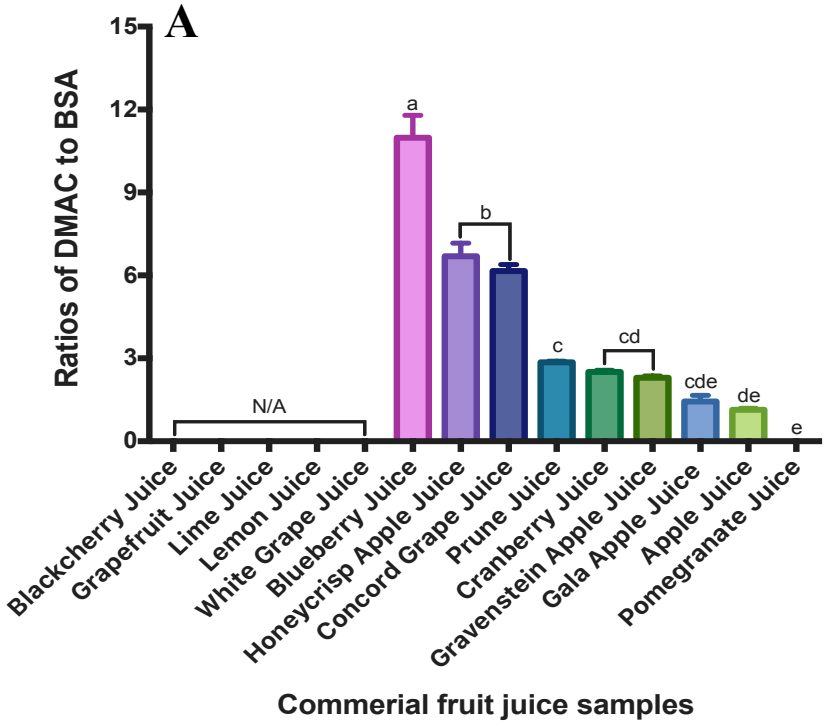
Samples	DMAC (mg/L PC B2 equivalents)	BSA (mg/L catechin equivalents)	P values	Ratio DMAC/BSA
Commercial fruit juices				
Apple Juice	18.23 ± 0.16	17.71 ± 0.83	0.065	1.0 ± 0.06
Blueberry Juice	195.3 ± 4.9	21.87 ± 1.9	<0.001	9.4 ± 0.8
Blackcherry Juice	143.7 ± 4.0	nd	<0.001	N/A
Cranberry Juice	221.5 ± 4.9	89.40 ± 0.50	<0.001	2.5 ± 0.06
Concord Grape Juice	416.6 ± 5.1	73.99 ± 3.3	<0.001	5.7 ± 0.2
Gala Apple Juice	12.56 ± 0.34	8.883 ± 1.6	0.112	1.4 ± 0.2
Grapefruit Juice	21.33 ± 0.62	nd	<0.001	N/A
Gravenstein Apple Juice	27.90 ± 0.21	12.17 ± 0.30	<0.001	2.3 ± 0.07
Honeycrisp Apple Juice	68.80 ± 0.34	9.922 ± 0.74	<0.001	7.0 ± 0.5
Lemon Juice	15.82 ± 0.36	nd	<0.001	N/A
Lime Juice	7.957 ± 0.23	nd	<0.001	N/A
Pomegranate Juice	154.8 ± 1.6	89307 ± 3698	<0.001	0.0017 ± 0
Prune Juice	30.58 ± 1.3	11.13 ± 0.49	<0.001	2.7 ± 0.05
White Grape Juice	2.427 ± 0.020	nd	<0.001	N/A
Apple juice samples of different cultivars				
Arkansas Black	47.29 ± 0.53	8.030 ± 0.99	<0.001	6.1 ± 0.7

Ashmead's Kernel	291.5 ± 0.52	3.110 ± 0.35	<0.001	96 ± 11
Black Twig	48.96 ± 1.3	8.030 ± 0.77	<0.001	6.2 ± 0.4
Cameo	48.90 ± 0.14	nd	<0.001	N/A
Fuji	26.39 ± 0.54	nd	<0.001	N/A
Gold Rush	30.86 ± 0.14	nd	<0.001	N/A
Golden Delicious	18.21 ± 0.078	10.18 ± 0.62	<0.001	1.8 ± 0.1
Golden Russet	22.96 ± 0.31	5.159 ± 0.67	<0.001	4.6 ± 0.5
Granny Smith	41.74 ± 0.32	nd	<0.001	N/A
Harrison 1	218.8 ± 3.8	35.83 ± 3.5	<0.001	6.2 ± 0.7
Harrison 2	212.7 ± 10	17.16 ± 2.0	<0.001	13 ± 2
Hewes	561.6 ± 31	180.8 ± 16	<0.001	3.1 ± 0.3
Ida Red	18.56 ± 0.98	nd	<0.001	N/A
Jonagold	21.09 ± 0.84	4.236 ± 0.10	<0.001	5.0 ± 0.3
King David	18.90 ± 0.54	8.851 ± 0.62	<0.001	2.2 ± 0.2
Manchurian	7.740 ± 0.052	nd	<0.001	N/A
Pink Lady	21.13 ± 0.51	nd	<0.001	N/A
Red Delicious	19.07 ± 2.1	10.18 ± 0.31	0.014	1.9 ± 0.2
Rome	40.68 ± 2.7	10.29 ± 0.88	<0.001	4.0 ± 0.1
Snowdrift	5449 ± 120	2433 ± 42	<0.001	2.2 ± 0.06
Virginia Gold	20.02 ± 0.052	nd	<0.001	N/A
York	27.94 ± 4.5	1.262 ± 0.31	0.004	25 ± 7
Commercial ciders				
1	38.51 ± 0.25	nd	<0.001	N/A
2	102.7 ± 0.43	nd	<0.001	N/A
3	39.98 ± 0.085	nd	<0.001	N/A
4	253.7 ± 5.1	152.6 ± 2.7	<0.001	1.7 ± 0.02
5	133.8 ± 0.51	nd	<0.001	N/A
6	257.6 ± 0.34	nd	<0.001	N/A
7	143.8 ± 0.78	nd	<0.001	N/A
8	41.51 ± 0.10	nd	<0.001	N/A
9	4.015 ± 0.020	nd	<0.001	N/A
10	18.45 ± 0.085	nd	<0.001	N/A
11	26.45 ± 0.29	nd	<0.001	N/A
12	33.74 ± 0.31	nd	<0.001	N/A
13	32.54 ± 0.13	nd	<0.001	N/A
14	26.39 ± 0.29	nd	<0.001	N/A
15	48.41 ± 0.52	nd	<0.001	N/A
16	24.23 ± 0.034	nd	<0.001	N/A
17	2065 ± 19	7.723 ± 1.2	<0.001	280 ± 42

The ratios of DMAC to BSA results also vary significantly among samples within each category (Table 3.6) ($p < 0.0001$). Overall, the lowest ratio of DMAC to BSA results was found in pomegranate juice (< 1), and the highest ratio in cider sample 17 (280). For pomegranate juice, the BSA result was much higher than that obtained by DMAC. Relative to other fruit juices evaluated in this study, pomegranate juice is unique due to its high content of high-molecular-weight flavanols (Akhtar, Ismail, Fraternale, & Sestili, 2015). High molecular weight flavanols are quantified by BSA, but give lower responses by DMAC compared to flavanols of

low molecular weight (Wang et al., 2016). This is a likely explanation for the very low ratio of DMAC to BSA results for pomegranate juice. For cider sample 17, made from Hewe's Crab apples, the high ratio is likely due to the very high concentration of flavanols detected by DMAC. It is interesting to note that the apple juice made from the crab apple cultivar Snowdrift also had an extremely high concentration of flavanols detected by DMAC. However, the Snowdrift juice sample had a much lower ratio of DMAC to BSA results because it also contained a very high value of flavanols as detected by BSA precipitation (2433 mg/L catechin equivalents, the second highest value observed in this study after pomegranate), reflecting the uniquely high polyphenol content of certain crab apples.

The variation in ratios of DMAC to BSA results (Figure 3.2A and 3.2B) is likely due to differences in polyphenol composition among the samples. For DMAC, DP of flavanols and flavanol linkage influence the quantitative results (Wang et al., 2016) (Supplementary Information Figure 3.2). For example, monomers give higher responses than oligomers by DMAC (Prior et al., 2010; Wallace & Giusti, 2010; Wang et al., 2016). For BSA, precipitates form between BSA and flavanols with DP ≥ 3. Furthermore, the ability of flavanols to precipitate BSA increases with increasing polymer size. Only 12.4% of trimers and 23.9% of tetramers are precipitated by BSA (Harbertson et al., 2014). Concentrations of ten polyphenols with DP 1 to 4 were quantified in a subset of our samples, listed in Table 3.5. Monomers (catechin and epicatechin) and dimers (PC B1, B2, and B5) represent the majority of flavanols, while oligomers (PC C1 with DP 3, and Cinn A2 with DP 4) are present only in trace amounts in all samples except for Snowdrift apple juice and cider made from Hewe's Crab, both of which are genetically different from the *Malus* × *domestica* cultivars included in this study. With the majority of polyphenols detected being monomers, dimers, and non-flavanol compounds, it is not surprising that flavanols are not detectable by BSA in many cider samples. Although not conducted in this study, a thiolysis method (Neilson et al., 2016) could be used to measure the mean DP of each sample to provide more insight into the relationship of flavanol DP and the results of these methods.



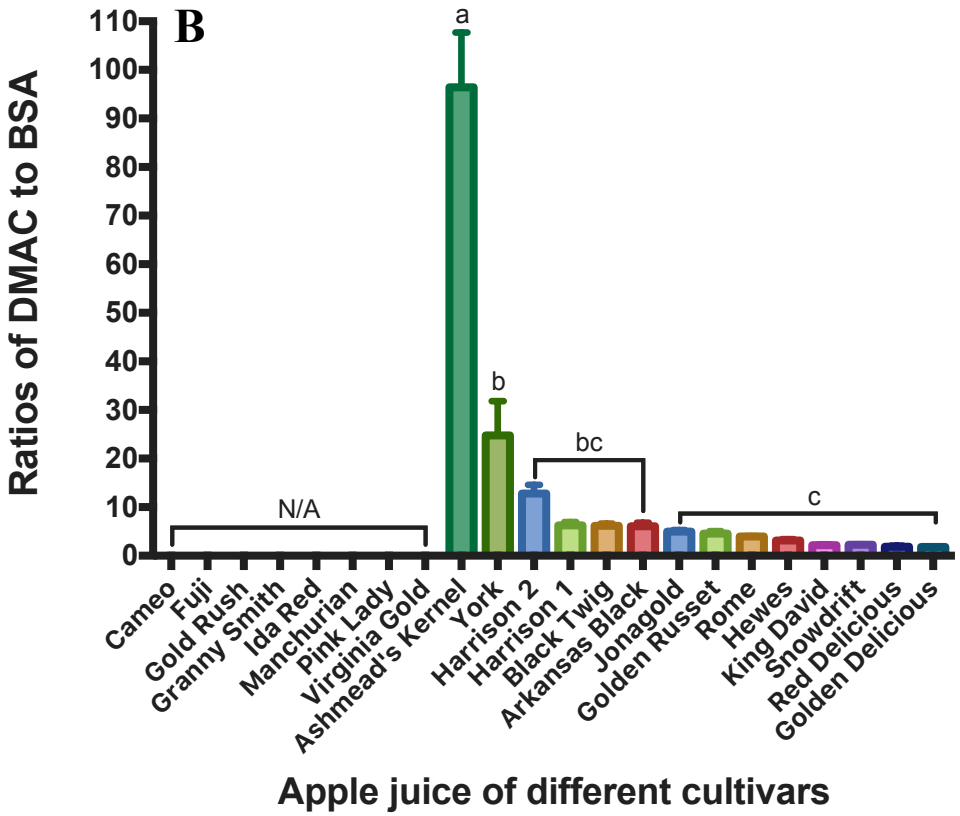


Figure 3. 2. Multiple comparisons of ratios of DMAC to BSA results among fruit juice samples (A) and apple juice of different cultivars (B). Bars represent the mean and error bars represent the SEM for n = 3 replicates. Lower case letters represent significant significant differences among the means. Significance was defined as $p < 0.05$. Many ratios of DMAC to BSA results could not be calculated (N/A) due to the non-detectable concentrations of flavanols by the BSA method being counted as zero for the ratio calculation.

3.4 Conclusion

L-P is preferable over F-C for samples of high polyphenol content with potassium metabisulfite and ascorbic acid present, despite the lack of accuracy observed for L-P. DMAC is more specific than BSA, and provides more accurate results for samples in which the majority of flavanols are monomers and dimers, even though BSA may better predict astringency. Additionally, ratios of results by L-P to F-C, and ratios of results by DMAC to BSA vary greatly, likely due to distinct polyphenol composition among samples. The results of this study provide a basis by which to make informed comparisons of polyphenol or flavanol concentrations obtained using the four methods evaluated. Furthermore, our results provide a basis for selection of the most appropriate of the four methods evaluated for a given application and fruit juice or beverage sample type.

3.5 Acknowledgement

The authors appreciate the apples donated by Silver Creek Orchards and Albemarle Ciderworks and for the ciders provided by Albemarle Ciderworks.

3.6 Author contributions

Sihui Ma designed the study, collected the data, interpreted the results and drafted the manuscript. Cathlean Kim assisted in data collection. Laura E. Griffin collected data in Supplementary Information Figure 3.2 and reviewed the manuscript. Andrew P. Neilson, Gregory M. Peck, Sean F. O'Keefe, and Amanda C. Stewart assisted in experimental design and revised the manuscript.

3.7 References

Akhtar, S., Ismail, T., Fraternale, D., & Sestili, P. (2015). Pomegranate peel and peel extracts: Chemistry and food features. Food Chemistry, 174, 417–425. https://doi.org/10.1016/J.FOODCHEM.2014.11.035

Aleixandre-Tudo, J. L., Buica, A., Nieuwoudt, H., Aleixandre, J. L., & du Toit, W. (2017). Spectrophotometric analysis of phenolic compounds in grapes and wines. Journal of Agricultural and Food Chemistry, 65(20), 4009–4026. https://doi.org/10.1021/acs.jafc.7b01724

Anastasiadi, M., Mohareb, F., Redfern, S. P., Berry, M., Simmonds, M. S. J., & Terry, L. A. (2017). Biochemical profile of heritage and modern apple cultivars and application of machine learning methods to predict usage, age, and harvest season. Journal of Agricultural and Food Chemistry, 65(26), 5339–5356. https://doi.org/10.1021/acs.jafc.7b00500

AOAC. (1995). Preparation and standardization of potassium permanganate solution, AOAC Method 940.35. Association of Official Analytical Chemists (AOAC) International.

Barua, D. N., & Roberts, E. A. (1940). Methods for the volumetric estimation of tea tannin in green-leaf and black tea. A new alkaline permanganate method. The Biochemical Journal, 34(12), 1524–1531. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16747284

Blainski, A., Lopes, G., & de Mello, J. (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from Limonium Brasiliense L. Molecules, 18(6), 6852–6865. https://doi.org/10.3390/molecules18066852

Bordenave, N., Hamaker, B. R., & Ferruzzi, M. G. (2014). Nature and consequences of non-covalent interactions between flavonoids and macronutrients in foods. Food and Function, 5(1), 18–34. https://doi.org/10.1039/c3fo60263j

Bors, W., & Michel, C. (2002). Chemistry of the antioxidant effect of polyphenols. Annals of the New York Academy of Sciences, 957(1), 57–69. https://doi.org/10.1111/j.1749-6632.2002.tb02905.x

Boulet, J.-C., Trarieux, C., Souquet, J.-M., Ducasse, M.-A., Caillé, S., Samson, A., … Cheynier, V. (2016). Models based on ultraviolet spectroscopy, polyphenols, oligosaccharides and polysaccharides for prediction of wine astringency. Food Chemistry, 190, 357–363. https://doi.org/https://doi.org/10.1016/j.foodchem.2015.05.062

Bridle, P., & Timberlake, C. F. (1997). Anthocyanins as natural food colours—selected aspects. Food Chemistry, 58(1–2), 103–109. https://doi.org/10.1016/S0308-8146(96)00222-1

Brossaud, F., Cheynier, V., & Noble, A. C. (2001). Bitterness and astringency of grape and wine polyphenols. Australian Journal of Grape and Wine Research, 7(1), 33–39. https://doi.org/10.1111/j.1755-0238.2001.tb00191.x

Budavari, S. (Ed.). (1996). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co., Inc.

Celeste, M., Tomas, C., Cladera, A., Estela, J. M., & Cerda, V. (1993). Thermometric titration of polyhydric phenols. Application to the determination of the tannin content of wines. Analyst, 118(7), 895–898. https://doi.org/10.1039/AN9931800895

Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). Thorough study of reactivity of various compound classes toward the Folin−Ciocalteu reagent. Journal of Agricultural and Food Chemistry, 58(14), 8139–8144. https://doi.org/10.1021/jf1005935

Ewing, B. L., Peck, G. M., Ma, S., Neilson, A. P., & Stewart, A. C. (2019). Management of apple maturity and postharvest storage conditions to increase polyphenols in cider. HortScience, 54(1), 143–148. https://doi.org/10.21273/HORTSCI13473-18

Feliciano, R. P., Shea, M. P., Shanmuganayagam, D., Krueger, C. G., Howell, A. B., & Reed, J. D. (2012). Comparison of isolated cranberry (Vaccinium macrocarpon Ait.) proanthocyanidins to catechin and procyanidins A2 and B2 for use as standards in the 4-(Dimethylamino)cinnamaldehyde assay. Journal of Agricultural and Food Chemistry, 60(18), 4578–4585. https://doi.org/10.1021/jf3007213

Food Chemicals Codex. (2019) (11th ed.). United States Pharmacopeia.

Hagerman, A. E., & Butler, L. G. (1978). Protein precipitation method for the quantitative determination of tannins. Journal of Agricultural and Food Chemistry, 26(4), 809–812. https://doi.org/10.1021/jf60218a027

Harbertson, J. F., Kennedy, J. A., & Adams, D. O. (2002). Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. American Journal of Enology and Viticulture, 53(1), 54. Retrieved from http://www.ajevonline.org/content/53/1/54.abstract

Harbertson, J. F., Kilmister, R. L., Kelm, M. A., & Downey, M. O. (2014). Impact of condensed tannin size as individual and mixed polymers on bovine serum albumin precipitation. Food Chemistry, 160, 16–21. https://doi.org/10.1016/J.FOODCHEM.2014.03.026

Hyman, M. R., Sansome-Smith, A. W., Shears, J. H., & Wood, P. M. (1985). A kinetic study of benzene oxidation to phenol by whole cells of Nitrosomonas europaea and evidence for the further oxidation of phenol to hydroquinone. Archives of Microbiology, 143(3), 302–306. https://doi.org/10.1007/BF00411254

Lea, A. G. H., & Arnold, G. M. (1978). The phenolics of ciders: Bitterness and astringency. Journal of the Science of Food and Agriculture, 29(5), 478–483. https://doi.org/10.1002/jsfa.2740290512

Lowenthal. (1877). Uber die Bestimmung des Gerbstoffs. Analytical and Bioanalytical Chemistry (Previous as Zeitschrift Für Analytische Chemie), 16, 33–48.

Ma, S., Neilson, A., Lahne, J., Peck, G., O'Keefe, S., Hurley, E. K., … Stewart, A. (2018). Juice clarification with pectinase reduces yeast assimilable nitrogen in apple juice without affecting the polyphenol composition in cider. Journal of Food Science, 83, 2772–2781. https://doi.org/doi:10.1111/1750-3841.14367

Margraf, T., Karnopp, A. R., Rosso, N. D., & Granato, D. (2015). Comparison between Folin-Ciocalteu and Prussian Blue assays to estimate the total phenolic content of juices and teas using 96-well microplates. Journal of Food Science, 80(11), C2397–C2403. https://doi.org/10.1111/1750-3841.13077

Mercurio, M. D., & Smith, P. A. (2008). Tannin quantification in red grapes and wine: Comparison of polysaccharide- and protein-based tannin precipitation techniques and their ability to model wine astringency. Journal of Agricultural and Food Chemistry, 56(14), 5528–5537. https://doi.org/10.1021/jf8008266

Neilson, A. P., O'Keefe, S. F., & Bolling, B. W. (2016). High-molecular-weight proanthocyanidins in foods: Overcoming analytical challenges in pursuit of novel dietary

bioactive components. Annual Review of Food Science and Technology, 7(1), 43–64. https://doi.org/10.1146/annurev-food-022814-015604

Nielsen, S. S. (Ed.). (2010). Food Analysis (4th ed.). Springer.

Nunes, C. A., Alvarenga, V. O., de Souza Sant'Ana, A., Santos, J. S., & Granato, D. (2015). The use of statistical software in food science and technology: Advantages, limitations and misuses. Food Research International, 75, 270–280. https://doi.org/http://dx.doi.org/10.1016/j.foodres.2015.06.011

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity, 2(5), 270–278. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2835915/

Payne, M. J., Hurst, W. J., Stuart, D. A., Ou, B., Fan, E., Ji, H., & Kou, Y. (2010). Determination of total procyanidins in selected chocolate and confectionery products using DMAC. Journal of AOAC International, 93(1), 89–96.

Prior, R. L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M. J., & Reed, J. (2010). Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. Journal of the Science of Food and Agriculture, 90(9), 1473–1478. https://doi.org/10.1002/jsfa.3966

Rothwell, J. A., Perez-Jimenez, J., Neveu, V., Medina-Remón, A., M'Hiri, N., García-Lobato, P., Manach C, Knox C, Eisner R, Wishart DS, Scalbert, A. (2013). Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Database, 2013, bat070-bat070. https://doi.org/10.1093/database/bat070

Shrivastava, A., & Gupta, V. (2011). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles of Young Scientists, 2(1), 21–25. https://doi.org/10.4103/2229-5186.79345

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In Methods in Enzymology (Vol. Volume 299, pp. 152–178). Academic Press. https://doi.org/http://dx.doi.org/10.1016/S0076-6879(99)99017-1

Smit, C. J. B., Joslyn, M. A., & Lukton, A. (1955). Determination of tannins and related polyphenols in foods. Analytical Chemistry, 27(7), 1159–1162.

Spanos, G. A., & Wrolstad, R. E. (1992). Phenolics of apple, pear, and white grape juices and their changes with processing and storage. A review. Journal of Agricultural and Food Chemistry, 40(9), 1478–1487. https://doi.org/10.1021/jf00021a002

Sun-Waterhouse, D. (2011). The development of fruit-based functional foods targeting the health and wellness market: a review. International Journal of Food Science & Technology, 46(5), 899–920. https://doi.org/10.1111/j.1365-2621.2010.02499.x

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. Nutrients, 2(12), 1231–1246. https://doi.org/10.3390/nu2121231

U.S. Food and Drug Administration. (2015). Analytical procedures and methods validation for drugs and Biologics. Guidance for Industry.

USDA Food Composition Database. (2019). Retrieved February 24, 2019, from https://ndb.nal.usda.gov/ndb/search/list?home=true

Wallace, T. C., & Giusti, M. M. (2010). Evaluation of parameters that affect the 4-dimethylaminocinnamaldehyde assay for flavanols and proanthocyanidins. Journal of Food Science, 75(7), C619–C625. https://doi.org/10.1111/j.1750-3841.2010.01734.x

Wang, Y., Singh, A. P., Hurst, W. J., Glinski, J. A., Koo, H., & Vorsa, N. (2016). Influence of degree-of-polymerization and linkage on the quantification of proanthocyanidins using 4-dimethylaminocinnamaldehyde (DMAC) assay. Journal of Agricultural and Food Chemistry, 64(11), 2190–2199. https://doi.org/10.1021/acs.jafc.5b05408

Weber, F., Schulze-Kaysers, N., & Schieber, A. (2014). Chapter 15 - Characterization and quantification of polyphenols in fruits - Watson, Ronald Ross. In Polyphenols in Plants (pp. 293–304). San Diego: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-397934-6.00015-2

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., & Nury, F. S. (1999). Sulfur dioxide and ascorbic acid. In Wine Analysis and Production (pp. 178–191). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4757-6967-8_10

3.8 Supporting information available

Supplementary Information Table 3.1. Sample number of commercial cider samples and their corresponding names.

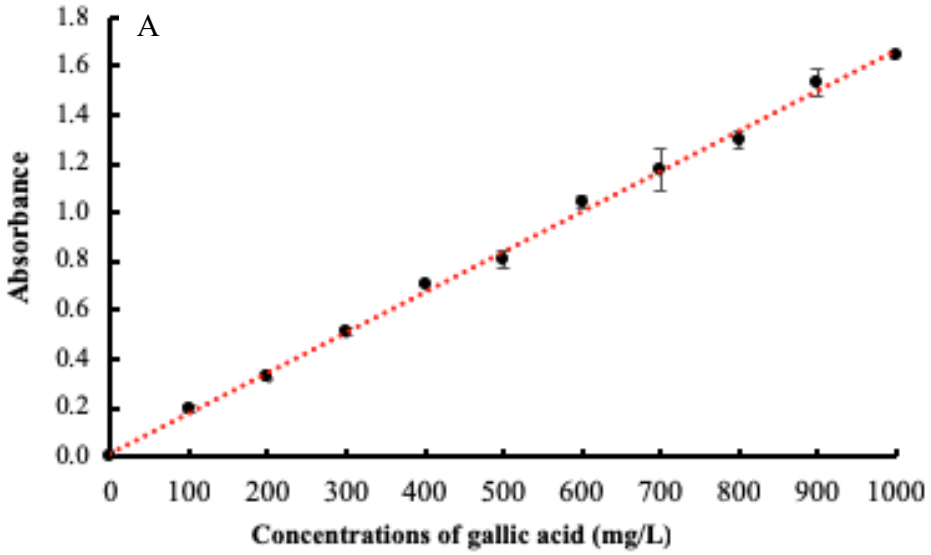
Sample number	Sample name
1	Bold Rock Hard Cider-Granny Smith Cider
2	Bold Rock Hard Cider Premium Dry
3	Castle Hill Cider Serendipity
4	Cidre de Normandie Brut brewed by Cidrerie Daufresne
5	Foggy Ridge Cider First Fruit
6	Foggy Ridge Cider Serious Cider
7	Foggy Ridge Cider Stayman Winesap
8	Potter’s Craft Cider Farmhouse Saison
9	The Standard Cider Company True Thirst Hard Apple Cider
10	Winchester Ciderworks Malice Hard Cider
11	Albemarle Ciderworks Arkansas Black
12	Albemarle Ciderworks Black Twig
13	Albemarle Ciderworks Gold Rush
14	Albemarle Ciderworks Harrison
15	Albemarle Ciderworks Jupiter's Legacy
16	Albemarle Ciderworks Red Hill
17	Albemarle Ciderworks Virginia Hewe's Crab

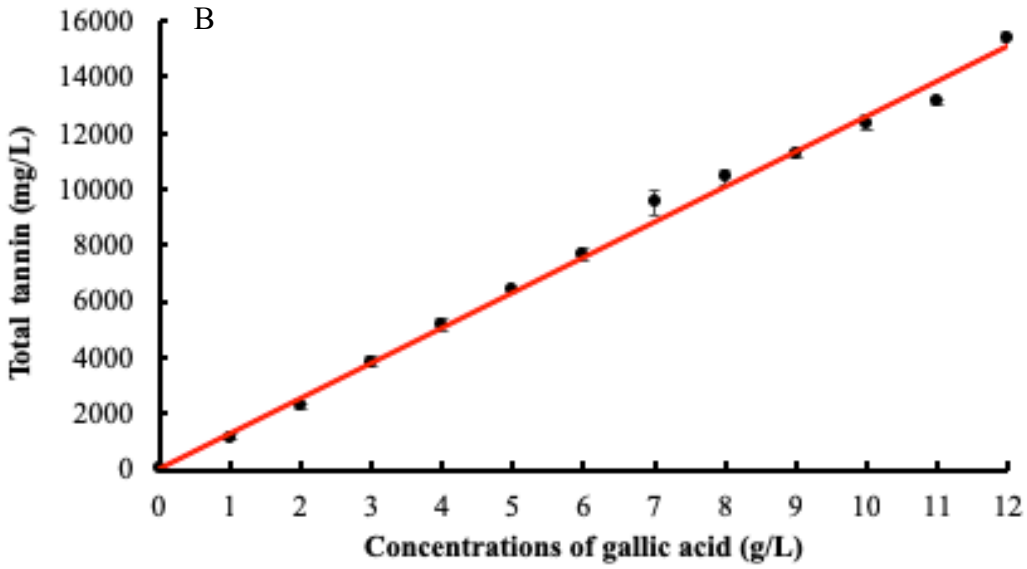
Supplementary Information Table 3.2. UPLC gradients for the separation of individual polyphenols

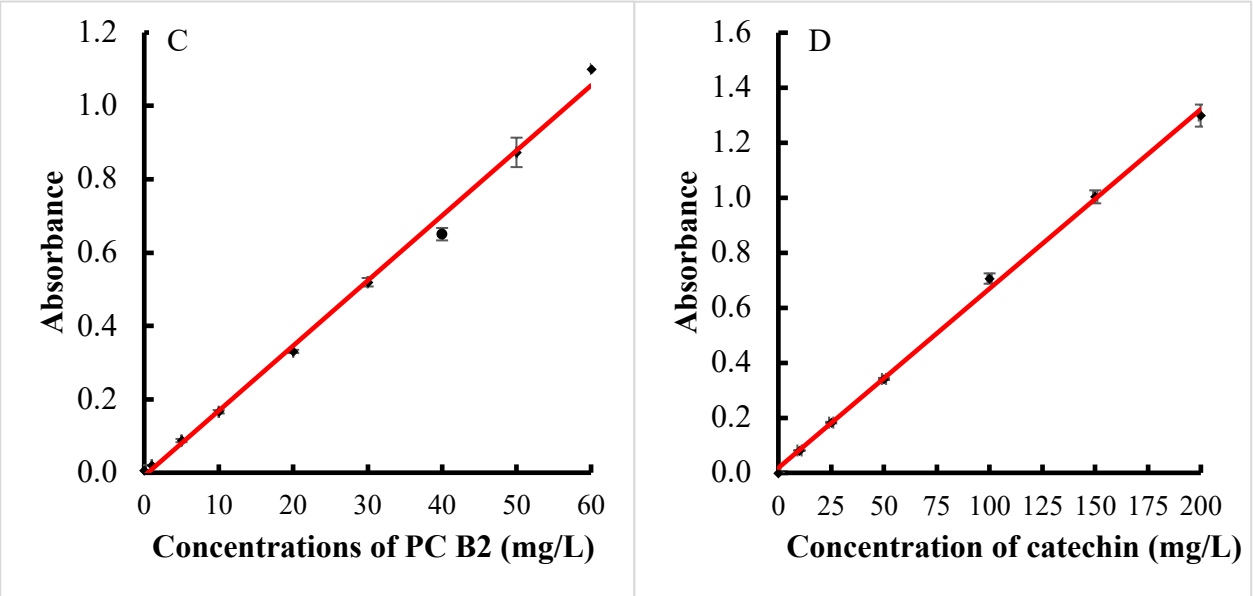
Time/minute	%A	%B
Initial	95	5
0.5	95	5
6.5	65	35
7.5	65	35
8.6	20	80
8.7	20	80
14.6	95	5

Supplementary Information Table 3.3. Retention times, molecular weights, and SIR channels for individual polyphenol compounds

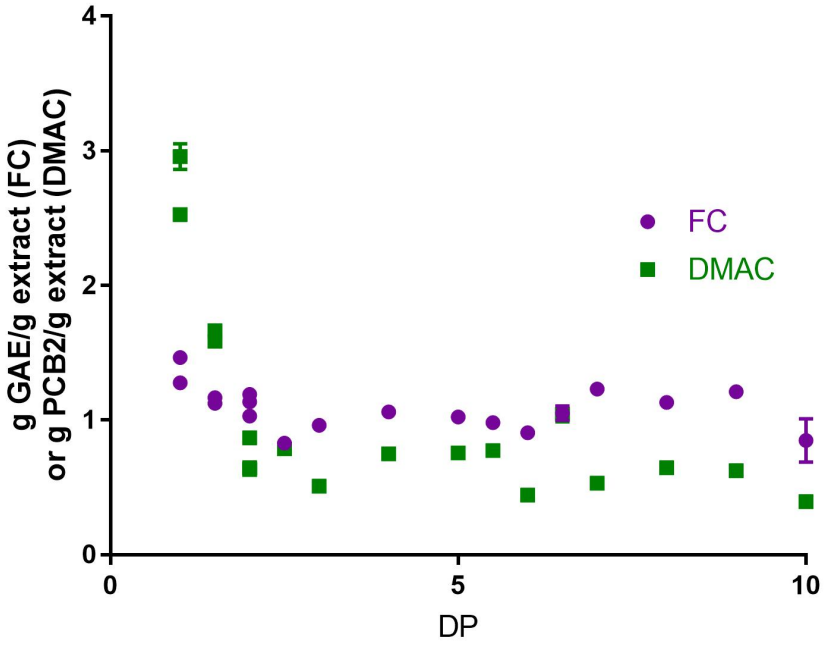
Compound	Retention time (min)	Molecular Weight (g/mol)	SIR (m/z)
PC B1	2.57	578.07	577.13
Catechin	2.89	290.09	289.09
Chlorogenic acid	2.93	354.31	353.06
PC B2	3.28	578.04	577.13
Epicatechin	3.55	290.09	289.09
PC C1	3.73	866.77	865.77
Cinn A2	3.89	1155.04	1154.04
PC B5	4.65	578.13	577.13
Quercetin	6.57	302.23	301.01
Phloretin	7.34	274.26	273.05







Supplementary Figure 3.1. Analytical curves for (A) F-C method; (B) L-P method (not used in quantification); (C) DMAC method; and (D) BSA precipitation method. Data points represent the mean for $n = 3$ replicates and error bars represent SEM.



Supplementary Figure 3.2. F-C and DMAC assay response as a function of flavanol DP. PC standards were analyzed at 0.1 mg/mL for FC and at 0.01 mg/mL for DMAC. Monomers though decamers were analyzed for each assay. 1:1 mixtures of EC + PCB_1, PCB_2 + PCC_1, PC pentamer + PC hexamer, and PC hexamer + PC heptamer were also analyzed to look at the assay responses of mDP 1.5, 2.5, 5.5, and 6.5, respectively. Each data point represents the means (n = 3) ± SEM for each PC standard in each assay.

CHAPTER 4 Contribution of individual polyphenol compounds and potential interfering compounds to the overall results of polyphenol quantification by spectrophotometric methods

Submitted to Journal of Food Science, Food Chemistry Section

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ABSTRACT

The contribution of individual polyphenol compounds (catechin, epicatechin, PC B2, PC pentamer, chlorogenic acid, phloretin, and quercetin) to polyphenol and flavanol quantification results using Prussian blue, Folin-Ciocalteu, 4-dimethylaminocinnamaldehyde (DMAC) and bovine serum albumin (BSA) protein precipitation methods were assessed. Additionally, the contribution and interactions of individual polyphenol compounds and potentially interfering compounds likely to be found in fruit and fruit products were evaluated using a full factorial design. Individual polyphenols evaluated were catechin (0.2, 30, 100 mg/L), PC B2 (1, 30, 100 mg/L), and chlorogenic acid (5, 200, 500 mg/L), and potential interfering compounds evaluated were ascorbic acid (1, 250, 2000 mg/L), glucose (10, 50, 200 g/L), and SO_2 (10, 100, 350 mg/L). The contribution of individual polyphenol compounds to common quantification methods differed up to thirteen-fold. Interactions among the polyphenol compounds catechin, PC B2, and CGA significantly impacted the quantification results of the four methods evaluated. Furthermore, ascorbic acid, glucose, and SO_2 interfered with the quantification results. The standardized coefficient (β) for all factors and interactions of polyphenol compounds varied from 0.347 to 129, and from near 0 to -46.8 for all factors and interactions of interfering compounds. Taken together, these findings illustrate that the choice of standards and the polyphenol and matrix composition of a given sample can cause significant disparity among the quantification results of polyphenols.

PRACTICAL APPLICATION

This study evaluated the contribution of individual polyphenol compounds and matrix constituents commonly present in fruit juice to four common polyphenol quantification methods: Prussian Blue, Folin-Ciocalteu, DMAC and BSA methods. For the same concentrations of two structurally different polyphenols, different results were obtained. Interaction effects between polyphenols and between matrix constituents were also observed. Therefore, the polyphenol standard used and sample matrix composition can cause significant disparity among the quantification results of polyphenols. This information should be helpful to researchers or producers interested in measuring or comparing values of polyphenol concentrations in fruit products.

4.1 Introduction

Quantification of total polyphenols is of research interest because of both the health benefits and sensory characteristics of polyphenols. The Prussian blue (P-B) and Folin-Ciocalteu (F-C) are two popular spectrophotometric methods for quantification of total polyphenols in fruits and fruit-based products (Margraf, Karnopp, Rosso, & Granato, 2015). A subgroup of polyphenols, flavanols, draws attention because they contribute important sensory characteristics including bitterness and astringency to fruits and fruit-based products. The 4-dimethylaminocinnamaldehyde (DMAC) and bovine serum albumin (BSA) protein precipitation methods are common spectrophotometric method for the quantification of total flavanols (Harbertson, Kennedy, & Adams, 2002; Wallace & Giusti, 2010).

The structures of polyphenol compounds vary tremendously, among the more than 8,000 known polyphenol compounds (Tsao, 2010). The four aforementioned analytical methods are all quantitative spectrophotometric methods, and the results are necessarily dependent on the chosen polyphenol standards. Gallic acid is the most commonly used standard in the P-B and F-C method (Margraf et al., 2015), while procyanidins (PC) dimers (such as A2, B1, and B2) are the most common standards for the DMAC and BSA precipitation methods (Harbertson, Kilmister, Kelm, & Downey, 2014; Wang et al., 2016). However, the structure of a given polyphenol is known to influence its absorbance response in spectrophotometric methods. For example, for the P-B method, the molar absorptivity of gallic acid is more than twice that of epicatechin (Budini, Tonelli, & Girotti, 1980). Differences in absorptivity have been observed among several polyphenol compounds, including gallic acid, catechin, pyrogallol and tannic acid using the F-C method (Blainski, Lopes, & de Mello, 2013). For the DMAC method, different absorption responses were observed for PC dimers and trimers with different inter-flavan linkages (Wang et al., 2016). Additionally, the type of PCs (A-type or B-type) and degree of polymerization (DP) also impacts the absorbance intensity for the DMAC method. In the BSA method, the binding ability between the polyphenol compounds and BSA was greatly influenced by the structure and molecular weights of the polyphenols (Harbertson et al., 2002). Overall, the choice of standards and the polyphenol composition would impact the results of polyphenol quantification using these methods. Using a polyphenol standard which is chemically different than the most prevalent compounds in a given sample would be expected to lead to inaccurate quantification. But few studies have evaluated the quantitative contribution of common polyphenol compounds present in fruit juices to each of these polyphenol quantification methods. Studying the contribution of individual polyphenol compounds to the quantitative results of these methods

will help us understand the relationship between polyphenol structure and quantification via these four spectrophotometric methods.

In general, the functionality of polyphenols may not be based solely on the effect of individual polyphenol compounds. For example, synergistic effects on inhibition of tumor growth have been found among tea polyphenols (George et al., 2011), and synergistically enhanced anti-carcinogenic effect was found between resveratrol and both ellagic acid and quercetin (Mertens-Talcott & Percival, 2005), while negative synergistic effects of the antiradical activity among catechin, resveratrol, and quercetin have been reported (Pinelo, Manzocco, Nuñez, & Nicoli, 2004). Such synergistic interactions among polyphenols could also influence the results obtained using the above methods for polyphenol quantification, although this has not yet been demonstrated. Assessing the extent to which such polyphenol interactions influence the quantification of total polyphenols and total PCs by these common analytical methods can provide relevant context for the interpretation of polyphenol concentration data and interpretation of studies evaluating polyphenol bioactivity in response to different polyphenol containing constituents, especially those with complex matrices such as whole foods or extracts of foods.

Polyphenols are present in fruits and vegetables along with other matrix constituents such as ascorbic acid (AA) and sugars (Le Bourvellec & Renard, 2012). They can also be affected by ingredients used during the processing of fruits and vegetables. For example, grape and apple polyphenols can interact with SO_2 in wine and cider making, respectively (Lea & Drilleau, 2003; Zoecklein, Fugelsang, Gump, & Nury, 1999). The ability of polyphenols to interact and bind with matrix constituents in food systems is associated with their health benefits and the sensory properties of the foods. Polyphenol structure, including DP, the number of external hydroxyl groups, and the number of terminal galloyl groups can impact the covalent and non-covalent binding ability of polyphenols (Le Bourvellec & Renard, 2012).

Additionally, various compounds from the food matrix or introduced to the food system during processing, including AA, sugars, SO_2, interfered with the quantification results of total polyphenols and total PCs. AA interfered with the P-B method (Margraf et al., 2015), while AA, sugars, SO_2 interfere with the F-C method (Everette et al., 2010). The DMAC method is very specific to PCs, thus non-target compounds in the sample matrix including AA, black raspberry anthocyanins, caffeine, chlorogenic acid (CGA), citric acid, gallic acid, polyvinylpyrrolidone, and rutin, do not contribute to the color development (Wallace & Giusti, 2010). However, other compounds present in the food matrix having structures of di- or tri-hydroxyl phenolic rings may still interfere with the quantification results (Wallace & Giusti, 2010). In the BSA precipitation method, the binding of BSA is not specific to polyphenols (Hagerman & Butler, 1978). The interaction between polyphenols and BSA was through hydrogen bonding and hydrophobic interactions, therefore other compounds in sample matrix which form covalent and non-covalent bonds with polyphenols or BSA could interfere with binding between polyphenols and BSA (Bordenave, Hamaker, & Ferruzzi, 2014). Because of the above potential interference from the sample matrix, there is a need to systematically quantify the contribution of the interfering compounds and the interaction among polyphenols and these compounds.

The objectives of this study are 1) to study the contribution of individual polyphenol compounds to the quantification results by P-B, F-C, DMAC, and BSA methods; 2) to study the interaction effects of individual polyphenol compounds to the quantification results by these methods; 3) to study the interaction effects of potentially interfering compounds and polyphenol compounds to the quantification results by these methods. The hypotheses are 1) individual

polyphenol compounds respond differently to these methods; 2) individual polyphenol compounds bring interaction effects to the quantification results by these methods; 3) potential interfering compounds and polyphenol compounds cause interaction effects to the quantification results by these methods.

4.2 Materials and methods

4.2.1 Chemicals and Standards

F-C's phenol reagent, gallic acid, sodium carbonate, PC B2, (+)-catechin, (−)-epicatechin, CGA, quercetin, phloretin, DMAC, ferric chloride hexahydrate, potassium ferricyanide(III) and sodium oxalate were purchased from Sigma-Aldrich (St. Louis, MO, USA); Analytical standard PC pentamer was purchased from Planta Analytica (Danbury, CT, USA); L-ascorbic acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA); BSA Fraction V was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

4.2.2 Analytical methods

4.2.2.1 P-B method

The P-B method was carried out according to the procedures described by Margraf, et al with modifications (Margraf et al., 2015). Reagent 1 was prepared by diluting ferric chloride hexahydrate in 0.01 mol/L HCl to 0.50 mmol/L. Standard solutions of gallic acid with concentrations of 0, 5, 10, 20, 30, 40, 50, 100 mg/L was made with water. A 300 μL Reagent 1 and 300 μL of properly diluted sample or the gallic acid standard solutions were mixed and reacted for two minutes. A 300 μL 0.50 mmol/L potassium ferricyanide (III) aqueous solution was added into the mixture, and mixed. The absorbance was read at 725 nm (GENESYS 10S UV-Vis spectrophotometer, Thermo Scientific, Waltham, MA, USA) after 15 minutes of incubation in dark.

4.2.2.2 F-C method

The F-C method was carried out following the procedures (Ma et al., 2018). The F-C's phenol reagent (2 mol/L) was diluted with water to 0.2 mol/L. Gallic acid standard solutions were prepared in the concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 g/L with water. A 50 μL of the appropriately diluted sample/gallic acid standard solution, 450 μL of water, and 1.25 mL of the 0.2 mol/L F-C's phenol reagent were added in cuvettes and mixed, following the addition of one mL of 75 g/L Na_2CO_3 aqueous solution. Absorbance was read at 765 nm on samples and standards after two-hour incubation under room temperature in dark. The concentration of total polyphenols was calculated based on the linear regression between absorbances vs. concentrations of gallic acid standard solutions. The results were expressed as mg/L of gallic acid equivalents.

4.2.2.3 DMAC method

The DAMC method was conducted as previously reported by Payne, *et al* (Payne et al., 2010). PC B2 standard solutions of 1, 5, 10, 20, 30, 40, 50 mg/L were made from combing the PC B2 stock solution (0.1 mg/mL in water) with water. A 200 μL of the appropriately diluted samples/standards was mixed with one mL of the 10 mg/L DMAC solution {DMAC was dissolved in acidified methanol [6 mL concentrated (36%) hydrochloric acid in 54 mL methanol]} in cuvettes. The absorbance was read at 640 nm (A). The concentration of total flavanols was calculated with A based on the linear regression conducted between A and the concentration of the PC B2 standard solutions. The results were expressed as mg/L of PC B2 equivalents.

4.2.2.4 BSA protein precipitation method

The BSA precipitation method was performed as reported by Harbertson, *et al* (Harbertson et al., 2002). Standard solutions of catechin of 0, 10, 25, 50, 100, 150 mg/L was made by combine catechin with water. A 500 µL appropriately diluted sample/standard was combined with 375 µL buffer [5% triethanolamine (v/v) and 10% sodium dodecyl sulfate (w/v)] and mixed. A 125 µL of the $FeCl_3$ reagent (0.01 mol/L $FeCl_3$ in 0.01 N HCl) was added and the absorbance was read at 510 nm after 10 min as A. The flavanol content for samples was obtained from the linear regression between A and the concentrations of the catechin standard solution. The results were reported in mg/L of catechin equivalents. The process of using BSA to precipitate polyphenols was not applied because of the limitation to obtain flavanols of DP > 4.

4.2.3 Dose-response curve

Individual polyphenol standards of catechin, epicatechin, PC B2, PC pentamer, CGA, phloretin, and quercetin were used to build the dose-response curve for each analytical method. Each standard was made in triplicate by combing the standards with water to achieve the concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8 ,9, 10 mg/L for the P-B method, concentrations of 0, 100, 200, 300, 400, 500 mg/L for the F-C method, concentrations of 0, 10, 20, 30, 40, 50 mg/L for the DMAC method, and concentrations of 0, 30, 60, 90, 120, 150 mg/L for the BSA precipitation method. Linear regressions were built between the mean absorbance and the concentrations of the individual polyphenol compounds, and R^2 and equations were obtained using Excel.

4.2.4 Contribution of individual polyphenol compounds and potential interfering compounds

4.2.4.1 Experimental design and sample preparation

To assess the contribution of individual polyphenol compounds, including catechin (0.2, 30, 100 mg/L), PC B2 (1, 30, 100 mg/L), and CGA (5, 200, 500 mg/L), and interactions thereof to the overall results of polyphenol quantification by P-B, F-C, DMAC, and BSA precipitation methods, twenty-seven different aqueous solutions were prepared in triplicate using a full-factorial design (Table 4.1). The concentrations of catechin, PC B2, and CGA were chosen based on the lowest, mean and highest values previously reported for fruit and fruit juice samples in the Phenol-Explorer database (Rothwell et al., 2013). After dissolving the phenolic compounds in DI water, the pH of each sample was adjusted to 2.5 using HCl.

Table 4. 1. Sample preparation for 27 samples to assess the interaction effects of catechin, PC B1, and CGA.

Sample number	Concentrations in mg/L		
	Catechin	PC B2	CGA
1	0.2	1	5
2	0.2	1	200
3	0.2	1	500
4	0.2	30	5
5	0.2	30	200
6	0.2	30	500
7	0.2	100	5
8	0.2	100	200
9	0.2	100	500
10	30	1	5
11	30	1	200
12	30	1	500

13	30	30	5
14	30	30	200
15	30	30	500
16	30	100	5
17	30	100	200
18	30	100	500
19	100	1	5
20	100	1	200
21	100	1	500
22	100	30	5
23	100	30	200
24	100	30	500
25	100	100	5
26	100	100	200
27	100	100	500

Similarly, to assess the effect of potentially interfering compounds including AA (1, 250, 2000 mg/L), glucose (10, 50, 200 g/L), and SO_2 (10, 100, 350 mg/L) to the overall results of polyphenol quantification by P-B, F-C, DMAC, and BSA precipitation methods, twenty-seven different aqueous model juice solutions were prepared in triplicate using a full-factorial design (Table 4.2). After dissolving the potentially interfering compounds in DI water, 30 mg/L catechin, 30 mg/L PC B2, and 200 mg/L CGA were added to each sample to approximate background levels of polyphenols in a typical fruit juice, and the pH was adjusted to 2.5 using HCl.

Table 4. 2. Sample preparation for 27 samples to assess the interaction effects of AA, glucose and SO_2

Sample number	Concentrations in mg/L		
	AA	Glucose	SO_2
1	1	10	10
2	1	10	100
3	1	10	350
4	1	50	10
5	1	50	100
6	1	50	350
7	1	200	10
8	1	200	100
9	1	200	350
10	250	10	10
11	250	10	100
12	250	10	350
13	250	50	10
14	250	50	100
15	250	50	350
16	250	200	10
17	250	200	100

18	250	200	350
19	2000	10	10
20	2000	10	100
21	2000	10	350
22	2000	50	10
23	2000	50	100
24	2000	50	350
25	2000	200	10
26	2000	200	100
27	2000	200	350

4.2.4.2 Statistical analysis

A three-way analysis of variance (ANOVA) with three factors (catechin, PC B2, and CGA) at three levels each (low, medium and high) with all interactions followed by Tukey's HSD multiple comparisons of means was performed to assess whether there were significant interaction effects among polyphenol compounds. A second three-way ANOVA with three factors (AA, glucose, and SO_2) at three levels each (low, medium and high) with all interactions followed by Tukey's HSD multiple comparisons of means was performed to assess whether there were significant interaction effects among the interfering compounds. Significance was defined as $p < 0.05$. To determine the contribution of each factor and interaction to the overall quantification results of each method, the standardized coefficients [a change in the independent variable measured in units of standard deviation (SD) caused by one SD change in one dependent variable holding all other variables constant] (Bring, 1994) and partial omega square (ω_p^2, indicator of effect size) were calculated (Lakens, 2013). Analysis was conducted using R Studio statistical software (version 1.1.383).

4.3 Results and discussion

4.3.1 Reactivity of individual polyphenol compounds

4.3.1.2 Reactivity of individual polyphenol compounds to the P-B and F-C methods

The dose-response curves built using individual polyphenol standards of catechin, epicatechin, PC B2, PC pentamer, CGA, phloretin, and quercetin for the P-B method are shown in Figure 4.1A. The chemical structure of the polyphenol compounds used in this study are shown in Figure 4.2. The R^2 and equations for the calibration curves are listed in Table 4.3. For all four spectrophotometric methods, the absorbances of the sample are directly related to the quantitative results of these methods according to the Beer-Lambert Law, which demonstrates the linear relationship between absorbance and concentration. The regression for all compounds tested using the P-B method were linear but the slopes of the curves were different. At the same concentration of 10 mg/L, the highest level tested in this study, quercetin had the highest absorbance, which was 13 times higher than the absorbance of phloretin. In the P-B method, polyphenols reduce the hexacyanoferrate (III) ion to hexacyanoferrate (II) ion, allowing it to then react with a ferric ion forming a complex of $Fe_4[Fe(CN)6]_3$ (Graham, 1992), which is blue in color. Calibration curves with different slopes were also reported by others for catechin and quercetin (Price & Butler, 1977). The differences in absorbance elicited by polyphenol compounds of the same concentration in the P-B assay may be due to differences in reducing power of the polyphenol compounds evaluated against the hexacyanoferrate (III) ion.

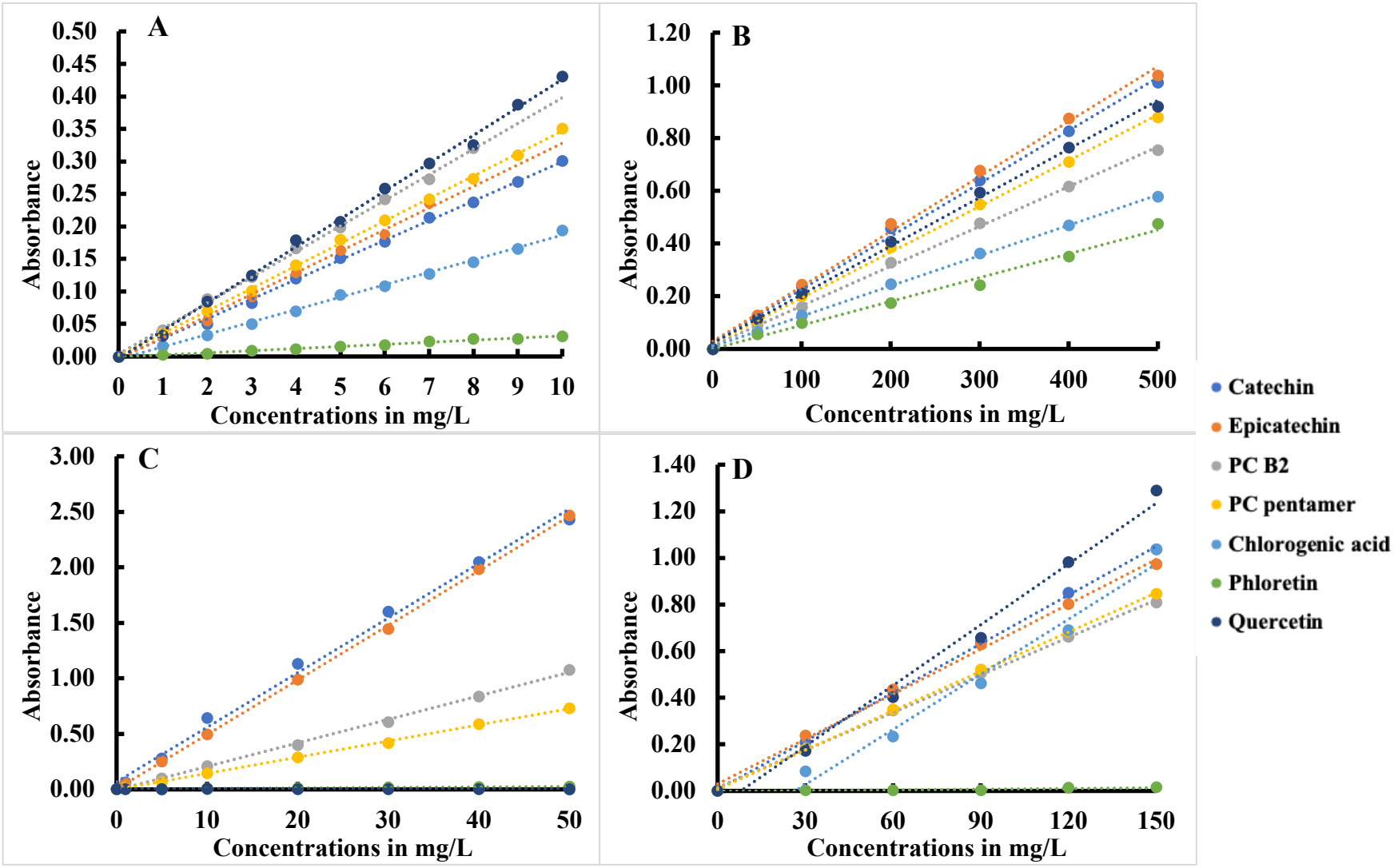
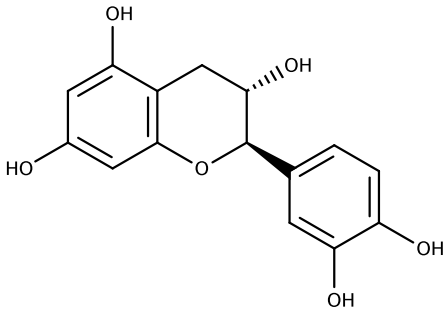
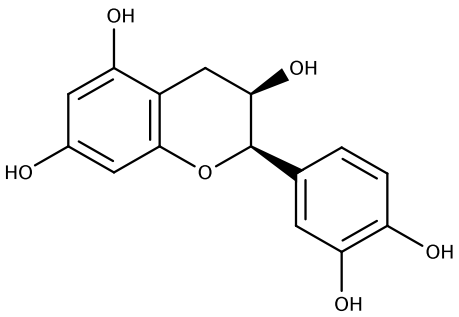


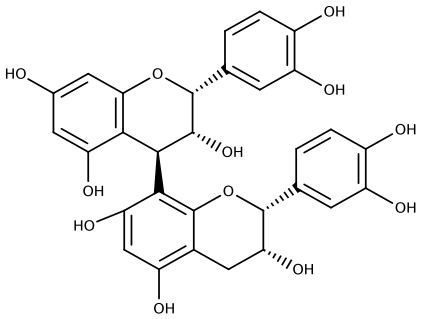
Figure 4. 1. Dose-response curves of four analytical methods (A) P-B, (B) F-C, (C) DMAC, and (D) BSA precipitation method. Each date point represents the mean of n = 3 replicates.



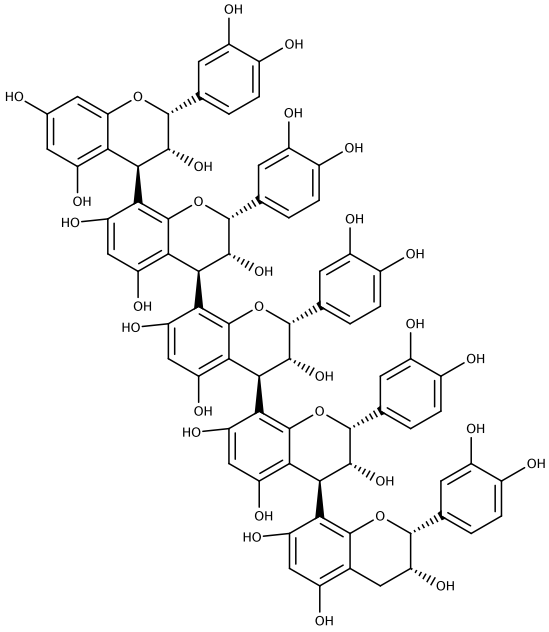
(A) (+)-Catechin



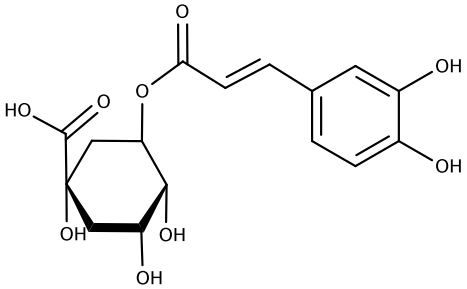
(B) (-)-Epicatechin



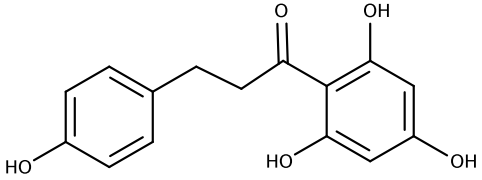
(C) PC B2



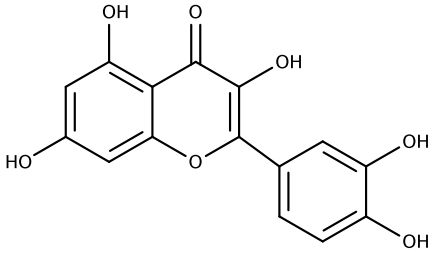
(D) PC pentamer (B type)



(E) CGA



(F) Phloretin



(G) Quercetin

Figure 4. 2. Chemical structures of (+)-catechin (A), (B) (–)epicatechin (B), PC B2 (C), PC pentamer (D), CGA (E), phloretin (F), and quercetin (G).

Table 4. 3. R^2 and equations of linear regression for polyphenol compounds using each method.

Compounds	Methods			
	P-B	F-C	DMAC	BSA
(+)-Catechin	$y = 0.0302x - 0.00228$ $R^2 = 0.998$	$y = 0.00201x + 0.0256$ $R^2 = 0.997$	$y = 0.0492x + 0.0678$ $R^2 = 0.994$	$y = 0.00697x + 0.00451$ $R^2 = 0.999$
(–)-Epicatechin	$y = 0.0404x - 0.0238$ $R^2 = 0.969$	$y = 0.00173x + 0.0198$ $R^2 = 0.998$	$y = 0.0146x - 0.00153$ $R^2 = 0.999$	$y = 0.00562x + 0.00695$ $R^2 = 0.999$
PC B2	$y = 0.0432x - 0.00717$ $R^2 = 0.989$	$y = 0.00151x + 0.0124$ $R^2 = 0.999$	$y = 0.0212x - 0.00567$ $R^2 = 0.999$	$y = 0.00537x + 0.0146$ $R^2 = 0.999$
PC pentamer	$y = 0.0346x + 0.000924$ $R^2 = 0.999$	$y = 0.00209x + 0.0283$ $R^2 = 0.996$	$y = 0.0492x + 0.00272$ $R^2 = 0.999$	$y = 0.00643x + 0.0300$ $R^2 = 0.997$
CGA	$y = 0.0191x - 0.00417$ $R^2 = 0.997$	$y = 0.00115x + 0.00900$ $R^2 = 0.999$	$y = -2.17\times10^{-5}x + 0.00346$ $R^2 = 0.0947$	$y = 0.00788x - 0.209$ $R^2 = 0.979$
Phloretin	$y = 0.00325x - 0.000894$ $R^2 = 0.991$	$y = 0.000903x - 0.000244$ $R^2 = 0.990$	$y = 4.30\times10^{-4}x + 0.00432$ $R^2 = 0.947$	$y = 9.84\times10^{-5}x - 0.00171$ $R^2 = 0.799$
Quercetin	$y = 0.0429x - 0.00311$ $R^2 = 0.998$	$y = 0.00184x + 0.0219$ $R^2 = 0.998$	$y = -1.82\times10^{-5}x + 0.00506$ $R^2 = 0.0592$	$y = 0.00869x - 0.0687$ $R^2 = 0.988$

Similarly, the regression for all compounds tested using the F-C method were linear but the slopes of the curves were different (Figure 4.1B). At the same concentration of 500 mg/L, the highest level evaluated in this study, epicatechin had the highest absorbance, which was 2 times higher than the absorbance of phloretin. The F-C method is another spectrophotometric method based on a redox reaction. In this method, polyphenols reduce the F-C's phenol reagent, which contains yellow heteropolyphosphotungstate-molybdate complexes [Mo(VI)], to a series of mixed complexes, such as $[(PMoW_{11}O_{40})^{4-}$ (Mo(V)), which are blue in color. Different reactivity of various polyphenol compounds in the F-C assay (quercetin, CGA, and gallic acid) was also reported by Everette *et al* (Everette et al., 2010), in agreement with our results. The differences in absorbance among polyphenol compounds of the same concentration may be due to the difference in their ability to reduce the Mo(VI) ion.

Both the P-B and the F-C methods are redox assays. As shown in Figure 4.2, catechin and epicatechin are isomers with a difference in the configuration of one OH group, and the reactivity of these two compounds to the P-B and F-C methods were similar. The DP of PCs influenced the reactivity of these two methods differently. The PC pentamer (DP = 5) had lower absorbance than PC B2 (DP = 2) in the P-B method, while the opposite was found for the F-C method. This may be due to the different reagent used in these two methods. Different polyphenols possess different reducing abilities against different reagents, which result in differences in in color development (Price & Butler, 1977). Phloretin belongs to the chalcone group with an open C ring, while quercetin belongs to the flavanol group with a closed C ring. Quercetin showed much higher absorbance than phloretin at the same concentration for both methods, which indicates that the difference in the C ring of flavanols greatly impacts the reducing ability of these compounds. CGA, a phenolic acid with a C6-C3 backbone different than other polyphenols tested in this study which are flavonoids with the backbone of C6-C3-C6, showed the lowest absorbance among all compounds for both the P-B and F-C methods, indicating that differences in the backbone of the polyphenols also impact their reactivity in these two methods. These findings are consistent with previous reports, wherein redox reactions were influenced by polyphenol structure, including the hydroxylation pattern and the DP of the polyphenols (Price & Butler, 1977). Differences in polyphenol structure may impact the electron supply to the reagents, the reduction of which determine the degree of color development in both the P-B and F-C assays.

A wide variation of absorbance values obtained using different polyphenol compounds of the same concentrations was observed. This indicates that values for total polyphenol concentration resulting from the P-B and F-C redox methods applied to samples containing mixtures of polyphenols, such as fruit and vegetable samples, should be interpreted and compared to each other with caution. The composition of polyphenols varies greatly depending on the botanical source of the sample. For example, green tea is rich in catechin (Chu & Juneja, 1997), while most apples are rich in chlorogenic acid (Anastasiadi et al., 2017). If the same actual total concentration of polyphenols was present in a tea sample and an apple sample, the quantitative results using P-B and F-C methods would be 1.5 to 2-fold higher for the green tea sample compared to the apple sample. Clearly, choosing the appropriate standards in building the calibration curve is of the utmost importance. Ideally, the dominant polyphenol compound in the samples should be used as the standard for more accurate quantification results. If this is not practical due to cost or availability, then a standard of similar structure to the most predominant polyphenol in the sample of interest should be sought. Furthermore, the use of different standards

in these redox methods will certainly lead to quantitatively different results, with differences of several fold in some cases.

4.3.1.2 Reactivity of individual polyphenol compounds to the DMAC and BSA precipitation methods

The dose-response curves built using individual polyphenol standards of catechin, epicatechin, PC B2, PC pentamer, CGA, phloretin, and quercetin for the DMAC and BSA precipitation methods are shown in Figure 4.1 C and D. The R^2 and equations are listed in Table 4.3. The regression for all PCs tested (catechin, epicatechin, PC B2, PC pentamer) using the DMAC method were linear but the slopes of the curves were different. At the same concentration of 50 mg/L, catechin and epicatechin had the highest absorbance by DMAC, more than two times higher than the absorbance of PC B2 and four times higher than the PC pentamer. These apparent differences in reactivity of flavanols with different DP and stereochemistry are consistent with prior reports (de Pascual-Teresa, Treutter, Rivas-Gonzalo, & Santos-Buelga, 1998). For example, others reported greater absorbance for catechin and epicatechin compared to oligomeric flavanols (Payne et al., 2010). However, the relationship between polyphenol DP and absorbance observed in our study was not consistent with prior reports. We found that higher DP led to lower absorbance in the DMAC assay, but Payne et al. reported variable results depending on the source of PC B2. In that study, trimers and tetramers had higher absorbance than PC B2 (a dimer) from one vendor, similar absorbance to PC B2 from another vendor, and lower absorbance than PC B2 from a third vendor. (Payne et al., 2010). This highlights the fact that purity or source of the standard could also cause substantial variation in the quantitative results. The non-flavanol compounds tested (CGA, phloretin, and quercetin) generated minimal color development with the DMAC reagent, as expected and as also reported by Payne *et al* (Payne et al., 2010). These findings could be explained by the operating principles of the reaction between flavanols and the DMAC reagent. Under acidic conditions, the DMAC reagent only reacts to the C8 position of the A ring at the terminal unit of the flavanols, specifically monomeric flavanols, such as catechin, epicatechin, and polymeric flavanols formed by catechins and epicatechins (Rohr, Meier, & Sticher, 2000), while quercetin, as one flavonol that has a double bond between C2 and C3 positions could not react with the DMAC reagent, nor did phloretin, as a chalcone which has a carbonyl at C4 position, nor did CGA, as a phenolic acid which does not have the meta-substituted dihydroxybenzene rings (Delcour & Varebeke, 1985). Therefore, the DMAC method is specific to flavanols but the choice of different polyphenol compounds as the standard could lead to variance in the quantification results on the same sample. Accurate measurement of flavanols can be achieved by using the appropriate standard, preferably the flavanol extract from the tested samples (Krueger et al., 2016). However, because of the complex composition of fruit and vegetables, the isolation, purification, and characterization of these standards are time and money demanding, and may not be successful depending on the sample matrix (Feliciano et al., 2012). A flavanol of similar DP and structure would be a more practical, if not ideal, choice of standard for accurate quantification of flavanols by DMAC.

Per the method described in 2.2.4, the individual polyphenols tested in this study were treated as standards and they did not bind with BSA, but rather they reacted with the $FeCl_3$ reagent in the presence of buffer [5% triethanolamine (v/v) and 10% sodium dodecyl sulfate (w/v)]. The regression for all compounds tested using the BSA precipitation method were linear with the exception of phloretin ($R^2 = 0.80$) (Figure 4.1D and Table 4.3). The slopes of the curves were different. At the same concentration of 150 mg/L, the absorbance of quercetin was 50% higher than the absorbance of PC B2. Previous research has shown that many different

polyphenols can reduce Fe^{3+} to Fe^{2+} but that this reducing capacity varied among compounds (Raquel Pulido, Laura Bravo, & Saura-Calixto, 2000; Yoshino & Murakami, 1998). These differences have resulted in quercetin absorbance readings twice as high as the absorbance generated by catechin at the same concentration of each compound (Raquel Pulido et al., 2000). In our study, the absorbance of quercetin was 30% higher than the absorbance of catechin; the differences in absorbance between quercetin and catechin were not as extreme. Several structural characteristics of polyphenols have been shown to increase their redox efficiency, including the degree of hydroxylation and the conjugation between the A and B rings (Raquel Pulido et al., 2000). This explains why the absorbance generated by catechin and epicatechin, which have the same number and location of the hydroxyl groups, were similar. Compared to catechin and epicatechin, quercetin has higher reducing capacity due to the conjugation between the A and B rings. Compared to quercetin, the missing o-dihydroxy structure in the B ring of phloretin may account for its lower reactivity with Fe^{3+}. Besides these differences in reactivity with Fe^{3+}, the BSA method has an additional potential source of error. Ideally, in any analytical method, the standard should be treated the same as the samples. In the case of the BSA precipitation method, a deviation from this general practice is required. It is difficult to obtain a standard that is comparable in structure to polyphenols present in the sample that will precipitate with BSA. Such standards are not widely commercially available and those that are available are prohibitively expensive for use in the quantities required in this assay. Monomers and dimers such as catechin or PC B2 are commercially available, but they do not form precipitates with BSA, and thus cannot be quantified using the BSA method. Catechin is, however, used as a standard for the BSA method. Known amounts of catechin are reacted with Fe^{3+} to generate a standard curve. This curve is then used to quantify the polyphenol concentration in the unknown samples. For samples, a precipitate is formed with BSA. This precipitate includes only the polyphenols DP ≥ 3, where precipitation efficiency increases with larger molecular weight of the PC (Harbertson et al., 2014). The precipitate is then resuspended, reacted with Fe^{3+}, and quantified using the standard curve made with catechin.

4.3.2 Contribution of interactions between individual polyphenol compounds on polyphenol quantification

4.3.2.1 Contribution of main effects of individual polyphenol compounds on polyphenol quantification

The quantification results for all samples evaluated using the P-B, F-C, DMAC, and BSA precipitation methods are shown in Table 4.4, and the results of the statistical analyses for the main effects are presented in Table 4.5, rows 1 to 3. The analysis of variance showed that the main effects were significant for catechin, PC B2, and CGA for all four methods (Table 4.5). Positive β values indicate that increases in concentrations of each of these compounds result in increasing quantification results for each of the four methods. However, the relative importance of the contributions of each polyphenol compound evaluated (catechin, PC B2, and CGA) differed (Table 4.5). These results are in agreement with our observation of high reactivity of catechin, PC B2 and CGA in these four methods (Figure 4.1), with one exception of low reactivity for CGA in the DMAC method. As evidenced by the values of β and ω_p^2, CGA (ω_p^2 = 0.99) contributed more than catechin and PC B2 (ω_p^2 = 0.33, 0.82 respectively) to the results of the P-B method. For both F-C and BSA methods, all three compounds contributed significantly to their quantification results, (ω_p^2 values = 1.0 for all). In contrast, for the DMAC method, catechin and PC B2 (ω_p^2 = 1.0 for both) contributed more than CGA (ω_p^2 value of 0.34) to the quantification results. For the DMAC method, β values for catechin and PC B2 (129 and 40.9)

were also higher compared to CGA (1.82). The small $\omega_p^2$and β for CGA in the DMAC method can be explained by the relatively low response of DMAC to CGA. These ω_p^2 values show that the variance in the quantification results by the DMAC are largely explained by the concentrations of catechin and PC B2 rather than CGA, likely due to interaction effects.

Table 4. 4. Quantification results of 27 samples listed in Table 4.1 by P-B, F-C, DMAC, and BSA precipitation methods to assess the interaction effects of (+)-catechin, PC B2, and CGA.

Sample number	Concentrations of polyphenols			
	P-B (mg/L of GAE)	F-C (mg/L of GAE)	DMAC (mg/L of PC B2 equivalents)	BSA (mg/L of catechin equivalents)
1	2.76 ± 0.0174	nd	0.564 ± 0.0286	nd
2	61.7 ± 3.76	112 ± 0.145	0.663 ± 0.0286	291 ± 1.10
3	158 ± 3.71	272 ± 0.251	0.713 ± 0.0286	207 ± 0.313
4	14.8 ± 0.405	22.6 ± 0.00	29.3 ± 0.351	12.6 ± 0.00
5	63.3 ± 0.474	134 ± 0.664	29.3 ± 0.119	323 ± 1.60
6	155 ± 9.44	291 ± 1.67	29.5 ± 0.248	282 ± 1.13
7	47.9 ±1.52	79.4 ± 0.145	95.6 ± 1.86	42.6 ± 0.138
8	90.6 ± 3.34	183 ± 0.632	94.1 ± 2.53	155 ± 0.271
9	155 ± 3.19	334 ± 0.664	93.0± 1.80	334 ± 0.313
10	13.9 ± 0.0603	30.1 ± 0.145	81.0± 0.472	17.0 ± 0.0521
11	59.0 ± 3.85	143 ± 1.24	82.9 ± 0.620	334 ± 0.276
12	171 ±1.00	300 ± 0.580	83.5 ± 0.446	310 ± 1.95
13	19.1 ± 1.44	53.3 ± 0.290	110 ± 0.955	28.7 ± 0.0521
14	53.2 ± 2.09	166 ± 0.251	117 ± 3.19	144 ± 0.413
15	156 ±1.40	321 ± 0.145	115± 0.693	328 ± 1.08
16	52.2 ± 0.348	107 ± 0.145	172 ± 1.18	113 ± 0.208
17	117 ± 0.577	212 ± 0.290	178 ± 1.18	164 ± 5.47
18	123 ± 3.76	360 ± 0.580	185 ± 0.681	350 ± 1.25
19	45.0 ± 0.165	103 ± 0.807	297 ± 2.70	54.4 ± 0.138
20	56.6 ± 0.385	214 ± 0.383	296 ± 2.07	168 ± 1.24
21	136 ± 2.10	366 ± 0.502	306 ± 2.50	349 ± 4.30
22	51.1 ± 0.400	124 ± 0.523	329 ± 1.32	65.6 ± 0.104
23	62.4 ± 2.94	233 ± 0.725	334 ± 1.51	181 ± 1.13
24	147 ± 3.42	382 ± 0.580	337 ± 0.330	365 ± 0.313
25	56.4 ± 0.287	173 ± 0.251	399 ± 1.31	185 ± 0.651
26	119 ± 1.73	279 ± 0.145	405 ± 1.31	591 ± 3.16
27	149 ± 2.04	423 ± 0.807	404 ± 1.98	388 ± 1.36

Table 4. 5. Statistical significance (p) of three-way ANOVA with interactions, standardized coefficients (β) and effect size (ω_p^2) for factors [polyphenol compounds: (+)-catechin, PC B2, and CGA] and their interactions for P-B, F-C, DMAC, and BSA precipitation methods based on the quantification results on samples listed in Table 4.1.

Sources of variation	Df	P-B			F-C			DMAC			BSA		
		p	β	ω_p^2	*p*	β	ω_p^2	*p*	β	ω_p^2	*p*	β	ω_p^2
Catechin	2	< 0.05	3.51	0.33	< 0.05	40.6	1.0	< 0.05	129	1.0	< 0.05	33.6	1.0
PC B2	2	< 0.05	10.2	0.82	< 0.05	28.6	1.0	< 0.05	40.9	1.0	< 0.05	29.9	1.0
CGA	2	< 0.05	48.3	0.99	< 0.05	108	1.0	< 0.05	1.82	0.34	< 0.05	104	1.0
Catechin × PC B2	4	< 0.05	1.19	0.19	< 0.05	-1.15	0.57	< 0.05	1.60	0.27	< 0.05	39.7	1.0
Catechin × CGA	4	< 0.05	-6.99	0.67	< 0.05	-1.49	0.70	< 0.05	1.17	0.25	< 0.05	1.27	0.97
PC B2 × CGA	4	< 0.05	-8.79	0.86	< 0.05	-2.73	0.87	0.26	0.177	0.017	< 0.05	-4.43	0.97
Catechin × PC B2× CGA	8	< 0.05	4.25	0.73	< 0.05	0.347	0.14	< 0.05	-0.442	0.19	< 0.05	-17.6	1.0

4.3.2.2 Contribution of interaction effects of individual polyphenol compounds on polyphenol quantification

Significant two-way and three-way interaction effects were found on all factors with the exception of the interaction effect of PC B2 and CGA on the quantification results of DMAC. Different contributions of interactions to the quantification results of the four methods were also found (Table 4.6). In general, the effect sizes of interactions were smaller compared to those of the main effects of individual polyphenol compounds, with the exception that the interaction of catechin × PC B2 contributed significantly to the quantification results of the BSA method. Significant interaction effects of different polyphenol compounds have also been reported by others. For example, synergism has been found among polyphenols from *Cistus salviifolius* extract for improved antimicrobial activity of mixtures compared to the same concentrations of individual compounds (Tomás-Menor et al., 2015). In contrast, negative synergism has been found among the polyphenols catechin, resveratrol, and quercetin, resulting in reduced antioxidant capacity (Pinelo et al., 2004) exhibited by mixtures of these compounds compared to the same compounds evaluated individually. However, the mechanisms behind these interactive effects in polyphenol quantification and antioxidant activity remain unclear. Our results indicate that the quantification result of mixtures of catechin, PC B2 and CGA is not equal to the sum of quantification results of the compounds when present in isolation, likely due to interactions among these compounds in aqueous solution. Advanced analytical instruments such as NMR could be used to help reveal these intramolecular and intermolecular reactions in the aqueous environment (Mistry, Cai, Lilley, & Haslam, 1991).

4.3.3 Contribution of potentially interfering compounds to polyphenol quantification

4.3.3.1 Contribution of main effects of potentially interfering compounds to polyphenol quantification

The quantification results of all samples evaluated using the P-B, F-C, DMAC, and BSA precipitation methods are shown in Table 4.6 and the results of statistical analyses for the main effects are presented in Table 4.7, rows 1 to 3. The analysis of variance showed that the main effects were significant for AA, glucose and SO_2 for all four methods. Specifically, for the P-B and method, our results are in general agreement with prior reports showing that AA increased quantification results and that glucose and SO_2 did not affect results. While our findings indicate that glucose and SO_2 had a statistically significant effect, the small values of β indicate that this may not be of practical significance. For the F-C assay, we found that AA decreased results, which is the opposite of our expectation and of prior reports. Because AA is a powerful antioxidant, we expected that it would lead to elevated results in a non-specific reducing assay, as has been reported by others (Everette et al., 2010). However, the high concentration of AA in our study of 2000 mg/L, while relevant in terms of AA concentrations in fruit products, far exceeded concentrations previously evaluated (up to 212 mg/L). We found that glucose and SO_2 had minimal but statistically significant effects on F-C results, decreasing and increasing the results, respectively. Prior reports found the opposite but similarly small effects of glucose and SO_2 on F-C (Margraf et al., 2015).

For the DMAC method, we found that the presence of AA, glucose and SO2 all decreased quantification results for total flavanols, although the contribution of AA was greater than that of glucose or SO_2. The DMAC method has been reported to be specific to flavanols (Wallace & Giusti, 2010) but the specificity of this method in the presence of AA, glucose and SO_2 had not been previously tested. Our findings for the BSA method were similar to those for the DMAC method. In the BSA method, color is generated by the reaction between reducing

compounds, including polyphenols, and the Fe^{3+}. AA, glucose and SO_2 are reactive with Fe^{3+} (Lovley, 1987). While we did not evaluate the mechanisms underlying these effects, the differences in the direction and magnitude of interference by these compounds commonly found in fruit matrices may be due to the differences in chemical structure and redox potential of these compounds.

Table 4. 6. Quantification results of 27 samples listed in Table 4.2 by P-B, F-C, DMAC, and BSA precipitation methods to assess the interaction effects of glucose, AA, and SO_2.

Sample number	Quantification results			
	P-B (mg/L of GAE)	F-C (mg/L of GAE)	DMAC (mg/L of PC B2 equivalents)	BSA (mg/L of catechin equivalents)
1	59.1 ± 0.230	177 ± 0.175	98.3 ± 0.180	276 ± 3.75
2	73.2 ± 0.903	237 ± 0.877	95.1 ± 0.548	282 ± 0.389
3	108 ± 0.605	407 ± 5.77	65.6 ± 0.650	238 ± 0.224
4	54.2 ± 1.81	170 ± 0.702	75.2 ± 1.73	275 ± 0.389
5	67.6 ± 0.573	228 ± 3.08	88.0 ± 0.974	254 ± 0.389
6	87.6 ± 1.54	346 ± 11.1	68.3 ± 1.87	211 ± 0.389
7	42.7 ± 0.326	114 ± 4.67	55.8 ± 0.949	162 ± 0.389
8	58.3 ± 0.110	162 ± 2.20	52.9 ± 0.785	144 ± 1.25
9	71.0 ± 0.459	207 ± 9.58	41.2 ± 0.468	103 ± 0.389
10	107 ± 0.674	361 ± 0.608	92.1 ± 0.785	281 ± 0.224
11	122 ± 1.18	424 ± 1.58	90.5 ± 1.06	271 ± 3.84
12	134 ± 2.39	529 ± 14.3	76.0 ± 0.631	228 ± 0.224
13	111 ± 0.555	346 ± 1.90	87.0 ± 0.393	256 ± 1.69
14	125 ± 2.70	411 ± 1.07	84.1 ± 0.477	241 ± 0.978
15	127 ± 2.69	407 ± 4.78	71.1 ± 0.360	204 ± 1.35
16	101 ± 1.29	276 ± 4.30	59.1 ± 0.180	148 ± 1.19
17	117 ± 1.61	327 ± 5.12	55.9 ± 0.180	126 ± 7.72
18	137 ± 0.972	331 ± 7.10	42.1 ± 0.393	84.7 ± 4.48
19	102 ± 0.168	155 ± 1.10	81.4 ± 0.393	231 ± 0.673
20	101 ± 0.320	163 ± 0.175	75.9 ± 0.238	223 ± 8.20
21	100 ± 0	169 ± 1.78	63.8 ± 0.548	174 ± 1.69
22	101 ± 0.241	157 ± 0.175	76.9 ± 0.413	205 ± 0.809
23	101 ± 0.110	160 ± 0.304	69.8 ± 0.325	186 ± 0.594
24	101 ± 0.191	158 ± 4.94	56.4 ± 0.312	145 ± 1.03
25	103 ± 0.0735	148 ± 1.56	40.7 ± 1.22	97.1 ± 0.449
26	101 ± 0.0972	152 ± 0.464	37.8 ± 0.770	81.6 ± 0.449
27	101 ± 0.230	147 ± 1.58	24.5 ± 0.156	43.9 ± 0.224

Table 4. 7. Statistical significance (p) of three-way ANOVA with interactions, standardized coefficients (β) and effect size (ω_p^2) for factors (interfering compounds: AA, glucose, and SO_2) and their interactions for P-B, F-C, DMAC, and BSA precipitation methods based on the quantification results on samples listed in Table 4.2.

Sources of variation	Df	P-B			F-C			DMAC			BSA		
		p	β	ω_p^2	*p*	β	ω_p^2	*p*	β	ω_p^2	*p*	β	ω_p^2
AA	2	< 0.05	9.81	0.99	< 0.05	-46.8	0.99	< 0.05	-5.96	0.96	< 0.05	-25.3	0.97
Glucose	2	< 0.05	-0.0336	0.72	< 0.05	-0.244	0.94	< 0.05	-0.205	0.99	< 0.05	-0.706	0.99
SO_2	2	< 0.05	0.0613	0.95	< 0.05	0.350	0.95	< 0.05	-0.0600	0.97	< 0.05	-0.159	0.96
Glucose × AA	4	< 0.05	0.0283	0.83	< 0.05	0.137	0.86	< 0.05	-0.00589	0.48	0.56	-0.0147	0.21
Glucose × SO_2	4	< 0.05	-0.0487	0.92	< 0.05	-0.242	0.92	< 0.05	0.00468	0.56	< 0.05	-0.0137	0.012
AA × SO_2	4	< 0.05	-3.09	0.36	< 0.05	-0.00120	0.79	< 0.05	7.08e-05	0.56	< 0.05	-6.23e-05	0.25
Glucose × AA × SO_2	8	< 0.05	4.26e-05	0.66	< 0.05	0.000754	0.64	< 0.05	-4.14e-05	0.78	< 0.05	0.000123	0.17

4.3.3.2 Contribution of interaction effects of potentially interfering compounds to polyphenol quantification

Significant two-way and three-way interaction effects were found on all factors for all methods evaluated with the exception of the interaction effect of AA × glucose on the quantification results of BSA. The contribution of interactions among AA, glucose, and SO_2 to the quantification results were smaller than the main effects of these compounds, evidenced by the lower values of ω_p^2, but their contribution still varied based on the type of interactions and the methods used. The interaction of glucose × SO_2 had a greater effect than other interactions for the P-B and F-C method as evidenced by the ω_p^2 values. Significant interaction effects between AA and SO_2 on oxidation have also been observed in wine (Peng, Duncan, Pocock, & Sefton, 1998), in agreement with our findings that this interaction effect significantly influenced the results of P-B and F-C, both redox methods.

For the DMAC method, the three-way interaction of AA × glucose × SO_2 was most influential, and the interaction of AA × SO_2 was the most significant for the BSA method, indicated by higher values of ω_p^2. Although there are few prior reports of interactions among these three compounds, the difference in redox potential and hydroxyl groups could lead to non-covalent binding among these compounds under acidic conditions. Since all three compounds are often found in fruit juice and beverages (Zielinski et al., 2014; Zoecklein et al., 1999), the potential for interference due to interactions of these constituents in the sample matrix should not be neglected in the quantification of polyphenols and flavanols using the F-C, P-B, DMAC, and BSA methods.

4.4 Conclusion

This study highlighted the differences in reactivity of polyphenol compounds, including catechin, epicatechin, PC B2, PC pentamer, CGA, phloretin, and quercetin, to common quantification methods of polyphenols and flavanols: the P-B, F-C, DMAC, and BSA methods. Significant interactions between catechin, PC B2, and CGA on the quantification results of the above four methods were also found. Furthermore, interference from matrix constituents often found in fruit juice and beverages were observed. AA, glucose, SO_2, and the interactions thereof influenced polyphenol quantification results. Our findings provide information on application of these common analytical methods to quantify polyphenols in fruit juice and beverages with complex chemical compositions. Extraction and isolation strategies of polyphenols and the use of representative polyphenol compounds as the standards in these assays are recommended for accurate measurement of polyphenols for predicting health and sensory attributes.

4.5 Acknowledgement

The authors want to thank the Graduate Teaching Scholar Program in the College of Agricultural and Life Sciences at Virginia Tech for providing the stipend for the graduate student on this project.

4.6 Author contributions

Sihui Ma designed the study, collected the data, interpreted the results and drafted the manuscript. Cathlean Kim collected the data. Jacob Lahne helped with the statistical analysis and draft the method section on statistical analysis. Andrew P. Neilson, Gregory M. Peck, Sean F. O'Keefe, and Amanda C. Stewart designed the study and revised the manuscript.

4.7 References

Anastasiadi, M., Mohareb, F., Redfern, S. P., Berry, M., Simmonds, M. S. J., & Terry, L. A. (2017). Biochemical profile of heritage and modern apple cultivars and application of

machine learning methods to predict usage, age, and harvest season. Journal of Agricultural and Food Chemistry, 65(26), 5339–5356. https://doi.org/10.1021/acs.jafc.7b00500

Blainski, A., Lopes, G., & de Mello, J. (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from Limonium Brasiliense L. Molecules, 18(6), 6852–6865. https://doi.org/10.3390/molecules18066852

Bordenave, N., Hamaker, B. R., & Ferruzzi, M. G. (2014). Nature and consequences of non-covalent interactions between flavonoids and macronutrients in foods. Food and Function, 5(1), 18–34. https://doi.org/10.1039/c3fo60263j

Bring, J. (1994). How to standardize regression coefficients. American Statistician, 48(3), 209–213. https://doi.org/10.1080/00031305.1994.10476059

Budini, R., Tonelli, D., & Girotti, S. (1980). Analysis of total phenols using the Prussian Blue method. Journal of Agricultural and Food Chemistry, 28(6), 1236–1238. https://doi.org/10.1021/jf60232a056

Chu, D. C., & Juneja, L. R. (1997). General chemical composition of green tea and its infusion. In Chemistry and applications of green tea (pp. 13–22).

de Pascual-Teresa, S., Treutter, D., Rivas-Gonzalo, J. C., & Santos-Buelga, C. (1998). Analysis of Flavanols in Beverages by High-Performance Liquid Chromatography with Chemical Reaction Detection. Journal of Agricultural and Food Chemistry, 46(10), 4209–4213. https://doi.org/10.1021/jf980255k

Delcour, J. A., & Varebeke, D. J. de. (1985). A New Colorimetric Assay for Flavanoids in Pilsner Beers. Journal of the Institute of Brewing, 91(1), 37–40. https://doi.org/10.1002/j.2050-0416.1985.tb04303.x

Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). Thorough study of reactivity of various compound classes toward the Folin−Ciocalteu reagent. Journal of Agricultural and Food Chemistry, 58(14), 8139–8144. https://doi.org/10.1021/jf1005935

Feliciano, R. P., Shea, M. P., Shanmuganayagam, D., Krueger, C. G., Howell, A. B., & Reed, J. D. (2012). Comparison of isolated cranberry (Vaccinium macrocarpon Ait.) proanthocyanidins to catechin and procyanidins A2 and B2 for use as standards in the 4-(Dimethylamino)cinnamaldehyde assay. Journal of Agricultural and Food Chemistry, 60(18), 4578–4585. https://doi.org/10.1021/jf3007213

George, J., Singh, M., Srivastava, A. K., Bhui, K., Roy, P., Chaturvedi, P. K., & Shukla, Y. (2011). Resveratrol and Black Tea Polyphenol Combination Synergistically Suppress Mouse Skin Tumors Growth by Inhibition of Activated MAPKs and p53. PLoS ONE, 6(8), e23395. https://doi.org/10.1371/journal.pone.0023395

Graham, H. D. (1992). Stabilization of the Prussian blue color in the determination of polyphenols. Journal of Agricultural and Food Chemistry, 40(5), 801–805. https://doi.org/10.1021/jf00017a018

Hagerman, A. E., & Butler, L. G. (1978). Protein precipitation method for the quantitative determination of tannins. Journal of Agricultural and Food Chemistry, 26(4), 809–812. https://doi.org/10.1021/jf60218a027

Harbertson, J. F., Kennedy, J. A., & Adams, D. O. (2002). Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. American Journal of Enology and Viticulture, 53(1), 54. Retrieved from http://www.ajevonline.org/content/53/1/54.abstract

Harbertson, J. F., Kilmister, R. L., Kelm, M. A., & Downey, M. O. (2014). Impact of condensed tannin size as individual and mixed polymers on bovine serum albumin precipitation. Food Chemistry, 160, 16–21. https://doi.org/10.1016/J.FOODCHEM.2014.03.026

Krueger, C. G., Chesmore, N., Chen, X., Parker, J., Khoo, C., Marais, J. P. J., … Reed, J. D. (2016). Critical reevaluation of the 4-(dimethylamino)cinnamaldehyde assay: Cranberry proanthocyanidin standard is superior to procyanidin A2 dimer for accurate quantification of proanthocyanidins in cranberry products. Journal of Functional Foods, 22, 13–19. https://doi.org/10.1016/J.JFF.2016.01.017

Lakens, D. (2013). Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. Frontiers in Psychology, 4, 863. https://doi.org/10.3389/fpsyg.2013.00863

Le Bourvellec, C., & Renard, C. M. G. C. (2012). Interactions between polyphenols and macromolecules: Quantification methods and mechanisms. Critical Reviews in Food Science and Nutrition. https://doi.org/10.1080/10408398.2010.499808

Lea, A. G. H., & Drilleau, J.-F. (2003). Cidermaking. (A. G. H. Lea & J. R. Piggott, Eds.), Fermented Beverage Production (2nd ed.). London: Springer US. https://doi.org/10.1007/978-1-4615-0187-9_4

Lovley, D. R. (1987). Organic matter mineralization with the reduction of ferric iron: A review. Geomicrobiology Journal, 5(3–4), 375–399. https://doi.org/10.1080/01490458709385975

Ma, S., Neilson, A., Lahne, J., Peck, G., O'Keefe, S., Hurley, E. K., … Stewart, A. (2018). Juice clarification with pectinase reduces yeast assimilable nitrogen in apple juice without affecting the polyphenol composition in cider. Journal of Food Science, 83, 2772–2781. https://doi.org/doi:10.1111/1750-3841.14367

Margraf, T., Karnopp, A. R., Rosso, N. D., & Granato, D. (2015). Comparison between Folin-Ciocalteu and Prussian Blue assays to estimate the total phenolic content of juices and teas using 96-well microplates. Journal of Food Science, 80(11), C2397–C2403. https://doi.org/10.1111/1750-3841.13077

Mertens-Talcott, S. U., & Percival, S. S. (2005). Ellagic acid and quercetin interact synergistically with resveratrol in the induction of apoptosis and cause transient cell cycle arrest in human leukemia cells. Cancer Letters, 218(2), 141–151. https://doi.org/10.1016/J.CANLET.2004.06.007

Mistry, T. V., Cai, Y., Lilley, T. H., & Haslam, E. (1991). Polyphenol interactions. Part 5. Anthocyanin co-pigmentation. Journal of the Chemical Society, Perkin Transactions 2, 0(8), 1287. https://doi.org/10.1039/p29910001287

Payne, M. J., Hurst, W. J., Stuart, D. A., Ou, B., Fan, E., Ji, H., & Kou, Y. (2010). Determination of total procyanidins in selected chocolate and confectionery products using DMAC. Journal of AOAC International, 93(1), 89–96.

Peng, Z., Duncan, B., Pocock, K. F., & Sefton, M. A. (1998). The effect of ascorbic acid on oxidative browning of white wines and model wines. Australian Journal of Grape and Wine Research, 4(3), 127–135. https://doi.org/10.1111/j.1755-0238.1998.tb00141.x

Pinelo, M., Manzocco, L., Nuñez, M. J., & Nicoli, M. C. (2004). Interaction among Phenols in Food Fortification: Negative Synergism on Antioxidant Capacity. Journal of Agricultural and Food Chemistry, 52(5), 1177–1180. https://doi.org/10.1021/jf0350515

Price, M. L., & Butler, L. G. (1977). Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. Journal of Agricultural and Food Chemistry, 25(6), 1268–1273. https://doi.org/10.1021/jf60214a034

Raquel Pulido, Laura Bravo, * and, & Saura-Calixto, F. (2000). Antioxidant Activity of Dietary Polyphenols As Determined by a Modified Ferric Reducing/Antioxidant Power Assay. https://doi.org/10.1021/JF9913458

Rohr, G. E., Meier, B., & Sticher, O. (2000). Analysis of procyanidins. Studies in Natural Products Chemistry, 21, 497–570. https://doi.org/10.1016/S1572-5995(00)80013-7

Rothwell, J. A., Perez-Jimenez, J., Neveu, V., Medina-Remón, A., M'Hiri, N., García-Lobato, P., … Scalbert, A. (2013). Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Database, 2013, bat070-bat070. https://doi.org/10.1093/database/bat070

Tomás-Menor, L., Barrajón-Catalán, E., Segura-Carretero, A., Martí, N., Saura, D., Menéndez, J. A., … Micol, V. (2015). The Promiscuous and Synergic Molecular Interaction of Polyphenols in Bactericidal Activity: An Opportunity to Improve the Performance of Antibiotics? Phytotherapy Research, 29(3), 466–473. https://doi.org/10.1002/ptr.5296

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. Nutrients, 2(12), 1231–1246. https://doi.org/10.3390/nu2121231

Wallace, T. C., & Giusti, M. M. (2010). Evaluation of parameters that affect the 4-dimethylaminocinnamaldehyde assay for flavanols and proanthocyanidins. Journal of Food Science, 75(7), C619–C625. https://doi.org/10.1111/j.1750-3841.2010.01734.x

Wang, Y., Singh, A. P., Hurst, W. J., Glinski, J. A., Koo, H., & Vorsa, N. (2016). Influence of degree-of-polymerization and linkage on the quantification of proanthocyanidins using 4-dimethylaminocinnamaldehyde (DMAC) assay. Journal of Agricultural and Food Chemistry, 64(11), 2190–2199. https://doi.org/10.1021/acs.jafc.5b05408

Yoshino, M., & Murakami, K. (1998). Interaction of Iron with Polyphenolic Compounds: Application to Antioxidant Characterization. Analytical Biochemistry, 257(1), 40–44. https://doi.org/10.1006/ABIO.1997.2522

Zielinski, A. A. F., Haminiuk, C. W. I., Nunes, C. A., Schnitzler, E., van Ruth, S. M., & Granato, D. (2014). Chemical Composition, Sensory Properties, Provenance, and Bioactivity of Fruit Juices as Assessed by Chemometrics: A Critical Review and Guideline. Comprehensive Reviews in Food Science and Food Safety, 13(3), 300–316. https://doi.org/10.1111/1541-4337.12060

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., & Nury, F. S. (1999). Sulfur dioxide and ascorbic acid. In Wine Analysis and Production (pp. 178–191). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4757-6967-8_10

CHAPTER 5 Free amino acid composition of apple juices with potential for cider making as determined by UPLC-PDA

Published in Journal of the Institute of Brewing, 2018, 124(4), 467-476.

https://doi.org/10.1002/jib.519

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ABSTRACT

Amino acids and ammoniumions constitute the yeast assimilable nitrogen naturally present in apple juice, with free amino acids being the major constituent. Little information is available on the extent to which free amino acid composition in apple (*Malus* × *domestica* Borkh.) juice varies among juices used for fermentation. Twenty amino acids were quantified by UPLC-PDA in juices from 13 apple cultivars grown in Virginia with potential use in cider making. The relative amino acid profile was significantly different among the apple juices evaluated. The total amino acid concentration ranged from 18 mg/L in Blacktwig juice to 57 mg/L in Enterprise juice. L-Asparagine, L-aspartic acid and L-glutamine are the principal amino acids observed inmost apple juices. Our results indicate that the relative concentration of amino acids in apples is different from that found in Vitis vinifera wine grapes, which are rich in L-proline and L-arginine. The impact of these differences on fermentation of apple juice by wine yeast strains warrants further research, as the amino acid composition of grape juice impactswine quality both directly and indirectly via yeast metabolism. These results will informfuture research on yeast metabolism and nitrogenmanagement during cider fermentation.

5.1 Introduction

Yeast assimilable nitrogen (YAN) refers to soluble nitrogen sources that can be utilised by yeast during fermentation. YAN is composed of primary amino acids and ammonium ions. YAN concentration and composition in apple juice is a rate-controlling factor in cider fermentation (1,2). Deficiency of YAN in juices causes slow or incomplete fermentation and the production of sulphur off-aromas (3,4). The kinetics of aroma production and themicrobiological stability of finished cider are also influenced by YAN concentration in the juice (1). The amino acid composition of apple juice is of interest in cider fermentation management as some amino acids are preferentially utilised by yeast than others, and because of their role as precursors for volatile aroma formation during fermentation (5,6). Wine yeast strains primarily utilise arginine, then serine, glutamic acid, threonine, aspartic acid and lastly lysine (7). The addition of aspartate and glutamate to apple juice prior to fermentation can result in the production of high concentrations of esters by yeast, imparting fruity aromas to the finished cider (6). The addition of methionine to apple juice prior to fermentation can decrease hydrogen sulphide production during cider fermentation, improving the sensory characteristics of the final cider (8).

YAN concentration in apple juice is largely determined by the concentration of yeast assimilable nitrogen in fresh apples, which is influenced by crop load (9). Soil, climatological factors and fertiliser application have been demonstrated to influence YAN in grapes (10), and may also impact on YAN concentration in apples. The concentration of yeast assimilable nitrogen can decrease during post-harvest maturation, storage, transportation and juice processing (11,12). Thus, quantification of YAN in apple juice immediately prior to fermentation is important.

Free amino acids account for the majority of YAN in apple juice (13). Analysis of the profile of amino acids in apple juice can provide useful information for cider fermentation management. The concentration and composition of amino acids in apples have been observed to differ among three cultivars grown in Northern Spain, and six cultivars grown in the UK (14). Based on information currently available, the amino acids have been classified into three categories based on their concentration in most apple cultivars: asparagine, aspartic acid and glutamine with the highest concentrations, serine, alanine, γ-aminobutyric acid (GABA), valine, isoleucine with medium to low concentrations, and other amino acids with the lowest concentrations (14–16). Lysine and sulphur containing amino acids, which are rarely found in plants, have been reported in Red Delicious, Golden Delicious, Ralls, Fuji, QinGuan, Jonagold, Granny Smith, and Orin (17). However, no information is available on amino acid composition in apple cultivars grown in North America with potential use in cidermaking, although this information would be useful to the growing cider industry in that region.

The purpose of this study was to characterise the amino acid composition in apples with potential use in cider making, and to assess the extent to which amino acid concentration and composition vary among juice samples. In this study, 20 amino acids were analysed in 13 apple juice samples fromcultivars grown in Virginia with potential for use in cider making.

5.2 Material and methods

5.2.1 Juice sample collection

Apples were harvested in 2014 from an experimental orchard at the Virginia Tech Alson H. Smith, Jr Agricultural Research and Extension Center located in Winchester, VA, USA. Apple cultivars, rootstocks and their planting years are reported in Table 5.1. All trees were subjected to dormant pruning annually and pests were controlled annually according to standard regional bestpractices (18). The trees had not received nitrogen fertiliser since 2010. Apples were

tested for maturity by the starch iodine test (19) and were harvested at commercial maturity, defined as a minimum of 60% of the flesh stained in the starch iodine test. Thirty apples of each cultivar were picked from trees with heights ranging from 1 to 2 m above the ground from all sides of the exterior canopy of each tree. The 30 apples of each cultivar were randomly separated into three groups, with 10 apples for each group representing an analytical replicate (n = 3). Each replicate was handled separately throughout the juicing process, sample preparation and storage. Fruit were juiced (Champion Juicer 2000, Lodi, CA, USA) on the day of harvest. Juice was then stored at −20°C in 15 mL centrifuge tubes and frozen juice samples were transported on ice from Winchester, VA to Blacksburg, VA (USA), where they were stored at −80°C until analysis.

Table 5. 1. Apple cultivars, rootstocks, and their year of establishments

Cultivar	Rootstock	Year of planting
Empire	MARK	1991
Enterprise	M.9	1995
Golden Delicious	M.9	2000
Shenandoah	M.9	2011
Rome	MM.111	1988
Arkansas Black	MM.111	1983
Blacktwig	MM.111	1983
Field Red	MM.111	1983
Granny Smith	MM.111	1983
Newtown Pippin (syn. Albemarle Pippin)	MM.111	1983
Northern Spy	MM.111	1983
Winesap	MM.111	1983
York	MM.111	1983

5.2.2 Amino acid analysis

Twenty amino acids were identified and quantified in the apple juice samples using a commercially available amino acid analysis kit (Waters UPLC® Amino Acid Analysis Solution, Milford, MA, USA) (20). Modifications were made to add analytes of interest to the calibration standard, specifically amino acids previously reported in apple juice, but not in the standard mixture provided which was designed for feed hydrolysate analysis. Three replicates were analysed for each of the 14 cultivars in the study, and results are reported as the mean values ± standard error of the mean (SEM) of these replicates.

5.2.3 Sample and standard preparation, and derivatisation

All juice samples and standards were thawed to 4°C, filtered through PTFE 0.22 µm membrane filters (Micro Solv, Eatontown, NJ, USA) at room temperature immediately prior to analysis and spiked with internal standard L-(+)-norvaline (Acros Organics, NJ, USA) to a final concentration of 2.5 mM in the juice. Waters Amino Acid Hydrolysate Standard was a mixture of protein hydrolysate standard, containing 2.5 mmM of each of the following amino acids dissolved in 0.1 N HCl (except as noted): L-alanine, L-arginine, L-aspartic acid, L-cystine (1.25 mM), L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine. Four additional amino acids – norvaline, L-glutamine (Sigma-Aldrich, St Louis, MO, USA), GABA (Sigma-Aldrich) and L-asparagine (Sigma-Aldrich) – were dissolved in 0.1M HCl solution to make four separate stock solutions with 5 mM concentration. Fifty microliters of each stock solution and 100 µL ofWaters Amino Acid Hydrolysate Standard were mixed with 700 µL ultrapure water (Millipore Milli-Q water purification system), to make the working standard, consisting of 0.25

mM of each of the amino acids, except for cysteine, which was at 0.125 mM. All juice samples and standards were derivatised using an AccQ•Tag Ultra Derivatisation Kit (Waters Corporation, Milford, MA, USA) according to the manufacturer's instructions (20). Both primary and secondary amino acids react with the Waters AccQ•Tag Ultra Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate), generating stable amino acid derivatives with UV absorbance character, and excessive reagent was hydrolysed to 6-aminoquinoline, which does not interfere with the detection of amino acids (20).

5.2.4 Analysis by UPLC/PDA

Chromatographic conditions were set according to the manufacturer's instructions for Waters UPLC-PDA cell culture, hydrolysates and alkylated cysteine analysis. A Waters AccQ•Tag Ultra Amino Acid Analysis Column (a certified BEH C18, 1.7 µm column specifically for amino acid analysis) was used on a Waters H-Class UPLC/PDA system. The following mobile phases (A–D) were used for the separation for a total run of 10 min per sample: A being 100% AccQ•Tag Ultra eluent A concentrate; B being 90:10 water–AccQ•Tag Ultra eluent B; C being 100% HPLC-grade water; and D being 100% AccQ•Tag Ultra eluent B. Amino acids were detected at the wavelength of 260 nm. Peaks were integratedand quantified by Empower™ Software, using the ApexTrack function (Waters Corporation, Milford, MA, USA).

5.2.5 Statistical analysis

The mean and SEM for the absolute concentration of each amino acid in each cultivar were calculated from the (n = 3) replicates. The total concentration of amino acids in a given samplewas calculated by summing the measured concentrations of individual amino acids observed in that sample. The mean and SEM for total amino acid concentration were then calculated for each cultivar from the (n = 3) replicates. For each analytical replicate (n = 3) within each juice sample (n = 13), the relative concentration of each amino acid was calculated. The relative concentration of a given amino acid refers to the proportion of the total amino acid concentration in a given sample represented by a particular amino acid. The mean and SEM of the relative concentration of each amino acid in each juice sample were then calculated from the replicates for each juice sample (n = 3) and the mean relative concentrations of each juice sample were plotted as a boxplot using GraphPad Prism v6.0e (GraphPad Software Inc., La Jolla, CA, USA).To determine whether relative amino acid profiles differed among juice samples, the profile data were submitted to discriminant correspondence analysis (DICA) (21), amultivariate analysis that determines whether the average relative amino acid profile of a juice sample differs in any important dimension from others. DICA is an extension of discriminant analysis (22) for nominal datasets (23) – such as the proportional data of the amino acid profiles discussed here. Discriminant analyses are themultivariate equivalents of univariate post hoc analyses, e.g. Fisher's least significan difference (22). In summary, these analyses create new variables to describe the observations as linear combinations of the original, measured variables, with the constraint that the new variables must separate treatment 'barycentres' (the mean vectors) as much as possible. Maps of the first two dimensions of the solution were produced for both the juice samples and the amino acids. For inference, 95% confidence boundaries around the cultivars were calculated (with Bonferroni corrections for multiple comparisons) by bootstrapping (1000 resamples), and the significance of the total variance in relative amino acid profile explained by juice sample type (cultivar; R^2) as evaluated by permutation tests (1000 permutations). The confidence ellipses were plotted directly onto the maps for the apple juice samples. When two confidence ellipses do not overlap, the groups are considered discriminable

at the chosen confidence level. All analyses were conducted in R using the ExPosition (24) and PTCA4CATA (25) packages.

5.3 Results and discussion

5.3.1 Juice amino acid profiles

With sample preparation by AccQ•Tag™ Derivatisation Chemistry and separation by UPLC, the Waters Acquity UPLC Amino Acid Solution is an analytical method that has been validated for quantification of amino acids in food with sufficient peak resolution and precise results (20, 26). It has been widely applied in the amino acid analysis of various food and biological samples (27) such as wine (28–30), apple juice (17), milk (31), royal jelly (32) and protein hydrolysates (33). L-Amino acids were of interest in this study because they are the form involved in cell metabolism (34).

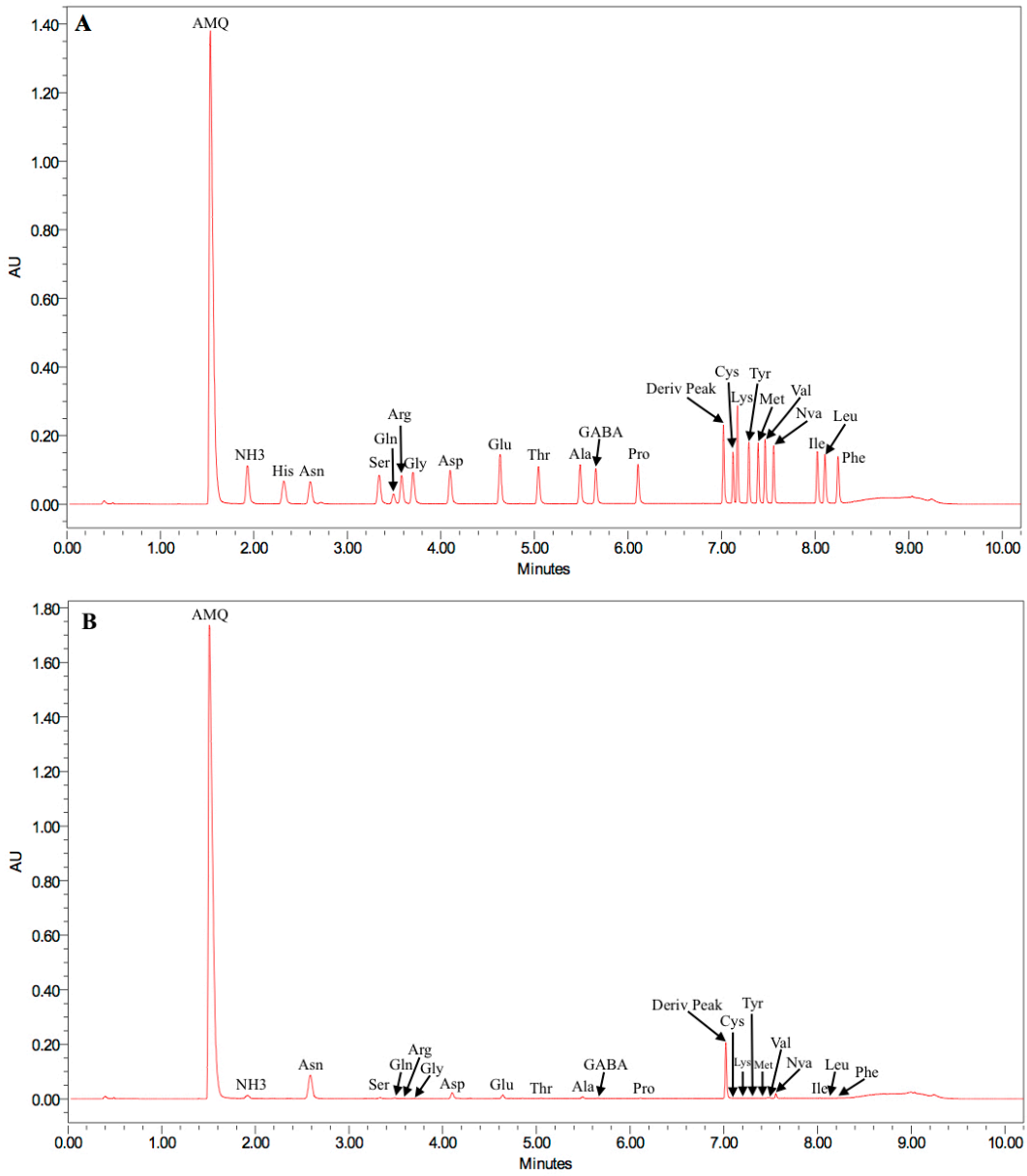

Figure 5. 1. Chromatograms of amino acids in (A) calibration standards and (B) juice sample of Enterprise diluted 10 times at the detection wavelength of 260 nm.

Twenty amino acids were analysed and quantified, and the chromatographic separation of the calibration standards and one juice sample (Enterprise, diluted 10 times) is shown in Fig. 5.1. The concentrations of each individual amino acid observed in each juice are shown in Fig. 5.2, and the values of concentrations of individual amino acid for each juice sample are presented in Table 5.1 of the Supporting Information. The means of the relative concentrations of each individual amino acid for each juice sample are presented in Fig. 5.3. Asparagine was the most abundant amino acid, on average, with the highest proportion being 49.7 ± 0.6% of the total amino acids in the Newtown Pippin sample, and the lowest proportion being 4.0 ± 0.04% of the total amino acids in the Golden Delicious sample. While the proportions of amino acids observed in a juice sample from a given cultivar may be influenced by growing season (13), crop load (9), site, rootstock and other factors (10), our data provide insight as to the extent of variation for each amino acid, and the ranges of relative concentrations of amino acids that could be expected in apple juice. The predominance of asparagine among all other amino acids was consistent with previous studies (15, 17, 35–37), although some prior research also reported threonine being the predominant amino acid (53.4%) in Fuji apple juice (38) and aspartic acid being the major amino acid (41.1%) in Brazilian dessert apples (39).

Phenylalanine was the second most abundant amino acid in most of the juices examined, with Rome having the highest proportion of phenylalanine with 49.2 ± 1.0% of the total amino acids, while Enterprise had the lowest proportion of phenylalanine with 4.8 ± 0.06% of the total amino acids. This finding differs from previous reports of phenylalanine representing only a small proportion of the total amino acids in apples, as low as 0.2–0.4% in dessert apples (17, 38, 39). Trace amounts of phenylalanine were found in British cider apples (15), and an average of 1.7–4% of the total amino acids was found in Spanish cider apples (36, 37). Since phenylalanine made a greater contribution to the total amino acid concentration for the juices in this study than previously reported, the potential impact merited consideration. Phenylalanine is a precursor to 2-phenylethanol, a volatile compound with a rose-like aroma (40,41), and to polyphenol compounds with demonstrated health benefits (42). Previous reports show that wine grapes contain greater concentrations of phenylalanine than apples (1.46% of the free amino acids on average in 11 grape varietals) (43), thus while effects of phenylalanine concentration in grape juice on wine composition have been demonstrated, this potential has not yet been examined in apples and cider.

Aspartic acid was also found to be a major amino acid in this study. The relative concentration of aspartic acid was as high as 16.01 ± 0.04% of the total amino acids in Winesap and as low as 4.82 ± 0.04% of the total amino acids in Rome. Our results were similar to those for Spanish cider apples, where the average relative aspartic acid concentration for six cultivars in one study was 12.51% (36) and for 17 cultivars in another study was 13.7% (37). The apples in our study contained a higher proportion of aspartic acid than was reported in a study of six British cider apple cultivars, in which the relative concentration of aspartic acid was 5.88 ± 1.63% on average (15). The apples in our study contained less aspartic acid compared with Fuji apples grown in China, in which the relative aspartic acid concentration was 26.5 %, and for Brazilian dessert apples, where 41.1% aspartic acid was reported (38, 39).

Glutamic acid is anothermajor amino acid in apples. The highest relative concentration of glutamic acid we observed was 9.99 ± 0.005% in Winesap and the lowest was 2.30 ± 0.04% of the total amino acids in Granny Smith. Here, apples contained more glutamic acid than British cider apples, in which the relative concentration of glutamic acid was 1.93 ± 1.48% on average (15), but much less than Spanish cider and Brazilian dessert apples, which the average relative

concentrations of glutamic acid were 13.5% across six cultivars (36), and 14.7% across nine cultivars (39), respectively. Our results are similar to the relative concentration of glutamic acid observed in Fuji apples grown in China (7.7%) (38) and some Spanish cider apples, for which the relative concentration of glutamic acid was 5.9% across 17 cultivars (37).

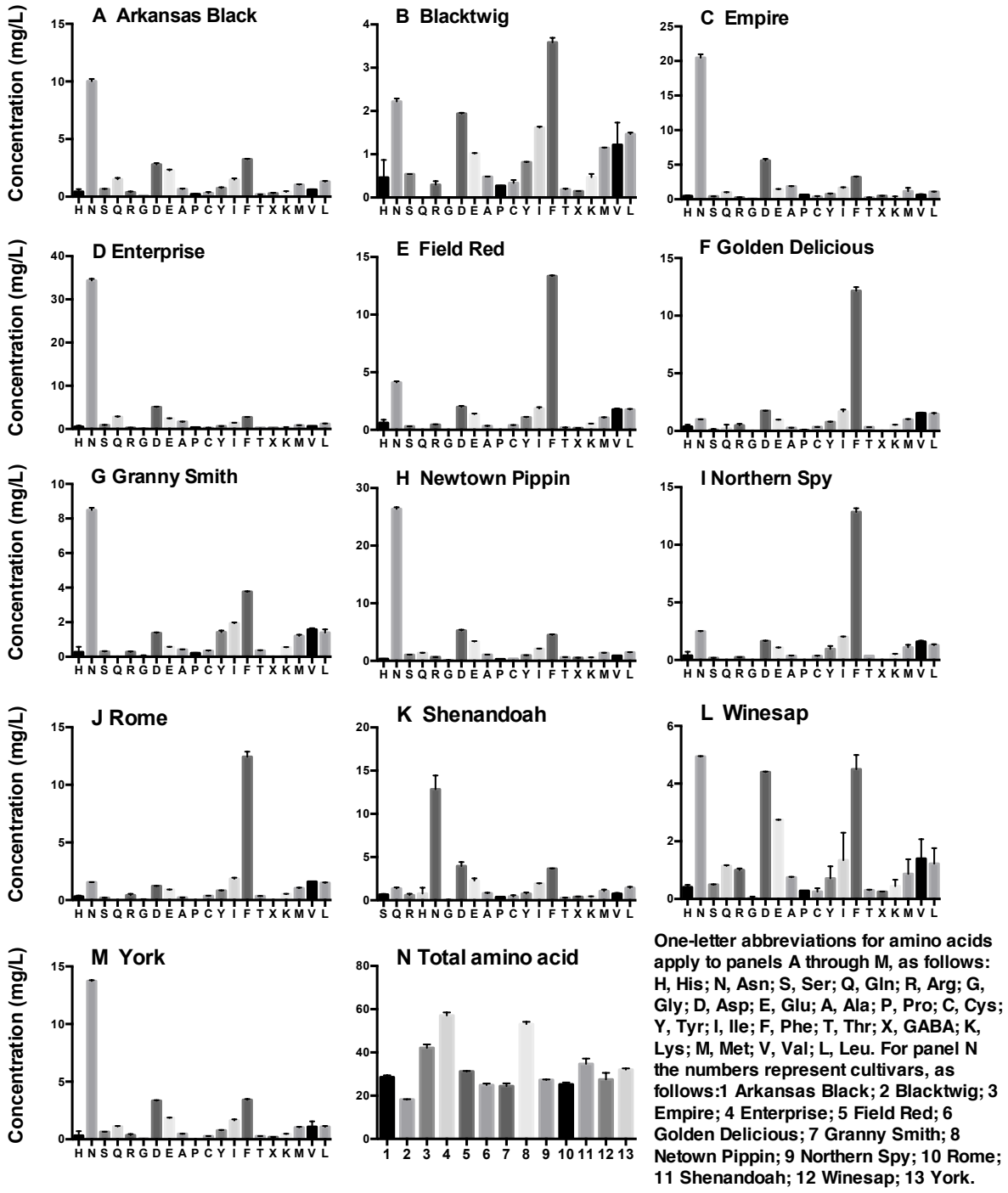

Figure 5. 2. Concentrations of individual amino acid in apple juices (A through M) and the concentrations of total amino acid in 13 apple juice samples (N). Values were plotted as mean and SEM for n = 3. Note: the scales on the Y-axis are not identical for all sub-figures.

The relative concentration of glutamine had high variation from cultivar to cultivar in our study, with Arkansas Black having the highest relative concentration of glutamine (5.28 ± 0.24%), while it was not detected in Blacktwig, Field Red, Granny Smith, Northern Spy and Rome. These results are similar to the relative concentration of glutamine reported in Spanish cider apples (2.4% on average across six cultivars) (36). Commercial apples grown in China and other Spanish cider apples contained slightly more glutamine than we observed, with relative concentrations of glutamine of 7.4% across eight cultivars and 6.5% across 17 cultivars, respectively (17, 37).

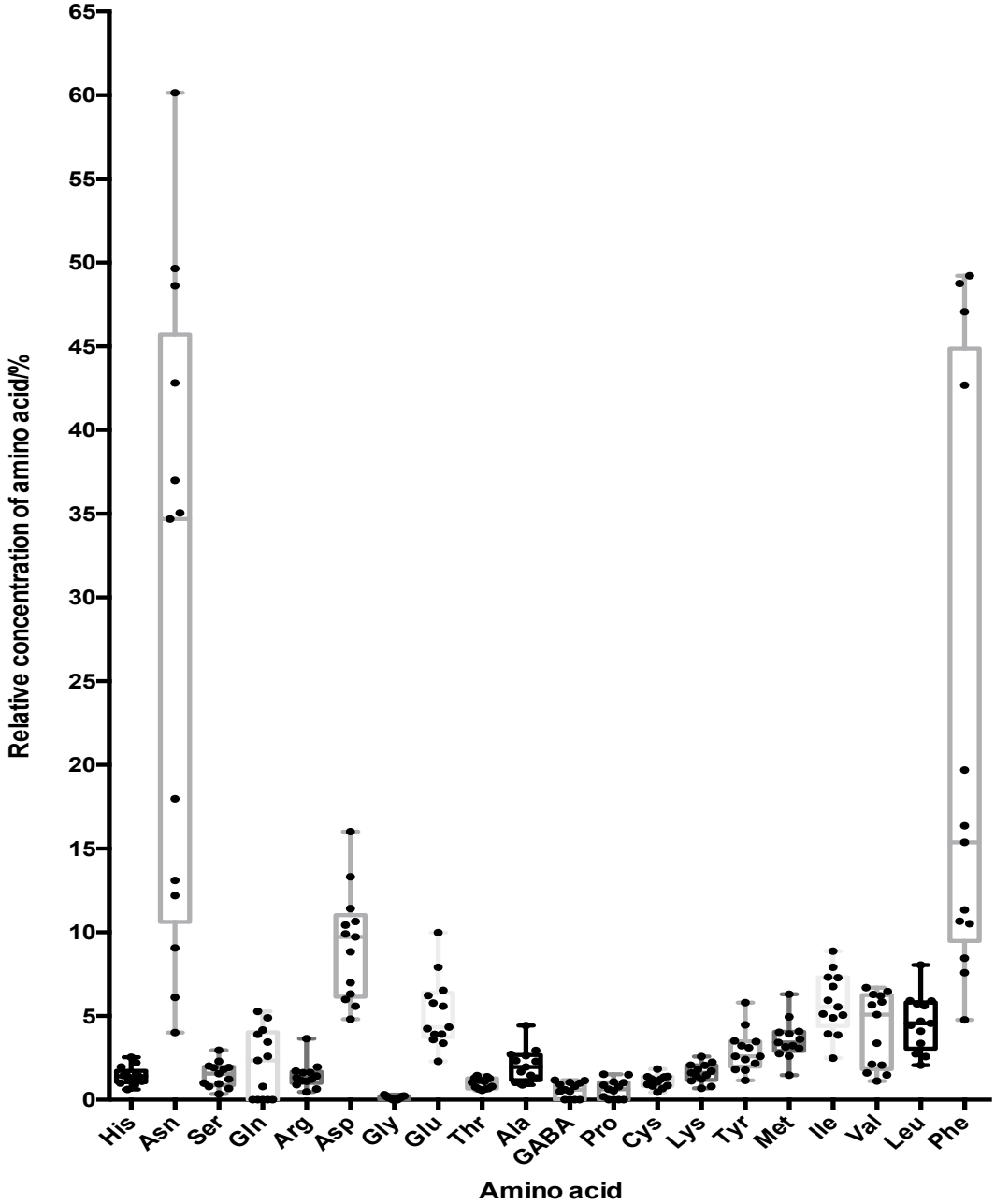

Figure 5. 3. Relative concentrations of individual amino acid in each apple juice sample for 20 amino acids. Values were plotted with the mean of each apple sample for n = 13 cultivars.

Serine, arginine, threonine, alanine, GABA, proline, lysine, tyrosine, methionine, valine, isoleucine and leucine were also present inmost apple juices examined, although at relatively lower concentrations than the other amino acids.

The relative concentrations of amino acids we observed in apples with potential for cider making were different than those of Vitis vinifera wine grapes. Others have reported that *Vitis vinifera* wine grapes are richest in proline and arginine (44–46). The concentration of arginine was 0.45 ± 0.05 mg/L across the 13 apple cultivars, making up only 1.51 ± 0.21% of the total amino acids analysed, in contrast to grape juice, where arginine contributed from 2 to 68%previous studies (30, 43, 45). The absolute concentration of arginine we observed (0.45 ± 0.05 mg/L) was much lower than previously reported in white grape *Vitis vinifera* cultivars grown in Washington State (107 ± 26 mg/L to 1646 ± 141 mg/L) (44), or the mean arginine concentration of Chardonnay juices from California (550 mg/L) and Washington State (389 mg/L) (46). The average concentration of proline was 0.23 ± 0.03 mg/L in the 13 apple juices, which made up 0.69 ± 0.16% of the amino acids observed. These values are much lower than the relative concentration range of proline observed in grape juice. Proline has been found to be the predominant amino acid in several grape juices, ranging from 4.3 to 32.2% of the total nitrogen (47). These differences in grape vs apple juice chemistry are notable, as the vast majority of the fruit beverage fermentation literature has been using Vitis vinifera grape where proline or arginine are usually by far the most prevalent yeast assimilable form of nitrogen. Wine yeasts, yeast nutrient supplements and wine making practices have been developed and optimised almost exclusively using grape systems, although these products and approaches are also commonly extrapolated to cider making. Different strains and species of wine yeasts can generate wines with different sensory characteristics owing to the diversity of secondary metabolites they produce. Furthermore, wine yeasts perform differently in grape must or juice with different composition, or in the presence of different yeast nutrient supplements (48). Amino acids in apple juice are the major contributors to the formation of higher alcohols and esters through the Erhlich pathway and esterification during cider fermentation. Eleutério dos Santos et al. (39) have suggested the following relationships between certain amino acids and cider flavour: asparagine, aspartic acid and glutamine generate the structure of cider flavour, while consumption of threonine, lysine and cysteine by yeast does not impact overall cider flavour (39). Pre-fermentation supplementation of apple juice with asparagine and glutamine has been shown to generate a greater concentration of esters during fermentation, resulting in cider with a fruitier flavour, compared with cider made from un-supplemented juice (6). Information on the initial amino acid concentration in apple juice, and how this composition varies by cultivar, could enable cider makers to make targeted adjustments to pre-fermentation amino acid composition with the goal of producing ciders with desired flavour and style (6). This potential, as well as the lack of information on yeast strain and yeast nutrient supplement performance in cider fermentation systems, suggests further research into these applications. Understanding the variation in amino acid composition among apple cultivars used for cider making provides the necessary foundation for this future work.

The relative amino acid profile of an apple juice is defined as the composition of amino acids present in a given apple juice sample. Thus, two apple juices with the same relative concentration of each amino acid but which differ in absolute concentrations of each amino acid would have the same relative amino acid profile. DICA, a multivariate method, allows the comparison of the profiles of amino acids across apple juice samples, simultaneously, rather than

comparing concentration of each individual amino acid among apple juice samples. These analyses are preferred to univariate treatments – like ANOVA followed by post hoc testing – because they take advantage of multicollinearity between observed variables that might be lost or even detrimental to univariate approaches, and do not suffer from familywise error inflation (22). The observations and/or treatment means can be visualised in two-dimensionalmaps using any pair of the new, discriminant variables to plot their positions. As in many multivariate analyses, proximity of two observations or two variables to each other indicates similarity in profile; unlike in these analyses, direct relation between observations and variables by spatial proximity is not possible because of their symmetrical roles in forming the space (49); the maps of the observations and variables are compared to infer relationships between the two. The quality and stability of this solution can be evaluated using computational methods like permutation tests and bootstrapping (50).

Apple juice sample group membership accounted for the majority of the variation in relative amino acid profile between the juice samples, and permutation tests on the data showed that this was unlikely to occur by chance (R^2 = 92%, p = 0.001). The first two dimensions of the DICA solution explained 91% of the variance in the mean profiles for the juice samples (see Figs 5.4 and 5.5). Dimension 1, which accounted for 82% of the variance in the juice samples, separated the juice samples into three groups driven by the relative concentration of phenylalanine and asparagine (see also Fig. 5.6: phenylalanine and asparagine contribute the most to the spatial configuration in dimension 1). Group 1 comprised Golden Delicious, Rome, Northern Spy and Field Red with high relative concentration of phenylalanine and low relative concentration of asparagine; group 2 was Blacktwig, Winesap, Arkansas Black, Shenandoah, Granny Smith and York with intermediate relative concentrations of phenylalanine and asparagine; and group 3 was Empire, Enterprise, and Newtown Pippin with low relative concentrations of phenylalanine and high relative concentrations of asparagine. In general, apple juice samples placed to the right in Fig. 5.4 had higher relative concentrations of phenylalanine, while juice samples on the left had higher relative concentrations of asparagine.

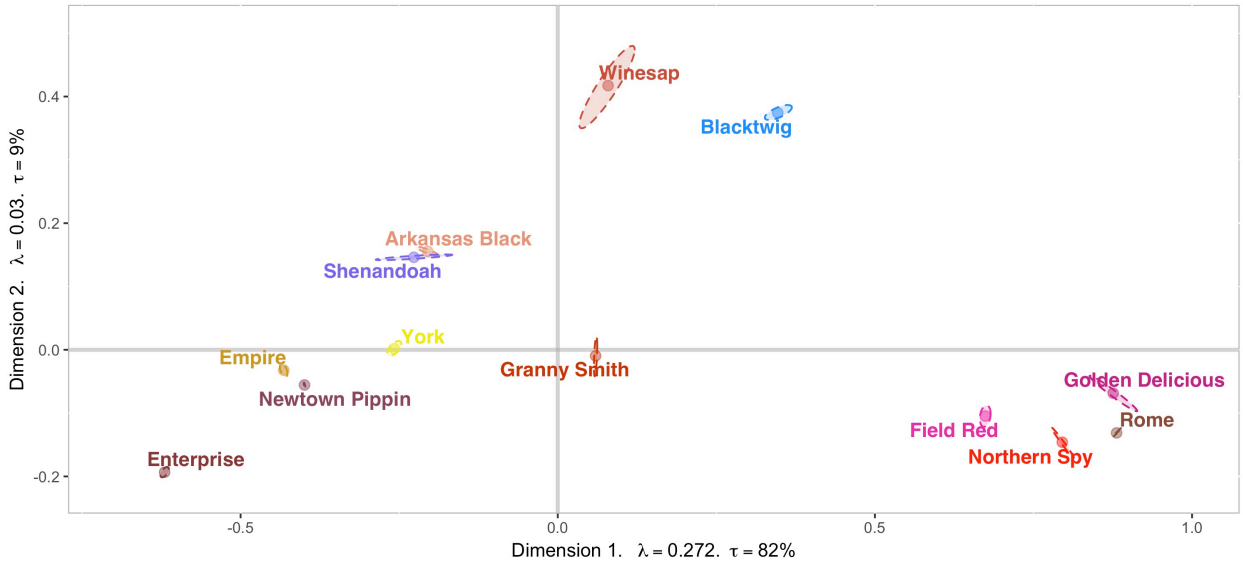

Figure 5. 4. Factor map of apple juice samples from DICA based on the amino acid profiles in apple juices of 13 apple cultivars. Confidence ellipses are based on bootstrapping (95% confidence level, 1000 replications). Filled circles represent the barycenters (treatment means) for each apple juice sample.

Dimension 2, which accounted for much less of the variance (9%), separated the relative amino acid profiles of apples mainly by the relative concentration of aspartic acid, glutamic acid, arginine, phenylalanine and asparagine (Figs 5.4 and 5.6). In general, cultivars with higher relative concentrations of asparagine, glutamic acid and arginine (such as Winesap, Shenandoah and Arkansas Black, in the upper quadrants of Fig. 5.4) were contrasted with those with higher relative concentrations of phenylalanine or asparagine (such as Empire and Northern Spy, in the lower quadrants of Fig. 5.4).

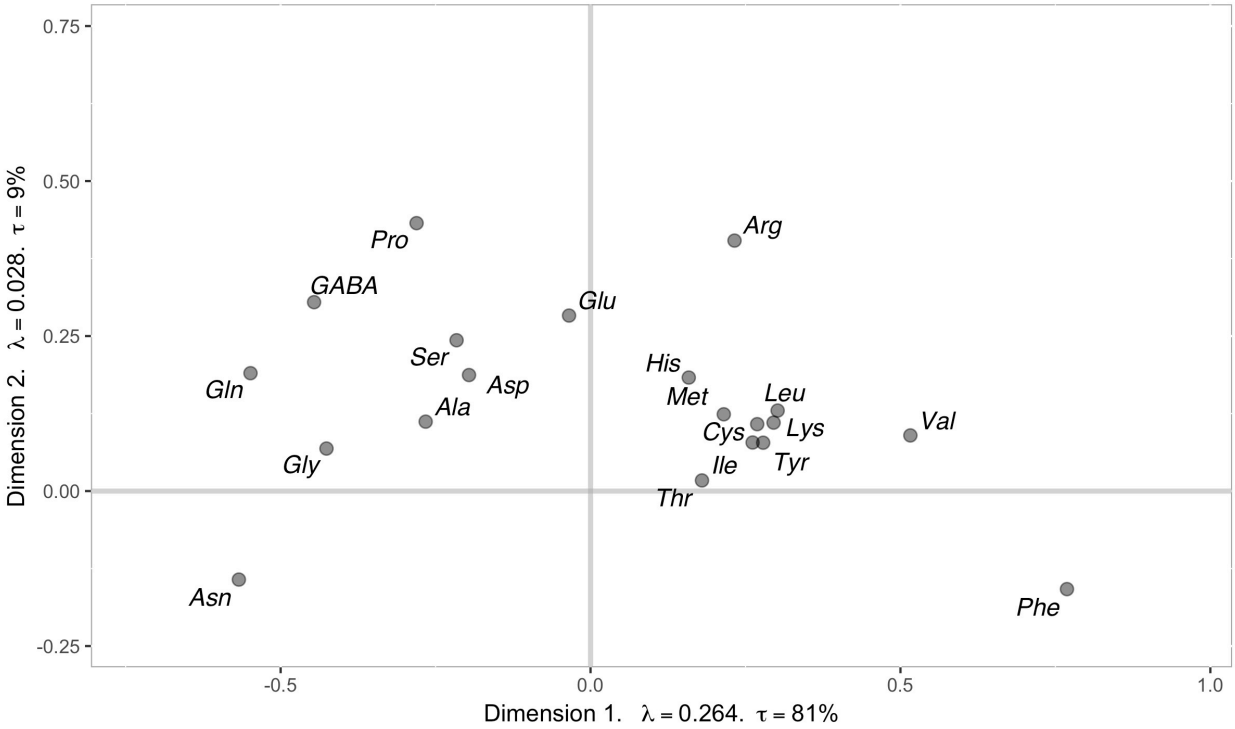

Figure 5. 5. Factor map of amino acids from DICA based on the amino acid profiles in apple juices of 13 apple cultivars.

Like asparagine, higher concentrations of aspartic acid and glutamic acid in apple juice prior to fermentation have also been linked to the generation of higher concentrations of volatile esters by yeast and may therefore impart positive sensory characteristics to the cider. While the relative concentrations of asparagine and glutamic acid did not play an important role in separating the apple juice samples in the first dimension, which accounted for the majority of variation in relative amino acid profiles, they are the major amino acids driving the separation of relative amino acid profiles in the second dimension, which accounted for a small but above-average amount of the variation. Thus, the juice samples on the top-left in Fig. 5.4 might be preferred over other juice samples to achieve fruity, ester-driven cider styles owing to higher relative concentrations of asparagine, aspartic acid and glutamic acid, which can generate pleasant and fruity aromas during fermentation. While these data provide a preliminary indication of which cultivars may be preferred in terms of volatile aroma production via amino acid metabolism, further research will be required to determine whether the results of this study can be generalised to the same cultivars grown under different conditions.

Confidence ellipses for relative amino acid profiles of each juice sample are presented in Fig. 5.4, but are in some cases are so small as to be difficult to visualise. Therefore – apart from

some confusion between Shenandoah and Arkansas Black – the relative amino acid profiles of each juice were easily discriminated. We conclude that the profiles of amino acids were different among the juice samples tested. Cider makers should be aware of the differences in relative amino acid profile between juices used for cider production, as these differences can result in different aromas and flavours of the finished ciders, and different outcomes resulting from yeast nutrient supplementation. One limitation of this study was that fruit from only one tree per genotype was included in the sample set, although a wide range of genotypes with potential for cider production would be evaluated. In the future, we plan to use the same approach to more thoroughly characterise the amino acid composition of apple cultivars specifically, in variety trials and/or agricultural experiments.

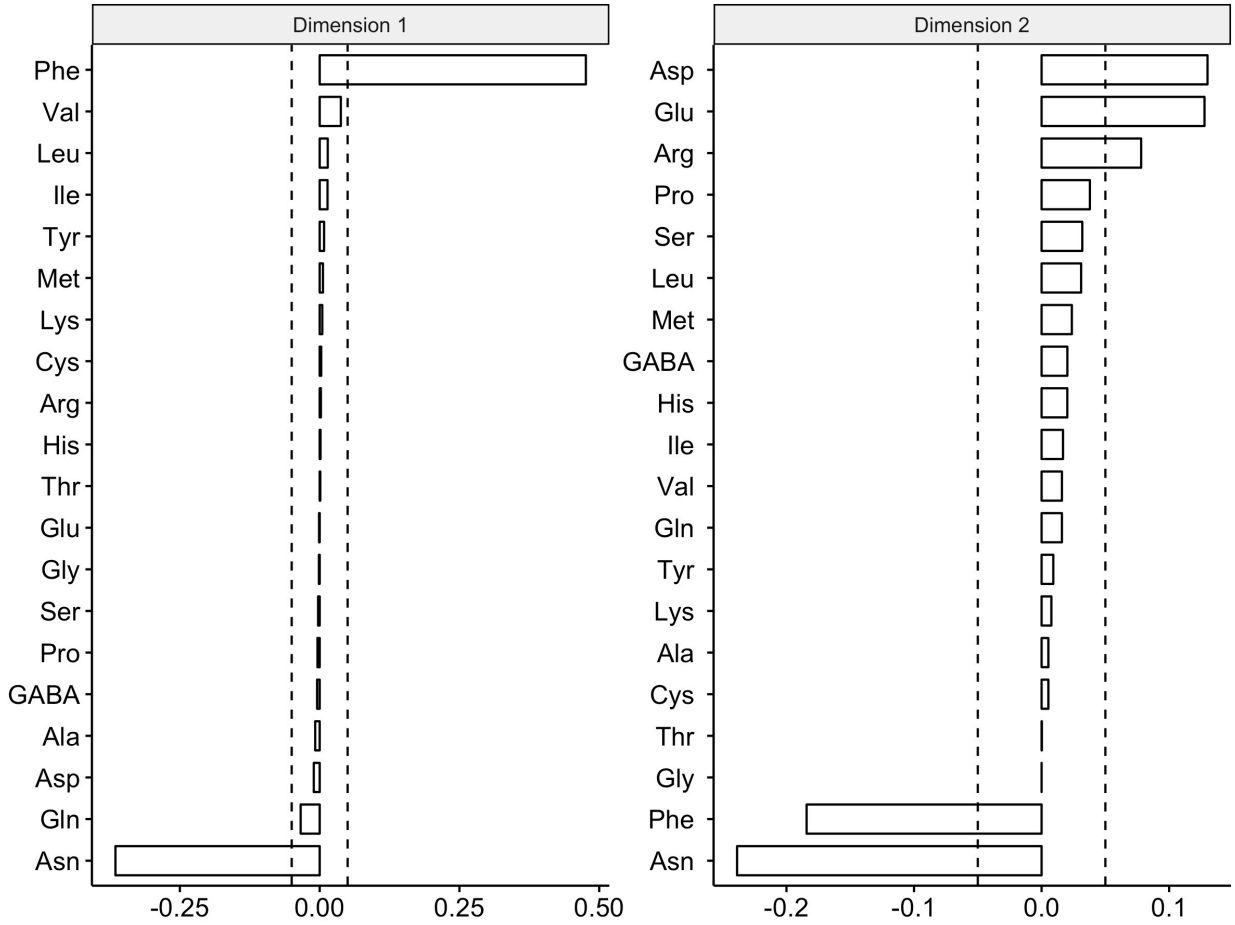

Figure 5. 6. Contributions of amino acids to each dimension in the DICA; dotted line indicates the expected (average) contribution. Contribution is defined as the proportion of explained variance in a dimension attributable to the variable in question. Sign (+/-) indicates association with positive or negative direction of the dimension (see Figure 5.5).

5.3.2 Total amino acids between juices

The total concentration of amino acids among samples are summarised in Supporting Information Table 5.1. Most of the total amino acid concentrations differed between juices, with some exceptions, as noted. Total amino acid concentration in the juices from 13 cultivars ranged from 18 ± 0.2 mg/L in Blacktwig to 57 ± 0.8 mg/L in Enterprise, with an average of 33 ± 1.5 mg/L. These results are lower than previously reported total amino acid concentrations in Brazilian dessert apple musts (from 192 mg/L to 431 mg/L) (39), Fuji apples grown in China

(617.81 mg/L) (38), Spanish cider apples (81.39 ± 39.14 mg/L) (37) and British cider apples (average of 63 mg/L for six cultivars) (15). These differences could be attributable to multiple factors including differences in nitrogen fertilisation practices among growing regions. Owing to this wide variation in amino acid concentration among apples, the initial YAN should be measured to determine whether yeast nutrient additions are required when using juices from different apple cultivars and growing regions.

5.4 Conclusions

The amino acid composition of 13 apple juice samples of cultivars grown in Virginia with the potential for cidermaking was reported. Relative amino acid profiles differed among juice samples, and the total concentration of amino acids also differed among juice samples in this study. The observed differences in relative amino acid profile have the potential to result in differences in cider flavour and aroma. This represents the first report of individual amino acid concentrations for apples with potential for cider making in North America. The multivariate statistical methods used in this work can be applied to future studies on the influence of cultivar and/or agricultural practices on relative amino acid profiles in apple juice. By identifying the ranges of amino acid concentrations in apple juices with potential for cider making, these results are also expected to inform future research on yeast strains and yeast nutrient supplement products specifically for cider fermentation.

5.5 Acknowledgments

The authors would like to thank Abby Kowalski, David Carbaugh, Melissa Wright and the staff of the Alson H. Smith, Jr Agricultural Research and Extension Center, Virginia Tech. The authors also would like to thank Dr Andrew Lee from Department of Food Science and Technology of Virginia Tech for his help with formatting the references.

5.6 Funding

This work was supported by the 2014-2016 USDA Specialty Crop Block Grant (grant number 301-15-046) awarded by Virginia Department of Agriculture and Consumer Services and salary
support for Drs. Stewart and Peck from the Virginia Agricultural Experiment Station.

5.7 References

1. Alberti, A., Vieira, R. G., Drilleau, J. F., Wosiacki, G., and Nogueira, A. (2011) Apple wine processing with different nitrogen contents, Braz. Arch. Biol. Technol. 54, 551–558.

2. Kelkar, S., and Dolan, K. (2012) Modeling the effects of initial nitrogen content and temperature on fermentation kinetics of hard cider, J. Food Eng. 109, 588–596.

3. Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., and Henschke, P. A. (2007) Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically definedmedium by *Saccharomyces cerevisiae* wine yeasts, Appl. Microbiol. Biotechnol. 77, 145–157.

4. Boudreau, T. F., Peck, G. M., O'Keefe, S. F., and Stewart, A. C. (2017) The interactive effect of fungicide residues and yeast assimilable nitrogen on fermentation kinetics and hydrogen sulfide production during cider fermentation, J. Sci. Food Agric. 97, 693–704.

5. Henschke, P. A., and Jiranek, V. (2002) Yeasts metabolism of nitrogen compounds, in Wine Microbiology and Biotechnology, (Fleet, G. H. Ed.), pp. 77–164, Taylor & Francis, London.

6. Santos, C. M. E. d., Alberti, A., Pietrowski, G. d. A. M., Zielinski, A. A. F., Wosiacki, G., Nogueira, A., and Jorge, R. M. M. (2016) Supplementation of amino acids in apple must for the standardization of volatile compounds in ciders, J. Inst. Brew. 122, 334–341.

7. Jiranek, V., Langridge, P., and Henschke, P. A. (1995) Amino acid and ammonium utilization by Saccharomyces cerevisiae wine yeasts from a chemically defined medium, Am. J. Enol. Vitic. 46, 75–83.

8. Boudreau, T. F., Peck, G. M., Ma, S., Patrick, N., Duncan, S., O'Keefe, S. F., and Stewart, A. C. (2017) Hydrogen sulphide production during cider fermentation is moderated by pre-fermentation methionine addition, J. Inst. Brew. 123, 553–561.

9. Peck, G., McGuire, M., Boudreau, T., and Stewart, A. (2016) Crop load density affects 'York' apple juice and hard cider quality, Hortscience 51, 1098–1102.

10. Bell, S.-J., and Henschke, P. A. (2005) Implications of nitrogen nutrition for grapes, fermentation and wine, Aust. J. Grape Wine Res. 11, 242–295.

11. Villière, A., Arvisenet, G., Bauduin, R., Le Quéré, J.-M., and Sérot, T. (2015) Influence of cider-making process parameters on the odourant volatile composition of hard ciders, J. Inst. Brew. 121, 95–105.

12. Lozano, J. E. (2006) Chemical composition of fruits and its technological importance, in Fruit Manufacturing, pp. 133–161, Springer, New York.

13. Boudreau, T. F., Peck, G. M.,O'Keefe, S. F., and Stewart, A. C. (2018) Free amino nitrogen concentration correlates to total yeast assimilable nitrogen concentration in apple juice, Food Sci. Nutr. 6, 119–123.

14. Blanco Gomis, D., Picinelli Lobo, A. M., Gutiérrez Alvarez, M. D., and Mangas Alonso, J. J. (1990) Determination of amino acids in apple extracts by high performance liquid chromatography, Chromatographia 29, 155–160.

15. Burroughs, L. F. (1957) The amino-acids of apple juices and ciders, J. Sci. Food Agric. 8, 122–131. 16. Beech, F. W., and Carr, J. G. (1977) Cider and perry, in Alcoholic Beverages, (Rose, A. H. Ed.), pp. 139–313, Academic Press, London.

17. Wu, J., Gao, H., Zhao, L., Liao, X., Chen, F., Wang, Z., and Hu, X. (2007) Chemical compositional characterization of some apple cultivars, Food Chem. 103, 88–93.

18. Pfeiffer, D. G., Bergh, J. C., Fell, R. D., Hooks, C. R. R., Peck, G. M., Walsh, C. S., Yoder, K. S., Biggs, A. R., Kotcon, J. B., Derr, J. F., Chandran, R. S., Weaver, M. J., Brown, A., and Parkhurst, J. (2014) Spray bulletin for commercial tree fruit growers, Virginia, West Virginia, and Maryland Cooperative Extension, VCE Publications, Virginia Tech, 456–419.

19. Blanpied, G., and Silsby, K. J. (1992). Predicting harvest date windows for apples, Cornell Cooperative Extension.

20. Waters (2012) Acquity UPLC H-Class and H-Class Bio Amino Acid Analysis System Guide, Waters Corporation, Revision B.

21. Abdi, H. (2007) Discriminant correspondence analysis, in Encyclopedia of Measurement and Statistics, (Salkind, N. Ed.), pp. 270–275, Sage, Thousand Oaks, CA.

22. Rencher, A. C. (2002) Discriminant analysis, in Methods of Multivariate Analysis, 2nd ed., pp. 270–298, John Wiley & Sons, New York.

23. Abdi, H., and Béra, M. (2014) Correspondence analysis, in Encyclopedia of Social Networks and Mining, (Alhajj, R., and Rokne, J. Eds.), pp. 275–284, Springer, New York.

24. Beaton, D., Fatt, C. C., and Abdi, H. (2014) An ExPosition of multivariate analysis with the singular value decomposition in R, Computational Statistics & Data Analysis 72, 176–189.

25. Abdi, H. (2017, in development). The development page for PTCA4CATA: Partial Triadic Analysis for Check All That Apply (CATA). Available from: https://github.com/HerveAbdi/PTCA4CATA (last accessed Jan 2018)

26. Salazar, C., Armenta, J. M., Cortés, D. F., and Shulaev, V. (2012) Combination of an AccQ·Tag-ultra performance liquid chromatographic method with tandem mass spectrometry for the analysis of amino acids, in Amino Acid Analysis: Methods and Protocols, (Alterman, A. M., and Hunziker, P. Eds.), 828, Humana Press, Totowa, NJ.

27. Armenta, J. M., Cortes, D. F., Pisciotta, J. M., Shuman, J. L., Blakeslee, K., Rasoloson, D., Ogunbiyi, O., Sullivan, D. J., and Shulaev, V. (2010) Sensitive and rapid method for amino acid quantitation in malaria biological samples using AccQ•Tag ultra performance liquid chromatography–electrospray ionization-MS/MS withmultiple reaction monitoring, Anal. Chem. 82, 548–558.

28. Aubin, A., Hynes, M., and Shocker, J. (2007) Acquity UPLC for the rapid analysis of amino acids in wine, Waters Corporation.

29. Fiechter, G., andMayer, H. K. (2011) UPLC analysis of free amino acids in wines: profiling of on-lees aged wines, J. Chromatogr. B 879, 1361–1366.

30. Kelly, M., Gill Giese, W., Velasco-Cruz, C., Lawson, L., Ma, S., Wright, M., and Zoecklein, B. (2017) Effect of foliar nitrogen and sulfur on petit manseng (*Vitis vinifera L.*) grape composition, Journal of Wine Research 28, 165–180.

31. Lu, W., Lv, X., Gao, B., Shi, H., and Yu, L. (2015) Differentiating milk and non-milk proteins by UPLC amino acid fingerprints combined with chemometric data analysis techniques, J. Agric. Food Chem. 63, 3996–4002.

32. Liming, W., Jinhui, Z., Xiaofeng, X., Yi, L., and Jing, Z. (2009) Fast determination of 26 amino acids and their content changes in royal jelly during storage using ultra-performance liquid chromatography, J. Food Compos. Anal. 22, 242–249.

33. Boogers, I., Plugge, W., Stokkermans, Y. Q., and Duchateau, A. L. L. (2008) Ultra-performance liquid chromatographic analysis of amino acids in protein hydrolysates using an automated pre-column derivatisation method, J. Chromatogr. A 1189, 406–409.

34. Murray, R. K., Granner, D. K., Mayes, P. A., and Rodwell, V. W. (2014) Amino acids & peptides, in Harper's Illustrated Biochemistry, 30th ed., pp. 15–24, McGraw-Hill, New York.

35. Zhang, Y., Li, P., and Cheng, L. (2010) Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in 'Honeycrisp' apple flesh, Food Chem. 123, 1013–1018.

36. Mangas, J. J., Moreno, J., Picinelli, A., and Blanco, D. (1998) Characterization of cider apple fruits according to their degree of ripening. A chemometric approach, J. Agric. Food Chem. 46, 4174–4178.

37. Garai-Ibabe, G., Irastorza, A., Dueñas, M. T., Martín-Álvarez, P. J., and Moreno-Arribas, V. M. (2013) Evolution of amino acids and biogenic amines in natural ciders as a function of the year and the manufacture steps, Int. J. Food Sci. Technol. 48, 375–381.

38. Ye, M., Yue, T., and Yuan, Y. (2014) Changes in the profile of volatile compounds and amino acids during cider fermentation using dessert variety of apples, Eur. Food Res. Technol. 239, 67–77.

39. Eleutério dos Santos, C. M., Pietrowski, G. d. A. M., Braga, C. M., Rossi, M. J., Ninow, J.,Machado dos Santos, T. P.,Wosiacki, G., Jorge, R.M.M., and Nogueira, A. (2015) Apple amino acid profile and yeast strains in the formation of fusel alcohols and esters in cider production, J. Food Sci. 80, 1170–1177.

40. Etschmann, M., Bluemke,W., Sell, D., and Schrader, J. (2002) Biotechnological production of 2-phenylethanol, Appl. Microbiol. Biotechnol. 59, 1–8.

5.8 Supporting Information

Supporting Information Table 5.1. Amino acid concentrations in apple juice from 13 apple cultivars in mg/L. Mean and SEM were calculated from the analytical replicates (*n*=3) for the absolute concentration of each amino acid in each cultivar. The letters nd indicates the analyte is not detected.

Amino Acid	Arkansas Black	Blacktwig	Empire	Enterprise	Field Red	Golden Delicious	Granny Smith	Newtown Pippin	Northern Spy	Rome	Shenandoah	Winesap	York
His	0.43±0.1	0.46±0.2	0.47±0.06	0.50±0.1	0.61±0.2	0.36±0.1	0.27±0.2	0.33±0.01	0.38±0.2	0.31±0.05	0.77±0.4	0.41±0.05	0.32±0.2
Asn	10±0.2	2.2±0.07	20±0.5	34±0.4	4.1±0.1	1.0±0.01	8.5±0.1	26±0.3	2.5±0.03	1.6±0.01	13±0.9	4.9±0.02	14±0.08
Ser	0.66±0.01	0.54±0.001	0.42±0.01	0.89±0.04	0.30±0.01	0.090±0.04	0.30±0.02	1.0±0.02	0.18±0.01	0.20±0.003	0.66±0.04	0.50±0.01	0.65±0.005
Gln	1.5±0.07	nd	0.99±0.02	2.8±0.07	nd	0.20±0.2	nd	1.4±0.02	nd	nd	1.4±0.1	1.1±0.02	1.1±0.03
Arg	0.38±0.04	0.30±0.05	0.27±0.01	0.27±0.02	0.44±0.02	0.49±0.07	0.28±0.02	0.65±0.01	0.25±0.01	0.41±0.07	0.59±0.09	1.0±0.03	0.36±0.06
Gly	0.09±0.001	nd	0.09±0.001	0.08±0.01	nd	nd	0.03±0.03	0.12±0.003	nd	0.030±0.03	0.03±0.03	0.030±0.03	0.070±0.001
Asp	2.8±0.08	1.9±0.01	5.6±0.14	5.1±0.07	2.0±0.06	1.8±0.01	1.4±0.02	5.3±0.09	1.6±0.03	1.2±0.01	4.0±0.3	4.4±0.01	3.4±0.03
Glu	2.3±0.05	1.0±0.01	1.4±0.04	2.4±0.03	1.4±0.04	0.97±0.004	0.56±0.01	3.3±0.06	1.1±0.01	0.91±0.004	2.3±0.2	2.7±0.001	1.9±0.02
Thr	0.20±0.003	0.19±0.01	0.27±0.01	0.32±0.01	0.20±0.005	0.31±0.003	0.35±0.02	0.62±0.03	0.35±0.0003	0.35±0.01	0.26±0.01	0.30±0.01	0.25±0.01
Ala	0.66±0.03	0.48±0.005	1.9±0.03	1.7±0.02	0.33±0.02	0.25±0.01	0.41±0.01	1.0±0.01	0.35±0.02	0.22±0.002	0.81±0.04	0.75±0.01	0.47±0.01
GABA	0.29±0.03	0.15±0.002	0.49±0.01	0.28±0.004	0.16±0.01	nd	nd	0.54±0.01	nd	nd	0.40±0.03	0.25±0.004	0.19±0.01
Pro	0.22±0.02	0.27±0.001	0.64±0.02	0.36±0.01	nd	0.050±0.05	0.22±0.01	0.29±0.005	nd	nd	0.37±0.02	0.28±0.001	nd
Cys	0.30±0.05	0.34±0.04	0.34±0.07	0.26±0.03	0.39±0.01	0.34±0.01	0.34±0.02	0.36±0.002	0.33±0.02	0.35±0.01	0.40±0.1	0.25±0.07	0.27±0.01
Lys	0.42±0.04	0.47±0.04	0.33±0.05	0.39±0.03	0.53±0.01	0.51±0.01	0.55±0.01	0.60±0.004	0.49±0.03	0.52±0.01	0.43±0.01	0.44±0.13	0.46±0.01
Tyr	0.74±0.04	0.81±0.01	0.75±0.05	0.66±0.01	1.1±0.03	0.77±0.02	1.4±0.06	0.96±0.02	0.96±0.2	0.81±0.02	0.75±0.09	0.71±0.2	0.77±0.02
Met	1.0±0.03	1.2±0.01	1.2±0.3	0.83±0.03	1.1±0.02	0.98±0.03	1.2±0.04	1.4±0.03	1.1±0.1	1.0±0.03	1.1±0.1	0.87±0.3	1.0±0.03
Val	0.59±0.02	1.2±0.3	0.62±0.05	0.64±0.003	1.8±0.05	1.6±0.03	1.6±0.04	0.86±0.01	1.6±0.05	1.6±0.02	0.74±0.06	1.4±0.4	1.1±0.3
Ile	1.5±0.07	1.6±0.01	1.6±0.06	1.4±0.03	1.9±0.06	1.7±0.1	1.9±0.03	2.1±0.03	2.0±0.02	1.8±0.06	1.9±0.03	1.3±0.6	1.6±0.06
Leu	1.3±0.03	1.5±0.02	1.1±0.02	1.2±0.06	1.8±0.04	1.5±0.05	1.4±0.1	1.5±0.04	1.3±0.05	1.5±0.02	1.4±0.08	1.2±0.3	1.1±0.05
Phe	3.2±0.03	3.6±0.06	3.2±0.06	2.7±0.04	13±0.04	12±0.2	3.8±0.02	4.5±0.08	13±0.2	12±0.3	3.7±0.03	4.5±0.3	3.4±0.04
Total	**29±0.5**	**18±0.2**	**42±0.9**	**57±0.8**	**31±0.1**	**25±0.4**	**24±0.7**	**53±0.6**	**27±0.1**	**25±0.5**	**35±1**	**27±2**	**32±0.3**

CHAPTER 6 Improved academic performance and student perceptions of learning through use of a cell phone-based personal response system

Published in Journal of Food Science Education, 2018, 17(1), 27-32.

https://doi.org/10.1111/1541-4329.12131

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ABSTRACT

Personal response systems, such as clickers, have been widely used to improve the effectiveness of teaching in various classroom settings. Although hand-held clicker response systems have been the subject of multiple prior studies, few studies have focused on the use of cell phone-based personal response system (CPPRS) specifically. This study explores students' academic performance and their perceptions of learning through the use of a CPPRS (TopHat) in an undergraduate Food Science class. In this study, students did not use the CPPRS during the first half of the semester-long course, but did during the second half. When CPPRS was used, students responded to 2 multiple-choice questions at 3 points during the class, (a) at the beginning of class, (b) in the middle of the class, and (c) at the end of the class. Student performance was measured by correctness rates on eight 10-item multiple choice quizzes, 4 quizzes each covering the class content that was delivered with compared with without CPPRS. A survey was conducted at the end of the semester asking (n = 28) students' perceptions of CPPRS. The average correctness rate for quizzes covering content delivered with CPPRS (85% ± 9%) was significantly higher than for content delivered without CPPRS (82% ± 10%) (P = 0.016). In addition, students perceived that CPPRS was easy to use (5.04 ± 0.58 on a 1 to 6 scale with 1 being strongly disagree and 6 being strongly agree) and positively impacted their learning (4.52 ± 0.99 using the same scale). When used correctly, CPPRS can facilitate student learning in lectures.

6.1 Introduction

In Food Science education, it is important to create instructional environments (classrooms) where students are actively involved and engaged in order to foster student learning. The paradigm for undergraduate education has shifted from faculty passively transferring knowledge to students to a student-centered learning environment where active learners are more engaged in the learning process (Barr and Tagg 1995). This paradigm shift toward increased student engagement in undergraduate education is increasingly reflected in Food Science programs.

Active learning strategies, such as the use of attention-grabbing headlines for lecture material, effective use of stories, and in-class activities increase student engagement and interest in Food Science classes (Harris and others 2015). Engaging students through integrating real world context in Food Science Education has also improved students' understanding of complex concepts, increases knowledge gain, and significantly changes behavioral intentions with regards to Food Safety (Alberts and Stevenson 2017).

Classroom response systems, such as "clickers," have been widely used to improve the effectiveness of teaching and learning by fostering students' active involvement and engagement in various classroom settings (Sevian and Robinson 2011). Clickers can fully engage students in the classroom, allow instructors to evaluate students' understanding of (or misconceptions of) material in realtime, and help instructors to identify students who may require additional assistance (Judson and Sawada 2002). The effectiveness of clickers can be explained by a constructivist approach which supports the concept that clickers may improve student outcomes by providing opportunities for experiential learning in a classroom setting (Savery 2006; Schmidt and others 2007; Strobel and Van Barneveld 2009; English and Kitsantas 2013).

Despite the advantages of clickers, their disadvantages are not negligible. The additional cost of clickers themselves and user frustration due to common technical problems associated with clickers limit the willingness of some institutions and instructors to incorporate their use into classes (Blasco-Arcas and others 2013). Moreover, instructors must invest additional time and effort to adopt and integrate clickers into the teaching and learning process during class. Nonetheless, the use of clickers is often well received by students. Students generally enjoy using clickers in class and believe that it helps them to become more active and engaged learners (Gauci and others 2009). Multiple studies have observed improvement in student performance (higher grades) with the use of clickers (Uhari and others 2003; Poirier and Feldman 2007; Morling and others 2008). However, the impact of clickers on students' cognitive learning outcomes and academic performance varies depending upon how these devices or applications are used (Hunsu and others 2016). Therefore, proper implementation of clickers is essential to achieve the desired impact (Freeman and others 2014). Furthermore, differences may exist between impacts of cell phone based personal response systems (CPPRS) as opposed to clickers for students' academic performance, however those differences have not been systematically evaluated.

CPPRS, such as TopHatTM, are purported to provide similar benefits to clickers with increased functionality (TopHat 2017). In addition to responding to multiple choice or true/false questions, CPPRSs allow the use of free-response queries, and interactive functions that make use of maps, graphics, and images. Many CPPRSs can be installed on smartphones as well as other Internet-connected devices like laptops or tablet computers, thus students do not typically need to purchase additional hardware to use CPPRS. Smartphones have permeated the academic environment, especially classrooms (Ali and others 2012). When used properly in the classroom,

smartphones can contribute to improved academic performance (Gikas and Grant 2013). However, when misused, smartphones cause distraction and can even enable student misconduct (Tindell and Bohlander 2012).

Clickers have been used in in Food Science courses to engage students and promote active learning (Intemann 2006). Recently, Shaw et al. demonstrated that incorporating clickers into a short course on Hazard Analysis Critical Control Points (HACCP) improved the pass rate of the course (Shaw and others 2015). However, the effectiveness of using CPPRS in Food Science higher education has not been systematically evaluated and a better understanding of students' perceptions of CPPRS used in Food Science education would provide useful insight into the potential for application of such tools. To address this gap, our study aimed to evaluate the impact of using CPPRS on academic performance and students' perceptions of learning in an upper-level undergraduate Food Science course.

6.2 Materials and Methods

6.2.1 Overview

The study was conducted in an upper level undergraduate Food Science/Horticulture (cross-listed) course in Fall 2016 at a Southeastern land-grant university. In this study, students did not use the CPPRS (TopHat) during the first half of the semester-long course, but did in the second half of the course. Student performance was measured by the correctness rates on four 10-item multiple choice quizzes covering the class content in which (1) they used TopHat (4 quizzes), and (2) did not use the TopHat (4 quizzes). A survey was conducted at the end of the semester to assess students' perceptions of using TopHat.

6.2.2 Course structure and participants

Students enrolled (n = 52) in "Wines and Vines" class in Fall 2016 at a Southeastern land-grant university participated in this study. "Wines and Vines" is a 3 credit hour upper-level undergraduate course designed to help students develop a working knowledge of world wine styles and growing regions, basic principles of grape and wine production, wine appreciation, and sensory evaluation of wine. Two 75-min lectures were offered by the same instructor each week over a 16-wk period (1 academic semester). Because of the requirement to learn and practice sensory evaluation of wine, all students enrolled in this course must be at least 21 y of age by the beginning of the semester. The experimental classroom represented a balance between constructivist and "traditional" didactic lecture styles. A didactic lecture style was used to provide background and factual information. Sensory evaluation of wine with associated class debates and discussion provided opportunities for experiential learning and skill development.

6.2.3 Procedures

Academic performance. Over the course of 1 semester, 22 lectures covering subject matter of relatively consistent type and level of difficulty were delivered. TopHat was not used in the first half of the semester (11 lectures), but was used in the last half of the semester (11 lectures). Lecture structure was consistent throughout the semester. In the second half of the semester, TopHat was used during 3 distinct episodes in each lecture period. Questions were designed to test the students' comprehension of the class content. The TopHat questions were incorporated into the lectures without interrupting the natural flow of the lecture. Two review questions (multiple choice) were asked during the first episode (1 to 10 min) of the class. Two formative questions (non-multiple choice) were asked during the second episode (20 to 30 min) of the class. Two closure questions (multiple choice) reviewing the same day's lecture content were asked during the third episode (40 to 50 min). The students were given adequate time to respond to the question and were instructed to respond independently, without discussing

questions with classmates. One minute was allotted to answer each question. Additional time was allowed upon request; however, all participant responses were generally entered in much less than 1 min. The instructor explained the correct answers for all items at the conclusion of each of the 3 question periods. Prior to the delivery of lecture material with TopHat, students were instructed to install the application and watch a demo video to learn and practice the application. Typically, TopHat requires a subscription fee, however for the purposes of this experiment, the student subscriptions were purchased by the University's Center for Teaching and Learning. Students were incentivized to use TopHat in class by the opportunity to earn course credit for entering correct responses to the in-class exercises. Out of a total of 1004 points possible throughout the semester, 144 points could be earned by answering in-class questions using TopHat. Of these 144 points, half could be earned for simply entering a response (participation), and the other half could be earned by entering the correct response (correctness).

Students took 8 quizzes throughout the semester, the first 4 covering content delivered without the use of TopHat, and the last 4 covering content delivered using TopHat.

All quizzes were announced at the beginning of the semester. Each quiz consisted of 10 multiple-choice questions: 6 intended to assess lower-order learning (Remembering, Understanding, and Applying) as defined by Bloom's taxonomy, and 4 intended to assess higher-order learning outcomes (Analyzing, Evaluating, and Creating). It was hypothesized that the use of TopHat would improve factual recall of lower order information as well as encourage critical thinking and expansion of knowledge and skills. To limit bias, all multiple-choice quiz items included 4 possible answer choices, which never included "all of the above" or "none of the above." Answer choices were listed alphabetically for each quiz item. Students were allowed 12 min to take each in-class quiz. Printed copied of the quizzes were provided to students in class, and we observed that 12 min was an adequate amount of time for all students to complete the quizzes.

At the end of the semester, the researcher (not the instructor) announced the recruitment statement (Appendix A) in class and distributed Informed Consent forms (Appendix B) to students. The recruitment statement described the basics of the study (for example, who is involved, the nature of the research, anonymity and confidentiality). Signed consent was voluntary, and indicated students' agreement that the PI/Co-PIs may access and analyze their quiz scores for research purposes. No personally identifying student data was attached to the quiz score data set. Thirty-nine students (75% of the total class enrollment of n = 52 students) consented to allow their quiz scores to be included in this study (n = 39, 25 females and 14 males).

Statistical analysis. This study employed a 2 × 2 factorial design. Mean correctness rates ± standard deviation are presented in Table 6.1 for (1) quiz items covering content delivered with compared with without TopHat (2) items designed to assess lower compared with higher order thinking, and (3) the overall quiz scores. Significance of the 2 main effects (TopHat use and lowercompared with higher-order quiz items) and their interactions was determined using repeated measures (within subjects) analysis of variance with a Greenhouse-Geisser adjustment. Significance was defined as $p < 0.05$. This analysis was conducted using IBM SPSS Statistics (IBM Corporation, Armonk, N.Y., U.S.A.). Effect size was calculated (Cohen's d) for both main effects and interactions using means, standard deviation and the correlation between the 2 means. The effect size is defined as large when the Cohen's *d* value is 0.8, medium when the value is 0.5, and small when the value is 0.2.

Survey to evaluate student perceptions of learning. At the end of the semester, a

survey was conducted to evaluate students' perceptions of TopHat use in class. The survey was delivered using Qualtrics™ (Qualtrics, Provo, Utah, U.S.A.), and a list of the survey questions for this study is provided in Appendix C. The students were asked to take the survey verbally by the researcher (not the instructor) in class, and also via email announcement. The survey was open for 2 wk after the initial announcement. Due to the anonymous nature of the survey, no incentive was offered for completion of the online survey. The survey items addressed students' perceptions and questions following 2 themes were used for this study: (1) the impact of TopHat use on learning and (2) ease of use. Additional questions addressed demographic information. Specific questions grouped by theme are listed in Table 6.2. Response options and values were: strongly agree (6), agree (5), somewhat agree (4), somewhat disagree (3), disagree (2), and strongly disagree (1). Demographic data of students (age, gender, major, and academic class standing) was also collected at the end of the anonymous voluntary survey. The average score and standard deviation for each item and for each theme were then calculated from the digitized responses (Table 6.2).

6.3 Results and Discussion

6.3.1 Academic performance

The mean ± standard deviation of the correctness rates on the items designed to assess lower- and higher-order of thinking, for quizzes covering content delivered with/without TopHat, and for all quiz items are presented in Table 6.1.

Table 6. 1. Mean ± standard deviation of correctness rates on items designed to assess: lower- and higher-order of thinking, content delivered with/without TopHat, and all items/all content. Voluntary responses were received from n = 39 students, representing 75% of the total class enrollment of n = 52.

Correctness rates*	Content delivered without TopHat	Content delivered with TopHat	All content
Lower-order questions	0.85 ± 0.10	0.88 ± 0.08	0.87 ± 0.08
Higher-order questions	0.77 ± 0.13	0.79 ± 0.12	0.78 ± 0.12
All questions	0.82 ± 0.10	0.85 ± 0.09	0.83 ± 0.10

* Note: The two main effect pairwise comparisons and three of the four simple effect pairwise comparisons were statistically significantly different ($p \leq 0.01$). The simple effect pairwise comparison of higher-order/without TopHat vs. higher-order/with TopHat was not statistically significantly different ($p = 0.20$)

As expected, students performed better on quiz items designed to assess lower order thinking concepts (0.87 ± 0.08) than on quiz items designed to assess higher order thinking concepts (0.78 ± 0.12), $p < 0.01$. The effect size was large, $d = 0.971$. This finding agrees with our intended design for lower- and higher-order questions in the quiz. Higher-order questions are expected to prove more difficult than the lower-order question as they require more cognitive processing skills. But difficult questions do not necessarily associate testing higher cognitive levels (Lemons and Lemons 2013). Assessments including a combination of items designed to assess both the lower- and higher-order of thinking and learning better facilitate students' learning and ultimately result in higher academic performance (Wilen and Clegg 1986).

Overall, students performed better on the content that was delivered with TopHat (0.85 ± 0.09) than on the content delivered without TopHat (0.82 ± 0.10), $p = 0.016$. The effect size was medium, $d = 0.436$. Furthermore, there were significant interactions of the 2 independent variables in our study (the use of TopHat and higher- compared with lower- order items). Specifically, for the content delivered without TopHat, the correctness rates were better for the

lower (0.85 ± 0.10) compared with higher-order (0.77 ± 0.13) quiz items $p < 0.001$. The effect size was medium, $d = 0.600$. Similarly, for the content delivered with TopHat, the correctness rates for the lower (0.88 ± 0.08) and higher-order (0.79 ± 0.12) quiz items were significantly different with $p < 0.001$. The effect size was large, $d = 0.919$. Within all lower-order quiz items, mean correctness rate for content delivered with TopHat (0.88 ± 0.08) were higher than for content delivered without TopHat (0.85 ± 0.1) ($p = 0.016$). The effect size was medium, $d = 0.408$. However, within the higher-order quiz items, mean correctness rate for content delivered with TopHat (0.79 ± 0.12) was not significantly different than for content delivered without TopHat (0.77 ± 0.13), $p = 0.207$. The effect size was small, $d = 0.206$.

By incorporating in-class exercises using TopHat into the lectures, students performed better overall and better on questions designed to assess lower-order thinking. However, in our class, TopHat use did not improve students' performance on quiz items designed to assess higher-order thinking and learning. Our findings generally agree with prior research that clickers can promote students' academic performance by increased student engagement and more interaction between the instructor and the students (Mayer and others 2009).

6.3.2 Students' perceptions of the effect of using TopHat on learning

Survey results from 28 students (n = 28) who voluntarily responded to our survey are reported in Table 6.2. Twenty-one out of 28 students used a cell phone primarily to answer TopHat questions during the lectures, while 7 of 28 responded primarily using laptop computers or tablets. All of the students who participated in the survey own smartphones. There were 6 female and 22 male respondents, ranging from 21 to 26 y f age (mean ± standard deviation for age = 21.8 ± 1.25 y). The majority of respondents were white, with 1 respondent identifying as Hispanic or Latino. Eight respondents were Food Science and Technology majors, 3 Horticulture majors (2 of those were Viticulture minors within the Horticulture major), and the remaining 15 survey respondents were from various majors including General Engineering, Animal and Poultry Sciences, Agribusiness, Accounting, Psychology, Applied Economics, Sociology, Marketing, Biological Sciences, Hospitality and Tourism Management, Computer Engineering, and Electrical Engineering. Thus, a fairly diverse group of majors and genders are represented, but ethnic or age diversity was not observed among our respondents.

Table 6. 2. Survey results summarizing students' perceptions of using TopHat. Voluntary responses were received from n = 28 students, representing 54% of the total class enrollment of n = 52. Response options and values were: strongly agree (6), agree (5), somewhat agree (4), somewhat disagree (3), disagree (2), and strongly disagree (1).

Survey questions	Average score ± standard deviation
Theme 1: Impact of TopHat use on learning	**4.52 ± 0.99**
Using TopHat improved my learning.	4.46 ± 1.20
Using TopHat made me think more during class.	4.82 ± 1.02
Using TopHat increased my focus on the class.	4.29 ± 1.15
Theme 2: Ease of use	**5.04 ± 0.58**
Using TopHat was easy.	5.18 ± 0.82
Using TopHat was common sense.	4.93 ± 0.60
Using TopHat was straightforward.	5.00 ± 0.77

As presented in Table 6.2, students agreed or somewhat agreed that TopHat positively

impacted their learning (mean score for Theme 1 was 4.52 ± 0.99), and they agreed that TopHat was easy to use (mean score for Theme 2 was 5.04 ± 0.58). The student perceptions that TopHat had a positive impact on learning generally align with the quiz score data in that students earned higher scores on the lower-order items when TopHat was used in the delivery of the content evaluated.

Currently, it is a challenge to inhibit the existence and use of smartphones in classrooms due to the ubiquity of multitasking with smartphones in daily life (Armstrong 2014). One strategy for adaptation to pervasive multitasking in the classroom is to turn smartphones into useful learning tools to foster teaching and learning. Our results indicate that having students actively use their phones to participate in an upper-level undergraduate Food Science course can improve academic performance and student perceptions of learning.

6.3.3 Limitations and future directions

Factors extraneous to our study such as holidays and demands from other courses, jobs, etc. could have impacted academic performance along the time course of the semester, in addition to the impact of our treatment. While the difficulty of course content and lecture format were designed to be consistent throughout the semester, different topics were necessarily covered as the semester progressed. Students' personal preference and academic preparation for specific topics is expected to introduce some variation in academic performance. Finally, our course was the first course in the Department of Food Science and Technology at our University to incorporate the use of TopHat in lectures, thus the students were likely new to using TopHat. In the future, it would be apappropriate to examine the use of CPPRS across a variety of content areas within Food Science, as well as across various pedagogical approaches. This approach would increase generalizability of the current findings.

6.4 Conclusions

TopHat, a CPPRS system, is a useful tool to improve students' academic performance and perceptions of learning in upper-level undergraduate Food Science classes. While improvement in academic performance on lower- order of learning assessment items was observed, improvement in performance on higher-order items was not. With the prevalence of smartphones among students, CPPRSs such as TopHat offer a strategy for turning ubiquitous phones into useful tools that can facilitate a collaborative teaching and learning environment through engagement.

6.5 Acknowledgments

The authors would like to thank Elizabeth Clark and Brianna Ewing for grading the quizzes as teaching assistants to the Wines and Vines class in Fall 2016. The authors also would like to thank the Center for Instructional Development and Educational Research (CIDER) at Virginia Tech for their support on teaching and learning.

6.6 References

Alberts CM, Stevenson CD. 2017. Development of a reality-based multimedia case study teaching method and its effect on students' planned food safety behaviors. J Food Sci Educ 16(1):10–8.

Ali AI, Papakie MR, McDevitt T. 2012. Dealing with the distractions of cell phone misuse/use in the classroom - a case example. Competit Forum 10:220–30.

Armstrong A. 2014. Technology in the classroom it's not a matter of 'if,' but 'when' and 'how'. Educ Digest 79(5):39–46.

Barr RB, Tagg J. 1995. From teaching to learning: A new paradigm for undergraduate education. Change 27(6):12–5.

Blasco-Arcas L, Buil I, Hern´andez-Ortega B, Sese FJ. 2013. Using clickers in class. The role of interactivity, active collaborative learning and engagement in learning performance. Comput Educ 62:102–10.

Cornell Chronicle. 2006. Clicking in class helps lecturers from appearing remote by using student remotes as instructional tool. Available from: http://news.cornell.edu/stories/2006/02/clicking-class-helps-lecturersand-students-connect. Accessed 2017 May 20.

English MC, Kitsantas A. 2013. Supporting student self-regulated learning in problem-and project-based learning. The Interdisciplinary Journal of Problem-Based Learning 7(2):6.

Freeman S, Eddy SL, McDonough M, Smith MK, Okoroafor N, Jordt H, Wenderoth MP. 2014. Active learning increases student performance in science, engineering, and mathematics. Proc Natl Acad Sci 111(23):8410–5.

Gauci SA, Dantas AM, Williams DA, Kemm RE. 2009. Promoting student-centered active learning in lectures with a personal response system. Adv Physiol Edu 33(1):60–71.

Gikas J, Grant MM. 2013. Mobile computing devices in higher education: student perspectives on learning with cellphones, smartphones & social media. Internet High Educ. 19:18–26.

Harris GK, Stevenson C, Joyner H. 2015. Taking an attention-grabbing "headlines first!" approach to engage students in a lecture setting. J Food Sci Educ 14(4):136–41.

Hunsu NJ, Adesope O, Bayly DJ. 2016. A meta-analysis of the effects of audience response systems (clicker-based technologies) on cognition and affect. Comput Educ 94:102–19.

Clicking in class helps lecturers from appearing remote by using student remotes as instructional tool. 2006. [accessed]. http://www.news.cornell.edu/stories/2006/02/clicking-class-helps-lecturers-and-students-connect.

Judson E, Sawada, Daiyo. 2002. Learning from past and present: Electronic response systems in college lecture halls. Journal of Computers in Mathematics and Science Learning 21(2):167–81.

Lemons PP, Lemons JD. 2013. Questions for assessing higher-order cognitive skills: it's not just bloom's. CBE Life Sci Educ 12(1):47–58.

Mayer RE, Stull A, DeLeeuw K, Almeroth K, Bimber B, Chun D, Bulger M, Campbell J, Knight A, Zhang H. 2009. Clickers in college classrooms: fostering learning with questioning methods in large lecture classes. Contemp Educ Psychol 34(1):51–7.

Morling B, McAuliffe M, Cohen L, DiLorenzo TM. 2008. Efficacy of personal response systems ("clickers") in large, introductory psychology classes. Teach Psychol 35(1):45–50.

Poirier CR, Feldman RS. 2007. Promoting active learning using individual response technology in large introductory psychology classes. Teach Psychol 34(3):194–6.

Savery JR. 2006. Overview of problem-based learning: Definitions and distinctions. The Interdisciplinary Journal of Problem-Based Learning 1(1):9–20.

Schmidt HG, Loyens SM, Van Gog T, Paas F. 2007. Problem-based learning is compatible with human cognitive architecture: commentary on kirschner, sweller, and clark (2006). Educ Psychol 42(2):91–7.

Sevian H, Robinson WE. 2011. Clickers promote learning in all kinds of classes–small and large, graduate and undergraduate, lecture and lab. J Coll Sci Teach 40:14–8.

Shaw A, Mendonca A, Daraba A. 2015. "Clickers" and HACCP: Educating a Diverse Food Industry Audience with Technology. Journal of Extension 53:6. Available from:

https://joe.org/joe/2015december/tt6.php. Accessed 2017 May 20.

Strobel J, Van Barneveld A. 2009. When is pbl more effective? A meta-synthesis of meta-analyses comparing PBL to conventional classrooms. The Interdisciplinary Journal of Problem-Based Learning 3(1):44–58.

Tindell DR, Bohlander RW. 2012. The use and abuse of cell phones and text messaging in the classroom: a survey of college students. J Coll Teach 60(1):1–9.

Tophatmonocle Corporation. 2017. Homepage. Available from: https://tophat.com/. Accessed 2017 May 20.

Uhari M, Renko M, Soini H. 2003. Experiences of using an interactive audience response system in lectures. BMC Med Educ 3(1):12. Available from: https://bmcmededuc.biomedcentral.com/articles/10.1186/1472-6920-3-12. Accessed 2017 May 20.

Wilen WW, Clegg AA. 1986. Effective questions and questioning: a research review. Theory Res Soc Educ 14(2):153–61.

6.7 Appendix A: Recruitment Statement

Script for Announcing the Survey to the Class by the researcher

We have been using cell phones as clickers in this course this semester. We would like to collect some data on your perceptions of the use of cell phones as clicker as part of a research study. We would use these data to present at conferences and write an academic article.

The research is being conducted by Peter Doolittle, Executive Director of the Center for Instructional Development and Educational Research; Amanda Stewart, your instructor, Assistant Professor of Food Science and Technology; Daniel Steger, graduate student and TA, Food Science and Technology; Sihui Ma, graduate student and TA, Food Science and Technology.

Anyone who has been in this class this semester who is at least 18 y of age is eligible to participate in the survey. The survey is online and should only take about 10 minutes to complete. Participation in this survey is voluntary and the results will be anonymous. We will not be linking who you are to your responses. Whether or not you participate is up to you and your participation, or not, will have no impact on your grade for this course. If you have any questions, you can ask them now or send me an email at sdaniel3@vt.edu.

6.8 Appendix B: Consent Form

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Informed Consent for Participants in Research Projects Involving Human Subjects
Title of Project: Student Distraction Following Cell-Phonebased Personal Response System Use
Investigator(s): Peter Doolittle, pdoo@vt.edu, 231–3954; Amanda Stewart, amanda.stewart@vt.edu, 231–0868

I. Purpose of this Research Project

The purpose of this research study is to explore students' use of cell phone-based personal response systems (i.e., clickers). The classroom learning experience is multifaceted, involving lectures, discussions, and student responses to questions. How do students use cell phones as 'clickers' during class? Do students find their use beneficial to their learning?

The results of this research study will be used to construct academic conference presentations and academic scholarly articles. The research study is open to all students in

FST/HORT 3114 Wines and Vines.

The survey will provide information regarding students' use of cell phone-based 'clickers' in class, their impact on students' learning, and their contribution to the overall learning environment.

II. Procedures

Should you agree, the survey engagement will involve:

(1) You will be provided in class with a description of the online survey's purposed.
(2) The online survey will address students' use of the cell phone-based 'clickers' and basic demographic information (e.g., age, gender, year at VT).
(3) You will be provided in class with a link to the online survey.
(4) Completion of the online survey will take no longer than 10 minutes.
(5) Completion of the online survey will be anonymous.

III. Risks

The risks of involvement in the survey are minimal and involve only the provision of one's perceptions related to the class and the use of cell phone-based 'clickers.' You may withdraw from the survey at any time and the survey questions will not focus on any potentially embarrassing or dignity threatening topics.

IV. Benefits

Participation in this survey will benefit future teachers, students, and society by clarifying how cell phone-based 'clickers' may be used more effectively in classes. This knowledge will allow teachers to construct more effective learning environments. Finally, no promise or guarantee of benefits has been made to encourage you to participate.

V. Extent of Anonymity and Confidentiality

No identifying information (e.g., name, email) will be collected during the survey and only general demographic will be collected (e.g., age, gender, year at VT). At no time will the researchers release identifiable results of the study to anyone other than individuals working on the project without your written consent.

The Virginia Tech (VT) Institutional Review Board (IRB) may view the study's data for auditing purposes. The IRB is responsible for the oversight of the protection of human subjects involved in research.

VI. Compensation

No compensation for participation will be provided.

VII. Freedom to Withdraw

It is important for you to know that you are free to withdraw from this study at any time without penalty. You are free not to answer any questions that you choose or respond to what is being asked of you without penalty.

Please note that there may be circumstances under which the investigator may determine that a subject should not continue as a subject.

Should you withdraw or otherwise discontinue participation, you will be compensated for the portion of the project completed in accordance with the Compensation section of this

document.

VIII. Questions or Concerns

Should you have any questions about this study, you may contact one of the research investigators whose contact information is included at the beginning of this document.

Should you have any questions or concerns about the study's conduct or your rights as a research subject, or need to report a research-related injury or event, you may contact the VT IRChair, Dr. David M. Moore at moored@vt.edu or (540) 231–4991.

IX. Subject's Consent

I have read the Consent Form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

_____

Subject signature Date

_____

Subject printed name

(Note: each subject must be provided a copy of this form. In addition, the IRB office may stamp its approval on the consent document(s) you submit and return the stamped version to you for use in consenting subjects; therefore, ensure each consent document you submit is ready to be read and signed by subjects.)

6.9 Appendix C: Survey

Welcome to our survey on the Impact of Cell Phone-based Personal Response System Use on Academic Performance. If you are interested, please continue.

For Q1 to Q6 response options and values were: strongly agree (6), agree (5), somewhat agree (4), somewhat disagree (3), disagree(2), and strongly disagree (1).

Q1 Using TopHat improved my learning.
Q2 Using TopHat was easy.
Q3 Using TopHat made me think more during class.
Q4 Using TopHat was common sense.
Q5 Using TopHat increased my focus on the class.
Q6 Using TopHat was straightforward.
Q7 How did you primarily answer the TopHat questions?

- ❑ Cell phone
- ❑ Laptop
- ❑ Other. Please specify:

Q8 Which of the following best describes your cell phone?

- ❑ Basic phone
- ❑ Smartphone
- ❑ I do not own a phone

Q9 What is your age?

- ❑ 21

- ❑ 22
- ❑ 23
- ❑ 24
- ❑ Other. Please specify:

Q10 To which gender identity do you most identify?

- ❑ Male
- ❑ Female
- ❑ Transgender
- ❑ Other. Please specify:

Q11 What is your ethnicity?

- ❑ White
- ❑ Hispanic or Latino
- ❑ Black or African American
- ❑ Native American or American Indian
- ❑ Asian/Pacific Islander
- ❑ Other. Please specify:

Q12 What is your major/minor?

- ❑ Food Science and Technology Major
- ❑ Horticulture Major
- ❑ Viticulture Minor
- ❑ Other. Please specify:

Chapter 7 Conclusions and future work

The goal of this study is to deepen our understanding of analytical methods used in fruit chemistry, especially for the quantification of polyphenols and amino acids. To better disseminate the findings from research in teaching and learning, the use of a mobile phone-based instructional technology in food science education was evaluated.

Comprehensive evaluation of common analytical methods for the quantification of polyphenols in a broad range of fruit juice samples evidenced that there is substantial variation in quantitative results obtained through different methods and for samples with different polyphenol composition. Further characterization of the contribution of different polyphenol compounds using these methods supported these findings, showing that the type of polyphenol evaluated influences quantitative results. Additionally, the evaluation of matrix effects in fruit juice and beverage samples revealed that interference with these methods due to non-target matrix constituents leads to inaccuracy in quantitative results. However, mechanisms of the reactions among polyphenols and potentially interfering compounds in the sample matrix with the reagent used in each method still warrant further research. In the future, development of mass spectroscopy and nuclear magnetic resonance spectroscopy, especially efforts to lower the difficulty and cost of operation, will make these technologies available to further explore the mechanisms of these chemical reactions. Overall, the influence of polyphenol composition and potential matrix interferences of fruit juice and beverage samples should be taken into consideration when choosing appropriate analytical methods for the quantification of polyphenols in food science, horticulture, and nutrition research. Quantitative results on polyphenol concentration among different sample types should be compared with caution, noting the variance caused by operating principle of the methods, as well as polyphenol composition and matrix of the samples. By helping to inform comparisons of the tremendous amount of available fruit polyphenol data that has been collected in the course of food science, horticulture and nutrition research in recent decades using the assays evaluated in this study, our findings have the potential to advance research on the relationships between polyphenol-rich foods and their associated health benefits. Finally, our results can also help to inform researchers and fruit juice and beverage producers as to which of these methods can best predict bitterness and astringency in their products, and can aid in making useful comparisons between data collected using different methods. This should help to achieve improved consumer acceptance and preference of fruit products.

Analysis of amino acids in apple juice made from Virginia-grown apples provides information useful for the development of hard cider fermentation strategies, allowing more precise manipulation of the cider flavor and style through adjustment of pre-fermentation amino acid composition. This work provides a basis for future study to advance the understanding of the complex relationships among amino acids in apple juice, fermentation, and the flavor of finished cider.

The use of a cellphone-based personal response system improved student academic performance in an upper-level undergraduate food science course. Its use also gained positive perception from students. With the improvement in effectiveness in teaching and learning by the incorporation of this instructional technology, active learning is fostered and student engagement is facilitated. This teaching practice should be evaluated further using different student groups and course content before being widely applied in food science education.

Overall, the work described in this dissertation contributes to the fulfillment of two missions of a land grant university: research and teaching. Our research findings serve to advance knowledge of how polyphenol composition and sample matrix influence the polyphenol quantification in fruit juice and beverage samples, the amino acid composition in apple juice from different apple cultivars. Furthermore, our findings from the scholarship of teaching and learning demonstrate the effectiveness of incorporating a cellphone-based personal response system in food science education. This work can help food science educators better convey our research findings and help students better apply food science knowledge for advanced personal growth and broader social impact. In closing, the work described in this dissertation will serve to inform further investigations in 1) research on dietary polyphenols and health or sensory outcomes, 2) impact of polyphenols and/or amino acids on cider fermentation, and 3) teaching practices for greater student engagement in higher education classrooms.