AN ABSTRACT OF THE THESIS OF

Ling Wen for the degree of Doctor of Philosophy in Food Science and Technology presented on June 18, 2001. Title: Pineapple Juice: Phenolic Composition and Enzymatic Browning Inhibition.

Abstract approved: Redacted for Privacy
Ronald E. Wrolstad

The phenolic profile of pineapple juice was analyzed by reverse-phase HPLC/Diode Array Detector, and 9 major peaks accounting for 70% of total peak area were characterized. None of these peaks are represented by the pineapple phenolics reported to be present by previous workers. Most of the major peaks are not typically considered as phenolic compounds by the commonly accepted definition, and these peaks are: two aromatic amino acids -- tyrosine and tryptophan, dimethylhydroxylfuranone (DMHF) and its β-glucoside, and serotonin. One of the major peaks is characterized as a *p*-coumaric acid-like compound because they have very similar UV spectra and retention times. This is possibly why *p*-coumaric acid has been reported in the literature. The other three peaks are sinapyl derivatives with amino acid or peptides, and they are: S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione. This is the first time to report their existence in nature. Their structures were elucidated from UV spectra, acid hydrolysis and subsequent amino acid analysis, mass spectrometry, and 1D/2D NMR Spectroscopy. These sinapyl derivatives are possibly unique to pineapple, and may serve as marker compounds for pineapple juice.

A phenolic compositional database of these 9 major peaks have been created from 54 commercial authentic pineapple juice concentrates, which consist of major commercial varieties and have been collected over 3 consecutive years from the most significant growing regions around the world. Means and standard deviations were as follows (mg/100 mL single-strength juice, normalized to 12.8 ^{0}Brix): tyrosine, 3.6(1.4); serotonin, 1.8(0.8); dimethylhydroxylfuranone, 1.4(0.7); dimethylhydroxylfuranone β-glucoside, 6.2(3.0); tryptophan, 2.2(0.9); S-sinapyl-L-cysteine, 1.1(0.6); N-γ-L-glutamyl-S-sinapyl-L-cysteine, 2.3(1.1); S-sinapyl glutathione, 5.4(1.4); and a *p*-coumaric acid-like phenolic compound (calculated as *p*-coumaric acid), 0.5(0.4). This information will be useful for evaluation of authenticity and quality.

Pineapple juice has been demonstrated to have enzymatic browning inhibition activity, but the information about the nature of the inhibitor(s) is very limited. In order to characterize the major inhibitor(s) in pineapple juice, three different pineapple juices, fresh, canned, and frozen concentrate, were investigated by using a combination of fractionation methods. It was found: The inhibition activity could be mainly contributed by protease in fresh juice, by artificially added ascorbic acid in canned juice, and possibly by a possibly very polar organic acid (neither ascorbic acid nor citric acid) in frozen concentrate.

Pineapple Juice: Phenolic Composition and Enzymatic Browning Inhibition

by

Ling Wen

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed June18, 2001
Commencement June, 2002

Doctor of Philosophy thesis of Ling Wen presented on June 18, 2001

APPROVED:

Major Professor, representing Food Science and Technology

Chair of Department of Food Science and Technology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Ling Wen, Author

ACKNOWLEDGMENTS

I want to express my deepest gratitude to Dr. Ronald E. Wrolstad, for his unconditional support and guidance during all of my years at Oregon State University. He is the most enjoyable person to work with, and he also gave me a lot of personal help beyond academia.

Thank you also to all the friends I met here at FST. Among them, a special thanks to Bob Durst for his excellent technical advise and friendship. He is a kind of the guy whom you can always rely on when something is broken in the laboratory. Also, thanks go to all my labmates, Monica Giusti, Lucho Rodrigues-Saoma, Eric Fan-Chiang, Xialing Dong, Jung-Min Lee, Myriam Kucza, Keith Klesk, Arusa Chaovanalikit, Maria Widyasari, Thanyaporn Siriwoharn, visiting scientists Gtete Skrede, Feryl Karadeniz, who gave me help and made my experience in the laboratory more enjoyable.

I also want to thank the FST faculty and staff, especially to Anita Brown for her great help.

I want to thank the Washington-Oregon Tree Fruit Commission for its financial support, the National Food Processor Association and Dole Food Company for providing pineapple juice samples.

Finally, I want to thank my wife, my children and my parents for their love and support.

CONTRIBUTIONS OF AUTHORS

Dr. Ronald E. Wrolstad was involved in the design, analysis, and writing of each manuscript. Dr. Victor L. Hsu ran most of NMR experiments and assisted in NMR data interpretation in chapter III.

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PINEAPPLE JUICE: PHENOLIC COMPOSITION AND ENZYMATIC BROWNING INHIBITION

CHAPTER I. INTRODUCTION

An earlier work from our laboratory (Lozano-de-Gonzalez *et al.*, 1993) demonstrated that pineapple juice was an effective enzymatic browning inhibitor in both fresh and dried apples, but we had very limited information as to the nature of the inhibitor(s). Since some phenolic compounds can act as the substrates for the enzymatic browning while some other ones act as inhibitors, it would be very helpful if the phenolic composition of pineapple juice were known. Coincidentally in 1996, the National Food Processor Association (NFPA) had just finished a collaborative study on the general composition of commercially made authentic pineapple juice concentrate, and the phenolic composition of these samples was highly desired to make this compositional database complete. NFPA asked our laboratory to help on this project. Because of the mutual benefits, we were pleased to participate in the project.

While pineapple juice is a major commercial product in international commerce, its phenolics profile has not been well characterized, and only a few papers have been published on the phenolic composition of pineapple fruit (Macheix et al, 1990), juice (Fernández de *Simón et al.*, 1992) and shell fiber (Larrauri *et al*, 1997). We found, however, in a preliminary investigation of pineapple juice phenolics by reverse phase HPLC/DAD, that the retention time and UV spectra of the major peaks did not match the phenolic compounds previously reported to be present. In this study, we conducted extensive analyses of the pineapple juice phenolic profile, and 9 major peaks accounting

for 70% of total peak area were characterized. A phenolic compositional database of 54 authentic p ineapple juice concentrates, which consisted of major commercial varieties and were collected over 3 years from significant growing regions around the world, was established.

In addition to the characterization and quantification of the phenolics, we wanted to know their possible role in inhibiting enzymatic browning. By using a combination of different fractionation methods, we proceeded to investigate what fractions and classes of compounds could potentially inhibit enzymatic browning.

CHAPTER II. LITERATURE REVIEW

PHENOLICS

Definition (Robards et al.,1999).

There is some controversy about the definition of phenolics. Phenolic compounds form one of the main classes of secondary metabolites with a large range of structures and functions, but generally possessing an aromatic ring bearing one or more hydroxy substituents. This definition is not entirely satisfactory, however, since it inevitably includes compounds such as oestrone, the female sex hormone that is principally terpenoid in origin. For this reason, a definition based on metabolic origin is preferable, the plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism.

Classification

Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. They occur primarily in conjugated form, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist. The associated sugars can be present as monosaccharides, disaccharides, or even as oligosaccharides, and glucose is the most common sugar residue. Associations with other compounds, such as carboxylic and organic acids, amines, and lipids, and linkage with other phenols are also common (Bravo, 1998).

According to Harborne (1989), polyphenols can be divided into at least 10 different classes depending on their basic chemical structure. Table II.1 illustrates the basic chemical structure of the main polyphenolic compounds. Flavonoids, which constitute the most important single group, can be further subdivided into 13 classes, with more than 6467 known flavonoids described by 1999 (Harborne and Baxter, 1999) (Table II.2). In order to simplify the drawing of chemical structures, only the skeleton structures are shown in Tables 1 and 2, and there should be at least one hydroxyl group attached to the aromatic ring.

Importance to juice

Fruit phenolic compounds are relevant in terms of quality, as they have a role in the visual appearance (pigmentation, browning and haze), taste (astringency) and health-promoting properties (free-radical scavengers) of different products (Synge, 1975; Siebert and Lynn, 1997; Tomás-Barberán and Robins, 1997). Because of the quality concerns, the effect of processing on phenolic composition have been extensively investigated (Spanos and Wrolstad, 1990a, 1990b; Spanos et al., 1990).

Because of their ubiquity, specificity and multiplicity, phenolic composition is very useful for the purpose of authenticity of fruit products and detection of adulteration (Kirkesy et al., 1995; Rouseff, 1988; Wrolstad et al., 1995).

Relationship to health

Bravo (1998) has given a general review on the nutritional significance of phenolics, and Harborne and Williams (2000) have given a review specifically on the medicinal

Table II.1. Main classes of phenolic compounds (adapted from Bravo et al., 1998)

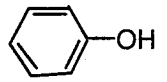
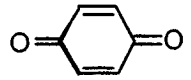
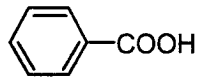
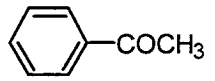
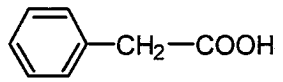
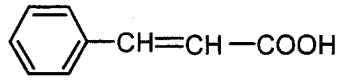
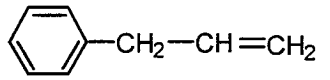
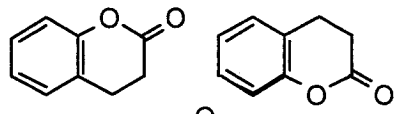
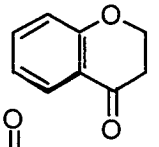
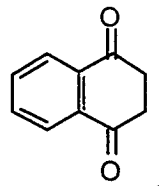
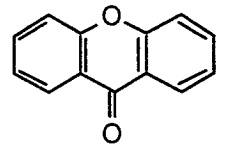
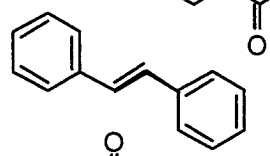
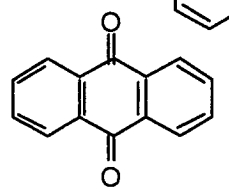
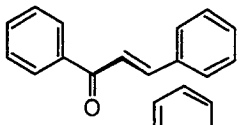
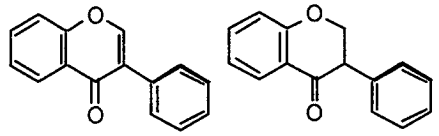
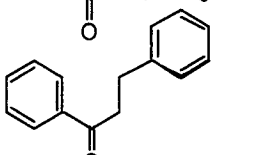
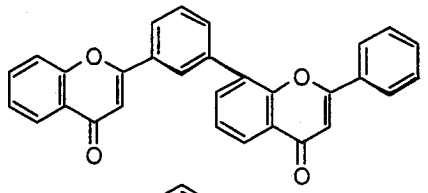
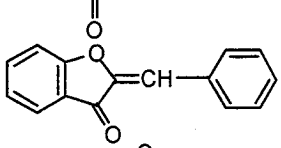
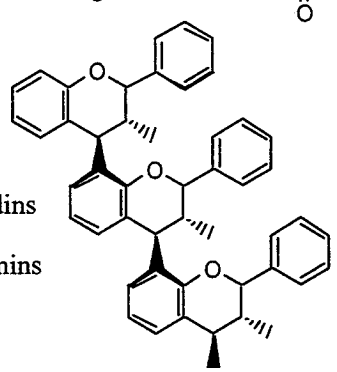
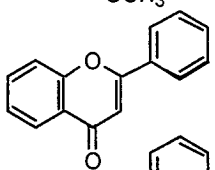
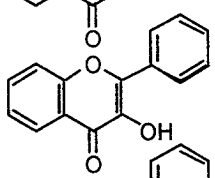
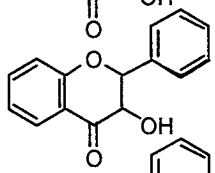
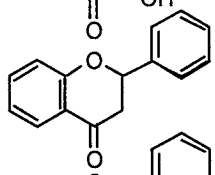
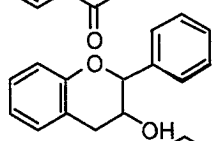
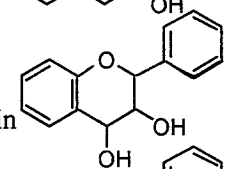
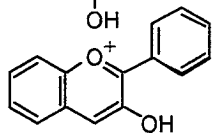
Class	Basic Skeleton	Basic Structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naftoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthroquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans, neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)$n	

Table II.2. Classification of flavonoids (adapted from Bravo et al., 1998)

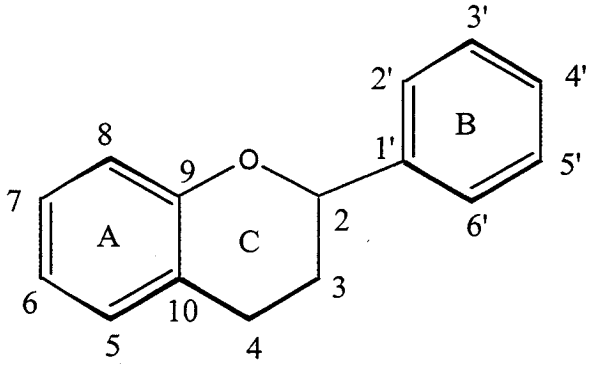
Flavonoid	Basic Structure	Flavonoid	Basic Structure
Chalcones		Isoflavonoids	
Dihydrochalcones		Biflavonoids	
Aurones		Proanthocyandins or condensed tannins	
Flavones			
Flavonols			
Dihydroflavonol			
Flavanones			
Flavanol			
Flavandiol or leucoanthocyanidin			
Anthocyanidin			

properties of flavonoids. The following discussion is mainly taken from these two papers. Polyphenols traditionally have been considered antinutrients by animal nutritionists, because of the adverse effect of tannins, one type of polyphenol, on protein digestibility. However, recent interest in food phenolics has increased greatly owing to their antioxidant capacity (free radical scavenging and metal chelating activities) and their possible beneficial implications in human health, such as in the treatment and prevention of cancer; cardiovascular disease, and other pathologies. Much of the literature refers to a single group of plant phenolics, the flavonoids (Rice-Evans and Packer, 1998; Frankel et al., 1995, Hertog et al., 1997; Hertog et al., 1995).

Phenolic antioxidants function as terminators of free radicals and chelators of metal ions that are capable of catalyzing lipid oxidation. However, under certain conditions (high concentrations of phenolic antioxidants, high pH, presence of iron), phenolic antioxidants can initiate an autooxidation process and behave like prooxidants (Shahidi and Wanasundara, 1992).

The efficiency of polyphenols as antioxidant compounds greatly depends on their chemical structure. Phenol itself is inactive as antioxidant, but ortho- and para-diphenolics have antioxidant capacity, which increases with the substitution of hydrogen atoms by ethyl or n-butyl groups (Shahidi and Wanasundara, 1992). Flavonoids are among the most potent antioxidants because they posses one or more of the following structural elements involved in the antiradical activity (Figure II.1): (1) an o-diphenolic group (in ring B), (2) a 2-3 double bond conjugated with the 4-oxo function, and (3) hydroxyl groups in positions 3 and 5 (Bors et al. 1990; Manach et al., 1996; Ratty and Das, 1988). Quercetin, a flavonol that combines all of these characteristics, is one of the

Figure II.1. Basic structure and numbering of flavonoids.



most potent natural antioxidants. Also, the antioxidant efficiency of flavonoids is directly related with their degree of hydroxylation (Ratty and Das, 1988), but there is some controversy about the relationship between antioxidant activity and glycosylation. Ratty and Das (1988) and Acker et al. (1996) found that aglycones are more effective than the corresponding glycoside, whereas Wang et al. (1997) and Tsuda et al. (1994) found that this was not necessarily true. The controversy could be caused by the different methods used to measurement antioxidant activity.

Analysis

Phenolics can be quantified as total, as subgroups, or as individual compounds. Total phenolics can be analyzed by the Folin reaction, vanillin reaction, titanium chloride and Prussian blue. The Folin reaction is the most general purpose while the other methods are either not general purpose, or the intensity of developed color depends on chemical structures (Shahidi and Naczk, 1995), although the last two methods can give comparable or more sensitive results than the Folin procedure in some cases (Harborne, 1989). The disadvantage of the Folin assay is the interference of reducing substances such as ascorbic acid (Shahidi and Naczk, 1995). Sometimes, measurement of a subgroup of phenolics, for example, anthocyanins, is desired. Guisti and Wrolstad (2001) gave an extensive description about the procedure of measuring total anthocyanins. If analysis of individual phenolics is needed, chromatographic methods such as HPLC, TLC and GC are used, with HPLC as the predominant method (Kirksey et al., 1995). When HPLC is used with a diode array detector, it can be used to resolve the coelution problem by checking peak purity (Spanos and Wrolstad, 1990a, 1990b; Spanos et al., 1990). Most

often, fractionation or purification is required to achieve satisfactory separation on HPLC because of the complexity of the phenolic profile found in most fruit and vegetable products. For example, to analyze procyanidins in apple, grape and pear juice, the other interfering phenolic compounds can be removed by using Sephadex LH-20 (Spanos and Wrolstad, 1990a, 1990b; Spanos et al., 1990).

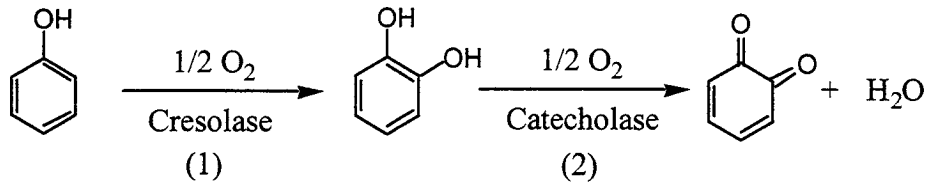
ENZYMATIC BROWNING

Basically, enzymatic browning can be defined as the initial enzymatic oxidation of phenols into slightly colored quinones (Nicolas et al.,1994). These quinones are t hen subjected to further reactions, enzymatically catalyzed or not, leading to the formation of pigments. With a few exceptions (prunes, raisins, dates, apple juice etc.), enzymatic browning is generally considered as degradation and lower quality with regard to appearance, taste and nutritional characteristics.

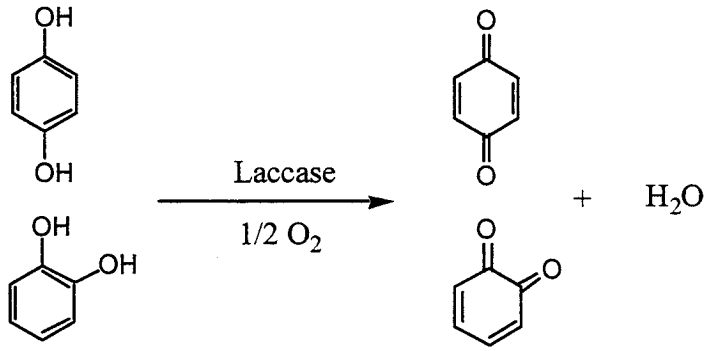
Mechanism

Enzymatic browning is mainly associated with polyphenol oxidases, which are able to act on phenols in the presence of oxygen. Two kinds of enzymes are classified under this trivial name (Nicolas et al., 1994). The first class, catechol oxidases (E.C. 1.10.3.1), catalyzes two distinct reactions (Figure II.2a): the hydroxylation of monophenols into o-diphenols (reaction 1) and the oxidation of o-diphenols into o-quinones (reaction 2). These two enzymatic reactions consume oxygen and are referred to as monophenolase (or

Figure II.2 Enzymatic browning reaction (Adapted from Nicolas et al., 1994).

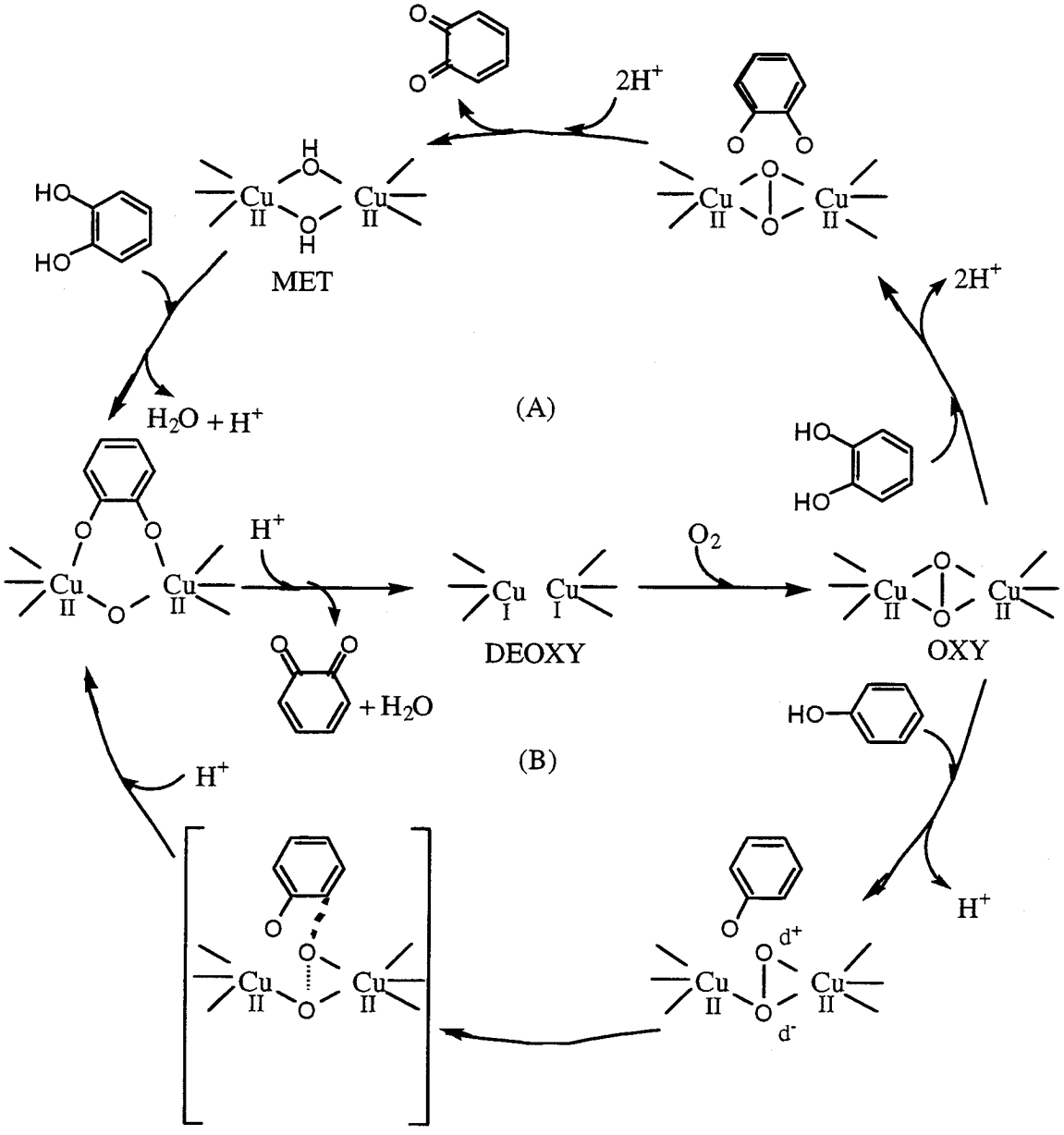


a) Reactions catalyzed by polyphenoloxidase (E.C. 1.14.18.1 and E.C. 1.10.3.1).
(1) Hydroxylation of monophenol to o-diphenol; (2) dehydrogenation of o-diphenol to o-quinone.



b) Reactions catalyzed by laccases (E.C. 1.10.3.2)

Figure II.3. Proposed kinetic scheme depicting the mechanism of oxidation of o-diphenol (catechol; top(A) and monophenol; bottom (B)) for Neurospora crassa polyphenol oxidase. (taken from Whitaker and Lee, 1995).



cresolase) activity and o-diphenolase (or catecholase) activity, respectively (the former is not always present). The second class, laccases (E.C. 1.10.3.2), oxidizes o-diphenols as well as p-diphenols (Figure II.2b), and it is not common in fruits although there are reports of its existence in peach and apricot (Walker, 1995).

The nomenclature of these enzymes is somewhat confusing since in addition to the two numbers E.C. 1.10.3.1 and E.C. 1.10.3.2, a third one exists, E.C. 1.14.18.1. It is referred to as monophenol monooxygenase (tyrosinase) and corresponds to the same enzymes as E.C. 1.10.3.1, which always catalyze the hydroxylation of monophenols (Nicolas et al., 1994).

Although peroxidases (EC 1.11.1.7) are distributed widely and they can also be considered as participating in enzymatic browning, they generally appear to be little involved in enzymatic browning of fruits and vegetables following a mechanical stress. The explanation could be that the peroxidase activity is limited by the internal level of hydrogen peroxide (Nicolas et al., 1994).

Inhibition

Because of its significance to the quality of fruit and vegetable products, the inhibition of enzymatic browning has been extensively studied. A few review papers (McEvily et al., 1992; Nicolas et al., 1994;) and a special book (Lee and Whitaker, 1995) have been published on this subject. Based on the enzymatic browning reaction mechanism, the inhibition approaches can be classified into five categories as shown in Table II.3. Most often, a combination of these approaches are taken (Vámos-Vigyázó, 1995).

Table II.3. Procedures used to limit browning (adapted from Macheix et al., 1990)

Mechanism	Experimental conditions
Inhibition of o-DPO	NaCl[1], $CaCl_2$[40], SO_2[3], low pH[37,38], high temperature[20,21], drying, NaOH[2,4], metal chelator (sodium diethyldithiocarbamate)[35], hormones (gibberellic acid, ethephon)[22], p-coumaric acid[6], 4-hexylresorcinol[15,16], unsaturated dicarboxylic acids[7,8], salicylhydroxamic acid[23], protease[15]
Exclusion of oxygen	Controlled atmospheres[25,26,27], high concentration of sugars[5], paraffin[36]
Reduction or trapping of quinones	Ascorbic acid and derivatives[14,16], glutathione[17,18], cysteine[17,18,19], N-acetylcysteine[11,12,13, 17, 18], SO_2 and metasulfites[15,17,18,39],polyvinylpyrrolidone[28,29], cyclodextrin[24]
Modification of substrates	methylation by O-methyltransferase[9], oxidative ring-opening by protocatechute-3,4,-dioxygenase[10]
Removal of browning	Unltrafiltration[31,32,33], resins[30], clarification[34], centrifugation[33]

1. El-Shimi, 1993.
2. Sapers and Miller, 1993.
3. Sayavedra-Soto and Montgomery, 1986.
4. Angel and Ben-Shalom, 1979.
5. Grncarevic and Hawker, 1971.
6. Walker and Wilson, 1975.
7. Janovitz-Klapp et al., 1990.
8. Son et al., 2000.
9. Finkel and Nelson, 1963.
10. Kelly and Finkle, 1969.
11. Buta et al., 1999.
12. Edwards and Wedzicha, 1997.
13. Friedman,1996.
14. Walker1977.
15. McEvily et al., 1992.
16. Sapers et al., 1989.
17. Molnar-Perl and Friedman,1990a
18. Molnar-Perl and Friedman,1990b.
19. Robert et al., 1996.
20. Chan and Yang,1971
21. Pardon et al., 1975.
22. Paulson et al., 1980.
23. Allan and Walker, 1988.
24. Billaud et al., 1995.
25. Smyth and Cameron, A.C., 1988.
26. Sapers and Miller, R.L. 1988.
27. Gunes and Lee, 1997.
28. Okamura and Watanabe, 1981.
29. Van Buren et al., 1976.
30. Kagami et al., 1968.
31. Gokmen et al., 1998.
32. Constenla and DLozano, 1995.
33. Sapers, 1992.
34. Dawes et al., 1994.
35. Wesche-Ebeling and Montgomery, 1990.
36. Bhat and Chandel, 1991.
37. Zemel et al., 1990.
38. Tronc et al., 1997.
39. Taylor et al.,1986.
40. Vámos-Vigyázó, 1995.

The PPO inhibitors can be further classified into three subcategories based on the inhibition mechanism: metal chelators such as sodium diethylthiocarbamate (removing the copper ion from PPO), substrate analog such as p-coumaric acid, and compounds such as sulfites which modify the protein structure of PPO.

Sulfites are very effective anti-browning agents, and they are the most widely used agents in foods to control enzymatic browning (Lambrecht, 1995). They inhibit enzymatic browning by inhibiting PPO and form a quinone-sulfite complex (Haisman, 1974). However, the concerns over the allergenic reactions in an ever-expanding asthmatic population promoted the Food and Drug Administration (FDA) to limit the use of sulfites in fresh fruit and vegetable products (Sapers et al., 1995).

Due to the limitation of sulfites, great efforts have been made to develop sulfite alternatives. One of such alternatives is 4-hexylresorcinol, which is a synthetic analog to the enzymatic browning inhibitors isolated from fig (McEvily et al., 1992). 4-Hexylresorcinol has been demonstrated to inhibit the enzymatic browning in apple (Buta et al., 1999; Luo and Barbosa-Canovas, 1995), and in pear (Dong et al., 2000; Sapers and Miller, 1995). However, there are concerns for the use of 4-hexylresorcinol. One concern is that it may have influence on sensory properties, and Dong et al. (2000) detected the flavor difference when comparing the treated samples and controls, although it is not clear if the difference was caused by 4-hexylresorcinol. Another concern is that it is a synthetic chemical and its future approval of use to fresh fruit and vegetable products is not clear, although it has been approved to inhibit the blackspot in shrimp (Lambrecht, 1995).

Both ascorbic acid and its isomer, erythorbic acid, have been used to prevent the enzymatic browning because of their ability to reduce the o-quinones back to diphenols. However, there are serious shortcomings of either ascorbic acid or erythorbic acid as an antibrowning agent because they can be easily oxidized by endogenous enzymes, as well as decomposed by iron or copper-catalyzed autooxidation (McEvily et al., 1992). To overcome this shortcoming, derivatives of ascorbic acid with increased stability have been developed, and these derivatives are: ascorbyl phosphate esters (Sapers and Miller, 1992; McEvily et al., 1992) and ascorbyl fatty acid esters (McEvily et al., 1992). The usage of these derivatives to fruit and vegetables need approval.

However, if avoiding or reducing these treatments during a process is desired, the long-term solution should be chosen, consisting of selecting and cloning cultivars which have low browning potential because of low phenolic substrate contents or weak o-DPO activity. Care must be taken that the other sensory qualities of the fruit and the agronomic qualities of the plants selected this way are not seriously altered (Macheix et al., 1990).

PINEAPPLE JUICE

Production and horticultural aspects (Abd Shukor et al., 1998)

The geographic origin of pineapple (*Ananas comosus* [L.] Merr.) is believed to be in South America, in the region encompassing central and southern Brazil, northern Argentina and Paraguay. It was rapidly spread in the 15th and 16th centuries by the

Portuguese to Africa, India, and later to China and Japan, and by the Spanish to Europe and the Philippines.

Besides banana, pineapple is perhaps the most popular fruit from the tropics. The world production of pineapple was estimated to be 11.5 million m.t. in 1992. Thailand was the largest producer with almost 2 million m.t., followed by the Philippines, China, Brazil and India.

The fresh pineapple trade varies from 500K to 600K m.t./year. The major exporters are the Philippines, Hawaii, Ivory Coast, and Mexico. The major importer is E.E.C, followed by Japan.

For canned pineapple fruit and juices, the trade volume was just over a million m.t./year. The major importer of canned pineapple was the USA followed by the E.E.C. The main exporters are Thailand, closely followed by Philippines.

Cayenne is the most important group of pineapple and it is grown for both canning and fresh fruit. More than 70% of the pineapple grown in the world is from this group. The majority of large producing countries like Thailand, Philippines, Indonesia and Australia grow Cayenne for fresh fruit as well as for their canning industry. Varieties or variants of Cayenne includes Smooth Cayenne, Hilo, Kew, Champaka and Sarawak.

The Queen cultivars are quite extensively cultivated over the world for fresh fruit. Varieties in this group include Moris, Mauritius, MacGregor, Ripley Queen and Alexandra.

The Spanish cultivars are not widely cultivated in the world. It is the main canning cultivar in Malaysia. The common varieties in this group are Singapore Spanish, Ruby,

red Spanish, Masmerah, Gandul, Hybrid 36, Selangor Green, Nangka and Betik. The Gandul is currently the most widely grown variety for canning in Malaysia.

General composition

Several papers have been published on the general composition of pineapple juice such as minerals, sugar profile, organic acid profile, and amino acid profile, and physical properties such as pH, Brix, %ash and titratable acidity (Elkins et al., 1997; Hodgeson & Hodgson, 1993; Pilando and Wrolstad, 1992; Wallach and Faethe, 1988). Three sugars, sucrose, glucose and fructose have been reported. As to amino acids, there is some controversy about presence of tryptophan. Except for Dizy et al. (1992), tryptophan was not reported being present by other investigators (Elkins et al., 1997; Hodgeson and Hodgson, 1993; Wallach and Faethe, 1988). Citric acid is the predominant organic acid (70.1%), followed by malic acid (28.6%) and quinic acid (1.2%) (Pilando and Wrolstad, 1992).

Phenolics

Although pineapple juice is a major commercial product in international commerce, its phenolic profile has not been well characterized, and only a few papers have been published on this subject. For pineapple fruit (Macheix et al.,1990), juice (Fernández de Simón et al., 1992) and shell fibers (Larrauri et al., 1997), only nonflavonoid phenolics were reported with the exception of myricetin in fiber. The reported phenolics in pineapple are summarized in Table II.4.

Table II.4. Summary of reported phenolic composition of pineapple

Phenolics	Category		
	Fruit[1]	Juice[2]	Shell fiber[3]
trans-Cinnamic acid			x
p-Coumaric acid	x	x	x
Caffeic acid		x	
Ferulic acid	x	x	
Sinapic acid		x	
p-Coumaroylquinic acid	x	x	
di-*p*-Coumaroylquinic acid	x		
p-Coumaroyl glucose	x		
Caffeoyl glucose	x		
Feruloylglucose	x	x	
Sinapoyl glucose	x		
p-Hydroxybenzoic acid		x	
p-Hydroxybenzoic aldehyde		x	
Syringic acid		x	
Salicylic acid			x
Tannic acid			x
Tyramine	x		
Myricetin			x

1. Macheix and others 1990
2. Fernández de Simón and others 1992
3. Larrauri and others 1997

Gardner et al. (2000) investigated the relationship between composition and antioxidant activity of several juices, orange, apple, pineapple, grapefruit and vegetable juice. It was found that the antioxidant activity of citrus juices is mainly (>66%) attributed to ascorbic acid, whereas ascorbic acid contributes less than 5% to the other products, and 0.8% to pineapple juice. Most of the antioxidant activity is believed to be due to phenolic compounds in pineapple juice.

Enzymatic browning inhibition activity

Lozano-de-Gonzalez et al. (1993) reported that pineapple juice was an effective browning inhibitor in both fresh and dried apples. Pineapple juice was fractionated using various size and charge separation procedures. All fractions inhibited enzymatic browning of crude apple extracts by at least 26%. The results indicate that the inhibitor is a neutral compound of low molecular weight.

CHAPTER III

CHARACTERIZATION OF SINAPYL DERIVATIVES IN PINEAPPLE (*Ananas comosus* [L.] Merill) JUICE

Ling Wen[1], Ronald E. Wrolstad[1]*, and Victor L. Hsu[2]

[1]Department of Food Science and Technology , Oregon State University
[2]Department of Biochemistry and Biophysics, Oregon State University
Oregon State University, Corvallis, OR 97331-6602.

An extract from a publication in Journal of Agriculture and Food Chemistry, 1999, 47(3):850-853

ABSTRACT

Three previously unidentified phenolic compounds were found in pineapple (*Ananas comosus* [L.] Merill) juice in substantial concentrations, and were isolated by semi-preparative reverse phase HPLC. The structures were elucidated from UV spectra, acid hydrolysis and subsequent amino acid analysis, mass spectrometry, and two-dimensional NMR Spectroscopy. The compounds are identified as: S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione.

KEY WORDS: Pineapple juice, *Ananas comosus* [L.] Merill, phenolics, sinapyl derivatives, glutathione

INTRODUCTION

While pineapple (*Ananas Comosus* [L.] Merill) juice is a major commercial product in international commerce, its phenolics profile has not been well characterized, and only a few papers have been published on the phenolic composition of pineapple fruit (Macheix et al, 1990), juice (Fernández de Simón et al, 1992) and shell fiber (Larrauri et al, 1997). We found, however, in a preliminary investigation of pineapple juice phenolics by reverse phase HPLC/DAD, that the retention time and UV spectra of the major peaks did not match the phenolic compounds previously reported to be present. Three late-eluting peaks had identical UV spectra with a maximum absorption at 285 nm, suggesting the possibility of their being derivatives of the same basic phenolic compound. This publication reports the structures of these three new phenolic compounds which were determined by mass spectrometric and heteronuclear two-dimensional NMR techniques combined with acid hydrolysis and subsequent amino acid analysis.

MATERIALS AND METHODS

Source

Authentic pineapple juice concentrate samples were provided by the National Food Processors Association (Washington, D.C.) and Dole Food Company, Inc. (Westlake Village, CA), and stored at -20 °C until analysis.

Extraction

Pineapple juice concentrate (400 g) was diluted 4 fold with de ionized w ater, a nd ultracentrifuged at 23,000 g for 20 min. The supernatant was collected and filtered through Whatman #1 filter paper with Celite diatomaeous earth filtration aid (Celite Corp., CA). The filtrate (100 mL) was passed through a MeOH activated C18 cartridge (5 g resin) from Alltech Associates (Deerfield, IL), followed by a 100 mL 0.01% aqueous HCl wash and then eluted with 50 mL MeOH. The MeOH eluate was combined and kept at -10^{0}C overnight, then passed through a 0.45 μm Millipore HA membrane to remove the white precipitate which had formed, presumably polysaccharides or proteins. The filtrate was rotary evaporated to dryness at 35 °C, then dissolved in 10 mL 0.01% aqueous HCl: MeOH (70: 30), and filtered through a 0.45 μm Millipore HV membrane prior to semi-preparative HPLC isolation.

Semi-preparative HPLC

Two Dynamax SD-300 pumps were used with a semi-preparative Microsorb C18 column (25 cm x 21.4 mm, 5 μm) from Rainin Instrument Co. (Woburn, MA). The end of the column was connected to a flow divider, which split 1 mL/min flow to a HP 1040A Diode Array Detector (DAD) with detection at 280 nm and the rest of flow (19 mL/min) to an o utlet f or m anual p eak c ollection. T he f ollowing g radient e mploying s olvent A

(methanol) and solvent B (0.07 M K-PO_4 buffer, pH 2.4) was used: 7 min from 35 to 45% solvent A, then 3 min from 45 to 48% solvent A and holding for 5 min. The peak purity was verified by analytical HPLC. Each peak collected from the preparative HPLC was rotary evaporated at 35 °C for 10 min to remove MeOH, then extracted with an Alltech C18 cartridge (5 g). The MeOH eluate from the cartridge was rotary evaporated to dryness and stored at -15 °C until analysis. Approximately 5 mg of compound 1, 50 mg of compound 2, and 50 mg of compound 3 were isolated.

Analytical HPLC

A Supelco LC-18 column (25 cm x 4.6 mm, 5 μm) was used with a HP 1040A DAD set at 280 nm. Flowrate: 1.0 mL/min; Injection: 50 μL. The following gradient employing methanol (solvent A), acetonitrile (solvent B) and solvent C (0.07 M K-PO_4 buffer, pH 2.4) was used: 10 min from 10% A/0% B to 22% A/0% B, then 25 min from 22% A/0% B to 22% A/25% B, the remainder being C.

Acid hydrolysis

About 0.5 mg each of compound 2 and 3 were acid hydrolyzed in a Teflon-lined screw-cap test tube with 5 mL 2 N HCl at 100 °C for 30 min in the dark (10 min N_2 flushing before hydrolysis), then cooled in ice-bath and extracted with a C18 Sep-Pak (360 mg resin) from Waters Associates (Milford, MA). The MeOH eluate of the Sep-Pak was rotary evaporated to dryness and dissolved in 0.01% aqueous HCl and subjected to HPLC analysis using the same conditions as stated above.

Amino acid analysis

200 μL of the MeOH eluate from the isolation step above was transferred to a 1.5 mL Teflon-lined screw-cap reaction tube, flushed dry with N_2, dissolved in 120 μL 6 N HCl, N_2 flushed for another min and then capped. The hydrolysis was performed in a heating

block at 95 °C for 3 hrs. The hydrolysate was subjected to amino acid analysis by PITC derivatization as described by Hagen et al. (1993), and the identity of hydrolyzed amino acids were confirmed by spiking the sample with the standard PITC derivatives prepared.

Mass spectrometry

Electrospray ionization MS (ESI-MS) were performed on a Perkin Elmer Sciex API III+ triple quadrupole ionspray mass spectrometer (Ontario, Canada) by pneumatically assisted electrospray. The operating conditions were standard. Tandem MS/MS were recorded on the same instrument with Ar-N_2 (9:1) as target gas. The collision energy was 15 or 25 V. Operating conditions were standard. The FAB-MS sample was prepared in a matrix of 0.1 N toluenesulfonic acid in 1:1 glycerol: 3-nitrobenzyl alcohol, and the positive spectra was recorded on a Kratos M550TC instrument (Manchester, UK) at a scan speed of 10 sec/decade using a resolution of 1100, with the gun producing a 7 KV beam of Xenon atoms.

NMR

^1H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded at 25 °C on a Bruker DRX 600 NMR Spectrometer (Billierica, MA). A mixed solvent was used (D_2O:CD_3OD:CF_3COOD - 60 :30:10 by volume) and the residual D_2O resonance was used as internal chemical shift reference. ^1H-^1H COSY and TOCSY, ^1H-^{13}C HMQC and HMBC experiments were performed using standard pulse sequence. Spectral widths of 10 and 200 ppm were used in the ^1H and ^{13}C dimensions, respectively.

S-sinapyl-L-cysteine (compound 1): A slightly yellow solid, ESI-MS m/z: 314.0 (MH^+), 192.8 (MH^+ - cysteine), 160.8. MS/MS of daughter ion (m/z 192.8, collision energy 25 volts) m/z (rel. int.): 192.8 (20), 161.2(100), 143.2 (15), 133.2(100), 115.0 (60), 105.2 (100), 79.0 (15), 55.0 (8). UV spectrum: see Figure 6; λ_{max} nm: 285 (mobile phase). ^1H NMR: see Table 1.

S-sinapyl glutathione (compound 2): A slightly yellow solid, ESI-MS m/z: 500.0 (MH^+), 308.0 (Glutathione•H^+), 192.8 (MH^+ - Glutathione). UV spectrum: the same as compound 1. ^1H and ^{13}C NMR: see Table 1 and 2. ^1H -^{13}C HMQC: see Table 3.

N-L-γ-glutamyl-S-sinapyl-L-cysteine (compound 3): A slightly yellow solid, ESI-MS m/z: 442.8 (MH^+), 250.8 (Glu-Cys•H^+), 192.8 (MH^+ - Glu-Cys). FAB-MS m/z 443.1473 (calculated for $C_{19}H_{27}O_8N_2S_1$: 443.1458; deviation : -3.4 ppm). UV spectrum: the same as compounds 1 and 2. ^1H NMR: see Table 1.

Sinapyl alcohol standard (Aldrich, Milwaukee, WI): ESI-MS m/z: 193.0 (MH^+-H_2O). MS/MS (collision energy 15 volts) m/z (rel. int.): 192.8 (16), 161.2 (80), 143.2 (5), 133.2 (83), 115.0 (45), 105.2 (100), 79.0 (10), 55.0 (5). UV spectrum: the same as other sinapyl derivatives (mobile phase).

RESULTS AND DISCUSSION

Figure 1 shows the analytical HPLC chromatogram of pineapple juice with peaks 1, 2 and 3 being the compounds investigated. The identities of other peaks and their concentration range for 54 authentic pineapple juice concentrate samples will be reported in another publication. Figure 2 is the semi-preparative HPLC chromatogram of pineapple juice after C18 cartridge fractionation which permitted collection of peaks 1, 2 and 3 without impurities as determined by analytical HPLC. Figure 3 shows the acid hydrolysis pattern of compounds 2 and 3. The acid hydrolysis of compound 2 generated compound 1, 3 and an additional compound with the same UV spectrum. Acid hydrolysis of compound 3 only generated compound 1. Compound 1 was no longer hydrolyzed by

Table III.1. ^1H NMR data for compounds1, 2 and 3 [ppm in D_2O:CD_3OD:CF_3COOD (600 MHz)]

H	1	2	3
2, 6	6.44 s	6.35 s	6.40 s
7	6.13 d(15.6)	6.04 d (15.6)	6.08 d (15.6)
8	5.78 dt (15.6, 7.8)	5.69 dt (15.6, 7.8)	5.73 dt (15.6, 7.8)
9	3.07 d (7.8)	2.94 d (7.8)	3.00 d (7.8)
1", 2"	3.54 s	3.47 s	3.51 s
2'	_	3.58 s	_
4'	3.95 dd (7.8, 4.2)	4.20 t (6.6)	4.26 dd (8.4, 4.8)
6'	_	2.18 t (7.2)	2.21 t (7.2)
7'	_	1.86 dt (7.2, 7.2),	1.90 dt (7.2, 7.2),
		1.80 dt (7.2, 7.2)	1.84 dt (7.2, 7.2)
8'	_	3.69 t (6.6)	3.74 t (6.6)
10'	2.86 dd (15.0, 4.2),	2.60 dd (13.8, 4.8),	2.69 dd (13.8, 4.8),
	2.71 dd (15.0, 7.8)	2.44 dd (13.8, 8.4)	2.52 dd (13.8, 8.4)

Coupling const. (J in Hz) given in parentheses
Chemical shifts assigned by comparison to chemical shift pattern of compounds 1, 2, 3 and glutathione standard, and also from COSY, TOCSY, HMQC and HMBC data

Figure III.1. Analytical C_{18} reverse-phase HPLC of pineapple juice. Peak identity: (1) S-sinapyl-L-cysteine; (2) S-sinapyl glutathione; (3) N-L-γ-glutamyl-S-sinapyl-L-cysteine.

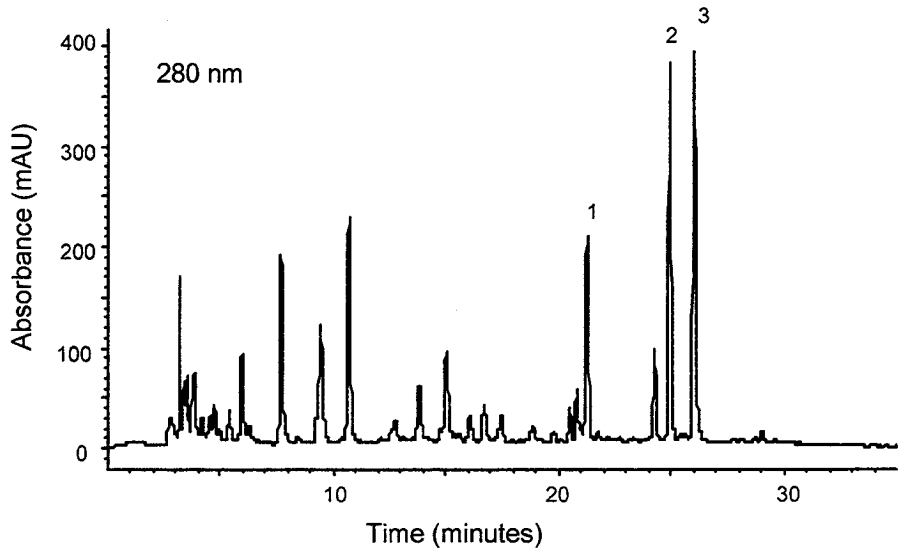


Figure III.2. Semi-preparative C_{18} reverse-phase HPLC of pineapple juice (after C_{18} cartridge fractionation). Peak identity: (1) S-sinapyl-L-cysteine; (2) S-sinapyl glutathione; (3) N-L-γ-glutamyl-S-sinapyl-L-cysteine.

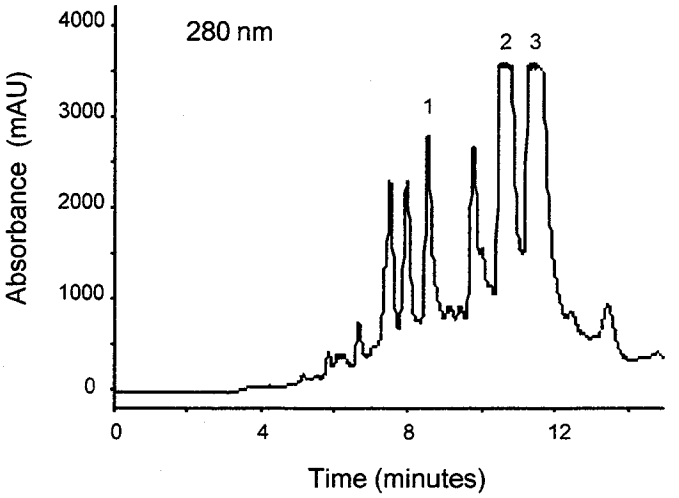
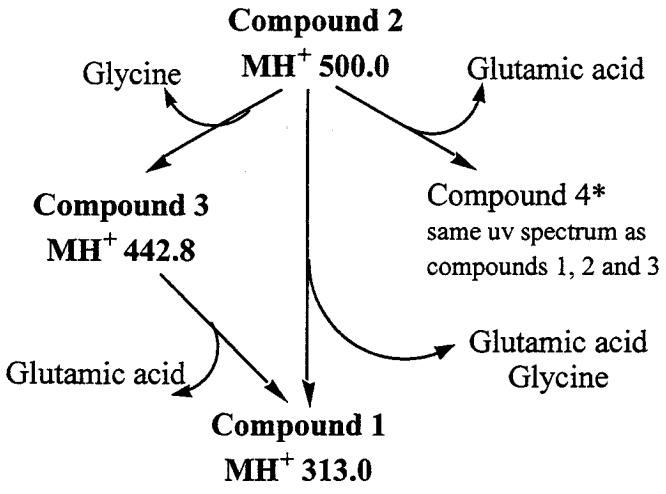
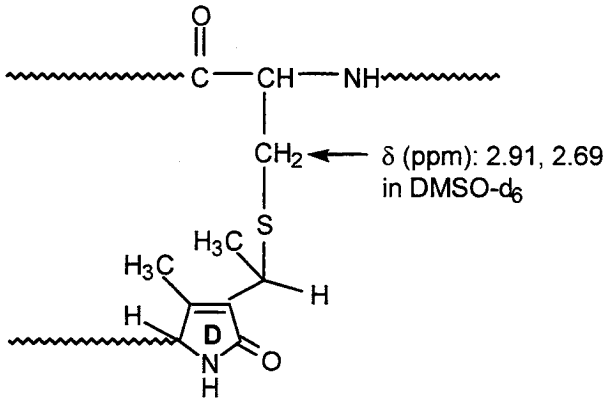


Figure III. 3. Acid hydrolysis pattern of compounds 2 and 3.



* not found in analytical HPLC of pineapple juice

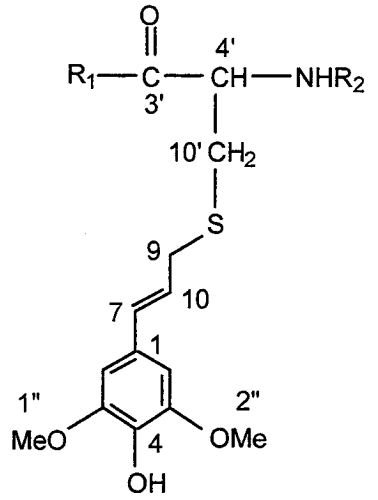
Figure III.4. Partial structure of doubly linked 15,16-dihydrobiliverdin. (Redrawn from Wemmer, D. E.; Wedemayer, G. J .; Glazer, A. N. 1993. Phycobilins of cryptophycean Algae. J. Biol. Chem. 268(3): 1658-1669).



acid. The odd-number in molecular weight differences between compounds 2 and 3, and also between compound 3 and 1, as determined by ESI-MS, suggested the differences were caused by amino acid residues - glutamic acid and glycine. The release of those same amino acids from compounds 2 and 3 with acid hydrolysis was confirmed by amino acid analysis. Compound 1 has an odd-number molecular weight, so it should contain an odd-number of nitrogens and it is also possibly an amino acid derivative of a basic phenolic c ompound. Gl utathione (Glu-Cys-Gly) is commonly found in plants, so it is possible that compound 2 is glutathionyl derivative of a phenolic compound, such as glutathionyl caftaric acid, which has been identified in grape juice (Cheynier et al, 1986). This structural proposal is in agreement with the ESI-MS fragmentation pattern of the three compounds. The ESI-MS shows a fragment dissociation of 121 units from compound 1 which is the molecular weight of cysteine, 307 units (glutathione) from compound 2 and 250 units (Glu-Cys) from compound 3.

The molecular weight of compound 3 as determined by FAB-MS is 443.1473, with several different formulas being possible. The formula ($C_{19}H_{27}O_8N_2S_1$, MH^+) was chosen based on the evidence for one sulfur atom (isotopic ratio ^{32}S: ^{34}S - 100:4.4) and the maximum UV absorption at 285 nm. The proton NMR spectra of compounds 1, 2 and 3 exhibit the characteristic pattern of Cys, Glu-Cys-Gly, Glu-Cys except that the two β methylene protons of Cys were split (see Table 1). However, this splitting has been previously reported for a conjugated di-peptide with similar type of structure (see Figure 4, Wemmer et al, 1993). The proton NMR spectra of these three compounds also exhibit the same resonances with chemical shifts at 6.4, 6.0, 5.5, 3.5 and 3.0 ppm. The 6.4 ppm chemical shift is the characteristic resonance of aromatic protons, while 6.0 and 5.5 ppm shifts are traits of double-bond protons. The final structure (Figure 5) was established by ^{13}C NMR (Table 2), HMQC and HMBC (Table 3). The sinapyl core structure was further confirmed by the same UV spectrum (Figure 6) and fragmentation pattern as sinapyl alcohol standard. It was found that the two protons at the C-2 and C-6 positions

Figure III.5. Structure of compounds 1, 2 and 3.



Compound	1	2	3
R_1	OH	HOOC—CH_2—NH— (1', 2')	HOOC—CH_2—NH— (1', 2')
R_2	H	—C(=O)—CH_2—CH_2—CH(NH)(COOH) (5', 6', 7', 8', 9')	H

Table III.2. ^{13}C NMR spectral data for compound 2
[ppm in D_2O:CD_3OD:CF_3COOD (150 MHz)]

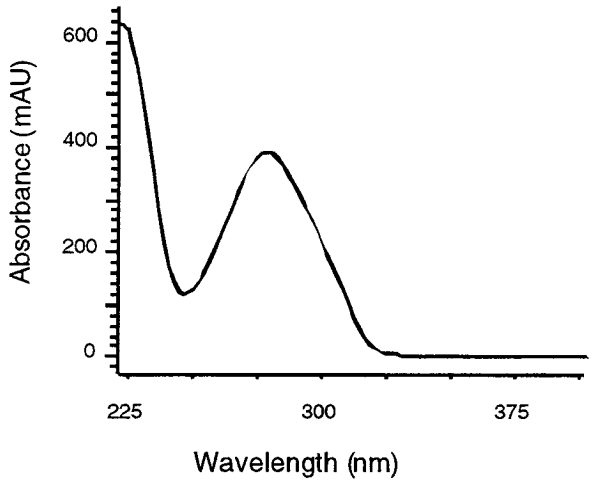
C	ppm
1	128.7
2	104.0
3	147.7
4	134.3
5	147.7
6	104.0
7	132.6
8	123.6
9	34.0
1", 2"	56.3
1'	172.6
2'	41.0
3'	172.7
4'	53.2
5'	174.0
6'	31.0
7'	25.5
8'	52.2
9'	171.2
10'	31.9

1' and 3' are overlapping

Table III.3. Cross-peaks in HMBC spectra of compound 2

	C	
H	3-bond	2-bond
2, 6	6, 2, 4, 7	3, 5
7	2, 6, 9	1
8	1	9
9	7, 10'	8
1", 2"	3, 5	
2'	3'	1' (?)
4'	5'	3', 10'
6'	8'	5', 7'
7'	5', 9'	6', 8'
8'	6'	7', 9'
10'	9, 3'	4'

Figure III.6. UV spectrum of compound 1. Compounds 2, 3 and sinapyl alcohol have the same UV spectrum as compound 2.



could undergo deuterium exchange under very acidic condition when kept at room temperature for over two days. This exchange was followed by ^{1}H NMR resulting in the almost complete loss of proton chemical shifts at 6.4 ppm. The deuterium exchange was further confirmed by 2 mass units increase in the fragment ion corresponding to the sinapyl moiety [m/z (without deuterium exchange): 193] in the ESI-MS. This deuterium exchange can be used to establish the fragmentation pathway during MS/MS by comparing the fragmentation pattern of the compound with that of the deuterium-exchanged compound.

Glutathione is a common reducing compound in biological systems. It is involved in biosynthesis and metabolism, and also plays a role in plant protection. For the glutathionyl derivative of caftaric acid identified in grape juice (Cheynier et al, 1986), glutathione was directly attached to the aromatic ring as a reducing agent after the oxidation of caftaric acid. The mechanism for the formation of the glutathionyl derivative of sinapyl alcohol in pineapple juice is not known. Furthermore, it is not clear if compounds 1 and 3 are formed independently or if they are the hydrolytic products of compound 1. To our knowledge, this is the first report of the presence of glutathionyl derivatives of sinapyl alcohol.

ABBREVIATIONS USED

COSY: correlation spectroscopy; TOCSY: total correlation spectroscopy; HMQC: heteronuclear multiple bond quantum correlation; HMBC: heteronuclear multiple bond correlation; ESI-MS: electrospray ionization mass spectrometry; FAB-MS: fast atom bombardment mass spectrometry.

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ACKNOWLEDGMENT

This work was funded by the Washington-Oregon Tree Fruit Commission, and the pineapple juice concentrate samples were supplied by the National Food Processors Association (NFPA) and Dole Food Company, Inc. This is technical paper no. 11,377 from Oregon Agricultural Experiment Station. We thanks D. A. Griffin for recording the ESI-MS and tandem MS/MS, B. Arbogast for recording the FAB-MS, R. W. Durst for help in HPLC, L. E. Rodriguez-Saona for help in amino acid analysis, S. Lam and S. Watts for recording some of the NMR spectra, M. M. Guisti for help in NMR data analysis, and J. F. Stevens for help in MS data analysis.

CHAPTER IV

PHENOLIC COMPOSITION OF AUTHENTIC PINEAPPLE JUICE

Ling Wen and Ronald E. Wrolstad

Department of Food Science & Technology, Oregon State University

Corvallis, OR 97331-6602

Accepted by Journal of Food Science

ABSTRACT

The phenolic composition of authentic pineapple juice concentrate was analyzed by HPLC. Nine major peaks accounting for 70% of total peak area were characterized and their concentrations measured in 54 commercial samples. Means and standard deviations were as follows (mg/100 mL single-strength juice, normalized to 12.8 ^{0}Brix): tyrosine, 3.6(1.4); serotonin, 1.8(0.8); dimethylhydroxylfuranone, 1.4(0.7); dimethylhydroxylfuranone β-glucoside, 6.2(3.0); tryptophan, 2.2(0.9); S-sinapyl-L-cysteine, 1.1(0.6); N-γ-L-glutamyl-S-sinapyl-L-cysteine, 2.3(1.1); S-sinapyl glutathione, 5.4(1.4); and a *p*-coumaric acid-like phenolic compound (calculated as *p*-coumaric acid), 0.5(0.4). This information will be useful for evaluation of authenticity and quality.

Keywords: pineapple juice, concentrate, phenolics, sinapyl, composition

INTRODUCTION

Although pineapple (*Ananas comosus* [L.] Merr.) juice is a major commercial product in international commerce, its phenolic profile has not been well characterized, and only a few papers have been published on this subject. For pineapple fruit (Macheix et al. 1990), juice (Fernández de Simón et al. 1992) and shell fibers (Larrauri et al. 1997), only nonflavonoid phenolics were reported with the exception of myricetin in fiber. The reported phenolics in pineapple are summarized in Table IV.1. The phenolic composition is very useful for establishing authenticity of juice (Kirksey et al. 1995) and evaluating the effect of processing on juice quality (Spanos and Wrolstad 1990a, 1990b; Spanos et

al. 1990). In addition, many phenolics are believed to provide healthy benefits as reviewed by Ho et al. (1992).

However, in our preliminary examination of pineapple juice phenolics by reverse-phase HPLC coupled with a photodiode array detector, we found that the retention times and UV spectra of the major peaks did not match the phenolic compounds previously reported to be present. Further investigation (Wen et al. 1999) identified 3 new sinapyl derivatives with amino acids as major compounds: S-sinapyl-L-cysteine, N-γ-L-glutamyl-S-sinapyl-L-cysteine and S-sinapyl glutathione.

Interestingly, some phenolic compounds have been reported in pineapple juice volatiles. Chavicol (*p*-allylphenol) was isolated and identified by Silverstein et al. (1965). Ethylphenol was tentatively identified by headspace GC/MS for whole intact fruit (Takeoka et al. 1989). Eugenol, vanillin and 4-allyl-2,6-dimethoxyphenol were identified, and phenol, 4-hydroxybenzaldehyde and syringaldehyde were tentatively identified in either free or glycosidically bound form or both forms at 10 ppb levels for fresh pineapple juice (Wu et al. 1991).

Table IV.1. Summary of reported phenolic composition of pineapple

Phenolics	Category		
	Fruit[a]	Juice[b]	Shell fiber[c]
trans-Cinnamic acid			x
p-Coumaric acid	x	x	x
Caffeic acid		x	
Ferulic acid	x	x	
Sinapic acid		x	
p-Coumaroylquinic acid	x	x	
di-*p*-Coumaroylquinic acid	x		
p-Coumaroyl glucose	x		
Caffeoyl glucose	x		
Feruloylglucose	x	x	
Sinapoyl glucose	x		
p-Hydroxybenzoic acid		x	
p-Hydroxybenzoic aldehyde		x	
Syringic acid		x	
Salicylic acid			x
Tannic acid			x
Tyramine	x		
Myricetin			x

a. Macheix et al. 1990
b. Fernández de Simón et al. 1992
c. Larrauri et al. 1997

Some compounds, which are not normally considered as typical phenolic compounds, appear as peaks in HPLC analysis of phenolics. They are benzaldehyde (Wu et al. 1991), tyrosine (Hodgson and Hodgson 1993; Elkins et al. 1997), dimethylhydroxylfuranone (Rodin et al. 1965; Ohta et al. 1987; Lee and Nagy 1987; Wu et al. 1991) and its β-glucoside (Wu et al. 1991), and hydroxylmethylfurfural (Dizy et al. 1992). Serotonin, the brain neurotransmitter, was also reported in pineapple juice (Bruce 1960; Feldman and Lee 1985). Many other aromatic nonphenolic compounds have been summarized by Hodgson and Hodgson (1993) for pineapple volatiles, and they are β-phenylethanol, cinnamic acid, 1-coumarilic acid, 2-phenylethyl acetate, ethyl benzoate and methyl benzoate.

The objective of this study was to characterize the major peaks in the HPLC of pineapple juice and to establish a compositional database for commercially produced pineapple juice concentrate. It is an integrated part of the National Food Processors Association (NFPA)'s effort to establish a compositional database for commercial pineapple juice concentrate (Elkins et al. 1997). The report by Elkins et al. (1997) summarized the measurements on pH, Brix, ash%, titrable acidity, minerals, sugar profile, organic acid profile, and amino acid profile for these same samples.

MATERIALS AND METHODS

Source

Authentic commercially produced pineapple juice concentrate samples (n = 54) were supplied by NFPA and stored at –15 ^{0}C until analyzed. The source of these samples is listed in Table IV.2. A total of 54 representative samples were collected over 3 years from significant growing regions around the world. The supplier was required to guarantee the authenticity of the sample. Some processing techniques, which may have significant effect on composition, such as ion-exchange, added pectin and/or the addition of acid, were not allowed for the production of the samples for this study. The detailed source and processing information can be found in Elkins et al. (1997). In addition, one additional juice concentrate and one canned juice were supplied by Dole Food Co., Inc. (Westlake Village, CA), and one fresh pineapple (c.v. Smooth Cayenne, Maui, Hawaii) was purchased from a local supermarket.

Sample preparation

The pineapple juice concentrate was diluted 4 times with deionized water, ultracentrifuged at 23000 g, and the supernatant was collected. Fresh pineapple was cut into cubes and blended into puree, then ultracentrifuged and the supernatant was collected. There is no need of ultracentrifugation for canned juice. Each sample was filtered through a 0.45 μm HA membrane (Millipore Corp., Bedford, MA) before HPLC analysis.

Table IV.2. Source of pineapple juice concentrate[a]

Variety	Growing region	Number of samples			Variety Code
		1st Yr	2nd Yr	3rd Yr	
Smooth Cayenne	Thailand; Philippines			6	1
Cayenne	Bukidnon, Philippines	7	9	3	2
Champaka	Maui, Hawaii	2	3	1	3
Cayenne Lisa	La Ceiba, Honduras	2			4
Cayenne F-200	Southern Philippines	2			5
N/A	Dominican Republic	4			6
N/A	N/A	7	8		7
		Total = 54			

[a] Some processing techniques such as ion-exchange, added pectin and/or acid addition were excluded in the production of samples. See Elkins et al. (1997) for details.

Standards

All chemical standards were purchased from Sigma Chemical Co. (St. Louis, MO), except that dimethylhydroxylfuranone (DMHF) was purchased from Aldrich (Milwaukee, WI) and N-γ-L-glutamyl-S-sinapyl-L-cysteine was isolated as described (Wen et al. 1999). Tyrosine (13.9 mg), serotonin (5.9 mg), DMHF (18.1 mg), tryptophan (6.2 mg), *p*-coumaric acid (6.7 mg) and S-sinapyl glutathione (10.6 mg) were dissolved in less than 5 mL MeOH, then brought up to 100 mL in a volumetric flask with 0.01% aqueous HCl and filtered through a 0.45 μm HA membrane. This is the 1st standard solution, which was subsequently diluted 8/12, 4/12 and 1/12 of the stock solution. A total of 4 standard solutions were used.

HPLC analytical system

A Perkin-Elmer Series 400 liquid chromatograph equipped with a 1040A Hewlett-Packard photodiode array detector and a Gateway 2000 computer system with Hewlett-Packard HPLC^{2D} Chemstation software was used. Juice samples (50 μL) were injected via a Beckman autosampler in double-injection mode. Peaks were monitored by their absorbance at 280 and 320 nm and quantified by their integrated peak areas using external standards. Absorbance between 200 and 400 nm was recorded for all peaks. To isolate the phenolics, a Supelco (Bellefonte, PA) LC-18 column (25 cm x 4.6 mm, 5 μm) was used with a flow rate of 1.0 mL/min at ambient temperature. The following gradient using methanol (solvent A), acetonitrile (solvent B) and solvent C (0.07 M K-PO4 buffer, pH 2.4) was used: 10 min from 10% A/0% B/90% C to 22% A/0% B/78% C, 25 min from 22% A/0% B/78% C to 22% A/ 25% B/53% C.

Characterization of major peaks

Peak assignments were made by co-chromatography with authentic standards (when available) and matching UV spectra. For some peaks, further characterization approaches such as enzymatic hydrolysis (see below) or retention behavior on ion-exchange resins (see below) were applied.

Characterization of DMHF-glucoside

The glucosidic DMHF was extracted from pineapple juice and enzymatically hydrolyzed using a slightly modified method from Wu et al. (1991). Pineapple juice supernatant (100 mL) was passed through a MeOH-activated C_{18} cartridge (5 g resin) from Alltech Associates (Deerfield, IL) under slight vacuum, and the direct eluate was collected. A total of 3 separations were conducted and 300 mL direct eluate was collected. The direct eluate was passed through 20 mL XAD-1180 (Alltech Associates) resin , which was packed in a 1 cm ID column that had been extensively washed with 200 mL MeOH followed by 200 mL deionized water before application of sample. The XAD resin was washed by 200 mL deionized water, followed by 300 mL hexane, and the DMHF-Glucoside was eluted with 300 mL MeOH. The methanolic eluate was rotary-evaporated to dryness at 35 °C and then dissolved in 5 mL 0.2 M citrate-phosphate buffer (pH 5.0) and 1 mL was added to 5.5 mg almond glucosidase (Sigma) and hydrolyzed overnight at 37 °C. The aglycone was confirmed by spiking with the standard.

Retention characterization by ion-exchange

CM Sephadex C-25 (Sigma Chemical Co., St Louis, MO) and BioRex-5 (chloride form; Bio-Rad Laboratories, Richmond, CA) resins were swollen in deionized water overnight, and slurry-packed in disposable Poly-Prep chromatography columns (0.8 x 4 cm graduated column with an integral 10-mL reservoir, Bio-Rad Laboratories) with 1.4 mL final wet volume for CM Sephadex C-25 and 1.2 mL for BioRex-5. The column bed was rinsed with 10 mL deionized water and 5 mL sample was carefully applied. The first 1.5 mL was discarded and the rest of the sample was collected and analyzed by HPLC.

Quantification of HPLC

External standards were used for calculation. The standard solutions were inserted into running sequence after every 4 samples. All samples and standards were kept frozen when not running. Except that *p*-coumaric acid was based on 320 nm absorption, all other peaks were based on 280 nm absorption. Tyrosine, tryptophan, serotonin, DMHF and S-sinapyl glutathione were each directly calculated from their standard curves. DMHF-GLU was calculated from the DMHF standard curve with the assumption of identical absorption behaviors. S-sinapyl-L-cysteine and N-γ-L-glutamyl-S-sinapyl-L-cysteine were calculated using S-sinapyl glutathione as standard with molecular weight correction. The *p*-coumaric acid-like compound was based on *p*-coumaric acid standard.

Antioxidant activity analysis

Antioxidant capacity (AOC) was measured using a Photochem photo-chemiluminometer (FAT, Berlin, Germany). Photo-chemiluminescent (PCL) assay is

based on continuous generation of free radicals (superoxide) in the solution of photo-sensitiser (luminol) by UV irradiation which is paralleled by simultaneous measurement of light emission produced by luminol during its interaction with superoxide radical causing delay in light output and/or decrease in its intensity which are proportional to the concentration of antioxidant. AOC is measured as an inhibition (I) of light output: I = 1-A/A0, where A is an area under the kinetic curve of light output in the presence of assayed sample, and A0 is an area of control (blank) sample. AOC of samples was expressed as Trolox equivalent of antioxidant capacity (TEAC), namely, calculated molar amount of Trolox with AC corresponding to 1 mole of assayed compound when plotted against Trolox standard calibration curve. PCL assay was conducted for both water-based and MeOH-based solution with triplicate measurements.

Statistical analysis

The analysis was conducted using SAS statistical software (SAS Institute Inc., Cary, NC) with the supplied ANOVA, PRINCOMP and DISCRIM procedures for analysis of variance, principal component analysis and canonical variable analysis, respectively.

RESULTS AND DISCUSSION

Fig. IV.2 is a typical HPLC chromatogram of pineapple juice phenolics. Nine major peaks were characterized, and their concentrations are summarized in Table IV.3 for all 54 samples. For comparison, an additional authentic pineapple concentrate, a canned

pineapple juice (single-strength) and a fresh pineapple juice were also analyzed. All the nine major phenolic peaks were also found in these 3 samples, and the concentration also fell within the range of the concentrate samples. The existence of all the major peaks in fresh pineapple means that none of these peaks was an artifact generated by processing. Since only one fresh juice sample was analyzed, no comparisons between fresh and concentrate samples are attempted in this discussion. Table IV.4 is a summary of the values reported in the literature for comparison with this investigation.

Sample preparation for HPLC

Cilliers and Van Niekerk (1984) described difficulties working with viscous tropical juices, such as pineapple juice, for HPLC analysis. Lee et al. (1986) reported that preliminary sample purification with the clarification agents (Carrez reagents: 30% zinc acetate and 15% potassium ferrocyanide) could remove the pulp, fat, protein and carotenoids. However, in this investigation, it was found that a pretreatment approach with ultracentrifugation and membrane filtration is sufficient, and no interference was observed for HPLC analysis of pineapple juice phenolics with continuous autosampler injection over 1 week.

Figure IV.1. Chemical structures of all the identified phenolic peaks in pineapple juice.

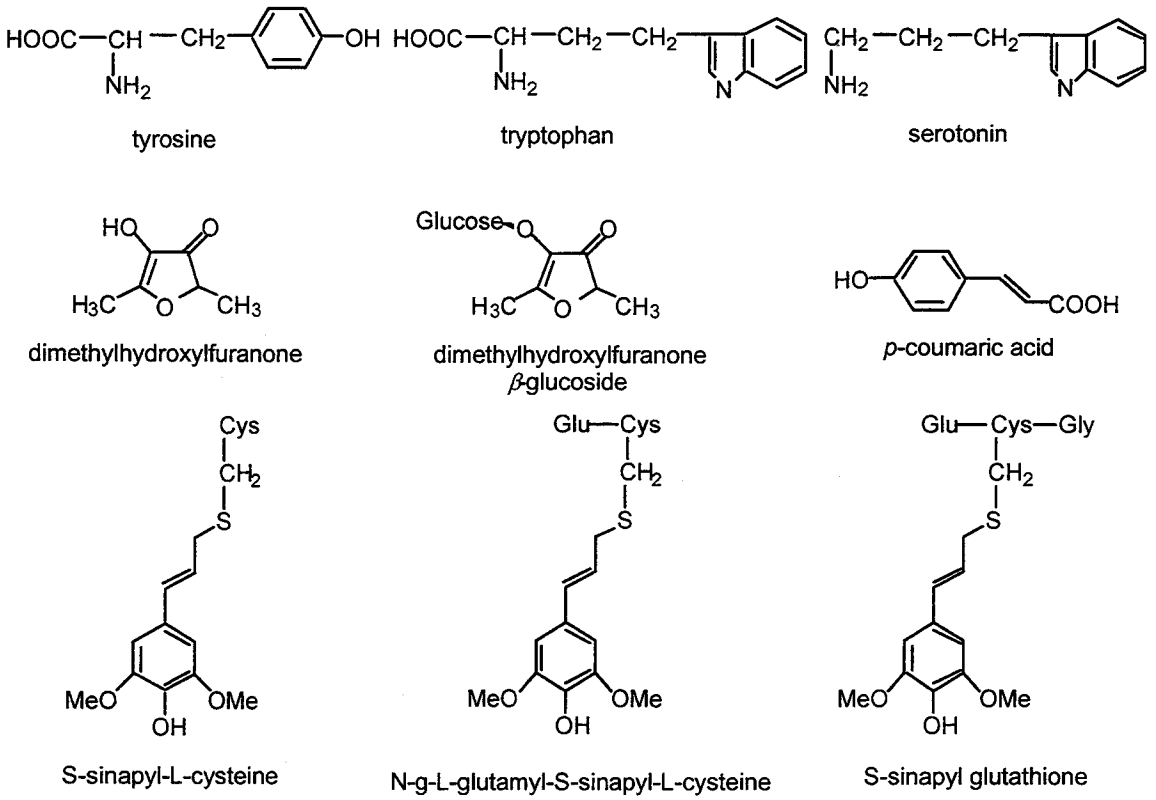

Figure IV.2. HPLC of pineapple juice concentrate.
Peak assignment: 1. tyrosine, 2. serotonin, 3. dimethylhydroxylfuranone β-glucoside, 4. dimethylhydroxylfuranone, 5. tryptophan, 6. S-sinapyl-L-cysteine, , 7. a *p*-coumaric acid-like phenolic compound, 8. N-γ-L-glutamyl-S-sinapyl-L-cysteine, 9. S-sinapyl glutathione.

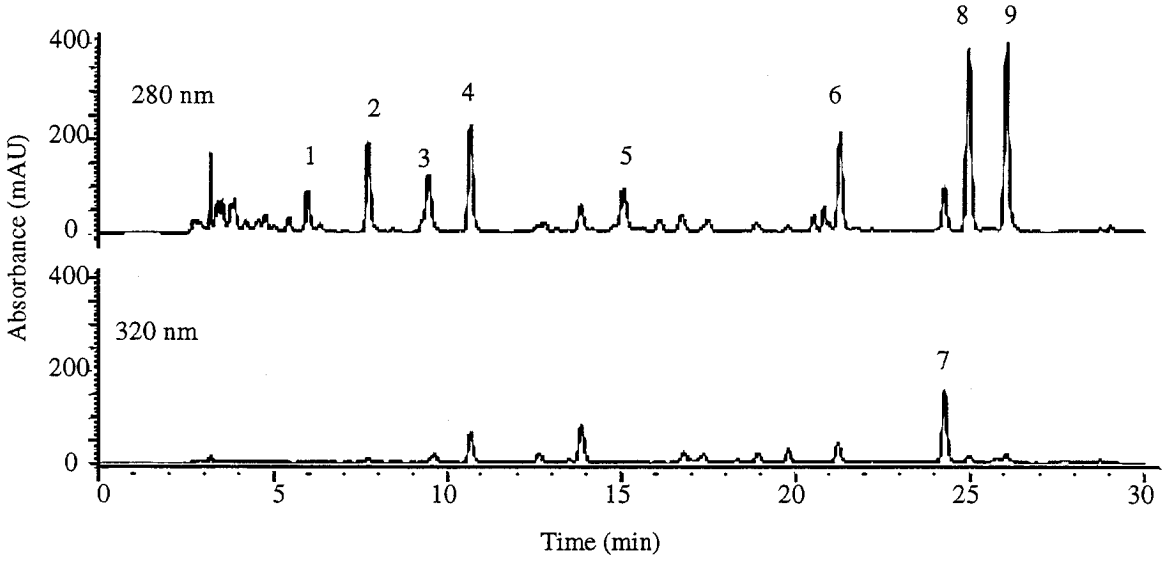

Table IV.3. Summary of phenolic content of pineapple juice (mg/100 mL single-strength juice, normalized to 12.8 ^{0}Brix)

Sample	P1	P2	P3	P4	P5	P6	P7	P8	P9	Year	Variety
1040	6.5	3.3	5.1	3.5	4.5	2.3	0.4	6.9	5.7	2	7
1036	4.3	1.3	4.6	0.9	2.1	1.5	0.5	5.4	2.8	2	7
1141	3.1	1.6	8.1	1.7	2.2	0.6	0.3	5.5	1.9	2	2
1042	3.0	1.5	7.0	1.3	2.1	0.9	0.4	5.4	2.0	2	2
1167	3.7	0.3	19.3	1.9	0.9	1.0	0.3	7.0	3.0	3	1
1033	7.2	2.2	3.7	1.0	3.4	2.6	1.7	5.3	4.0	2	7
964	2.7	1.6	7.5	1.7	2.6	0.6	0.1	4.2	1.6	2	3
1041	2.4	1.2	7.3	1.4	1.8	0.8	0.4	5.8	2.0	2	2
1171	5.2	1.2	4.2	0.9	2.3	2.1	0.8	5.3	3.0	3	1
900	2.8	1.4	5.2	1.0	1.5	0.6	0.2	5.6	1.2	2	2
1169	3.1	1.1	2.9	0.8	1.4	0.9	0.6	4.3	2.0	3	1
1170	4.2	2.3	3.9	2.2	3.5	1.7	0.3	4.5	4.7	3	1
1172	5.3	1.2	2.7	0.5	2.4	1.6	1.4	3.1	2.0	3	1
1035	6.1	1.8	3.2	1.2	3.4	2.8	2.1	5.0	3.5	2	7
1034	7.4	3.6	4.6	3.4	4.7	2.8	0.7	6.0	6.3	2	7
1043	2.9	1.7	5.3	1.0	2.0	0.9	0.3	4.5	1.7	2	2
1179	2.9	2.0	5.7	1.3	2.3	0.7	0.2	4.5	1.7	3	2
901	2.6	1.2	3.9	0.6	1.1	0.5	0.1	4.3	1.0	2	2
759	3.1	1.7	4.2	0.6	1.1	0.5	0.2	4.4	1.1	2	2
1180	3.4	2.2	6.4	1.1	2.3	0.7	0.2	3.9	1.5	3	2
1140	3.9	2.2	6.2	1.2	2.2	0.8	0.4	5.9	1.7	2	2
1168	3.4	1.0	9.2	2.3	1.9	1.0	0.3	5.5	2.5	3	1
1039	4.0	1.2	6.4	1.2	1.9	1.8	1.0	7.6	3.9	2	7
1365	2.0	1.1	5.7	1.4	1.3	0.4	0.1	4.8	1.4	3	3
1139	2.8	1.8	5.9	1.3	1.8	0.6	0.2	5.2	1.6	2	2
1181	3.3	1.8	4.5	0.9	2.2	0.2	0.1	3.6	1.2	3	2
1094	2.0	1.0	5.8	1.8	1.6	0.4	0.2	3.0	1.4	2	3
1037	5.0	0.9	4.7	1.1	1.5	1.5	1.0	6.2	3.1	2	7
1038	3.7	0.9	2.5	0.7	1.7	1.4	0.6	3.5	1.8	2	7
963	2.8	1.5	3.8	1.1	2.2	0.5	0.1	3.2	1.3	2	3
580	7.4	4.4	8.0	2.7	4.3	1.5	0.6	5.6	3.7	1	6
666	3.0	2.7	8.2	1.3	2.3	0.8	0.4	5.5	1.5	1	2
696	3.1	1.6	4.8	0.8	1.6	1.3	0.9	6.1	2.6	1	7
667	3.2	0.5	7.8	0.8	1.4	0.7	0.2	4.1	1.2	1	2
649	2.9	2.1	5.4	1.0	2.0	0.5	0.2	9.4	1.3	1	4
9878	3.8	2.5	7.3	2.0	3.3	1.3	0.3	6.6	3.2	1	3
664	3.2	3.0	8.6	2.5	3.0	0.9	0.3	8.5	3.2	1	5
679	2.6	3.1	7.8	2.2	2.5	0.8	0.3	7.6	2.5	1	5
697	5.1	1.6	4.3	0.7	2.2	1.9	1.0	4.9	2.6	1	7
581	5.0	1.8	16.8	2.6	2.3	1.3	0.7	5.0	3.1	1	6
582	2.8	2.0	7.9	1.9	3.0	0.8	0.2	5.1	1.8	1	3

Table IV.3 (Continued)

693	3.2	1.6	5.2	0.9	1.6	1.0	0.7	6.2	2.2	1	7
694	3.6	1.5	11.5	2.2	2.3	1.3	0.4	7.5	3.2	1	7
699	2.5	1.3	6.2	1.2	2.1	0.7	0.3	4.7	1.6	1	2
691	3.3	1.2	5.8	0.8	1.5	1.0	0.7	5.0	1.8	1	7
698	3.1	2.1	5.8	1.1	2.5	0.8	0.3	4.8	1.6	1	2
692	2.7	1.4	6.0	1.0	1.6	1.2	0.7	6.3	2.3	1	7
665	2.4	0.8	7.7	1.6	1.7	0.9	0.5	5.1	1.7	1	2
701	5.1	3.4	5.9	1.8	3.7	1.1	0.5	5.1	2.8	1	6
695	3.1	1.6	5.9	1.1	1.7	1.3	0.9	6.0	2.3	1	7
650	3.1	2.6	5.9	1.3	1.8	0.6	0.4	9.5	1.5	1	4
702	3.5	2.9	6.0	1.5	1.6	0.7	0.3	5.0	2.1	1	6
668	2.6	2.9	6.6	0.9	1.2	0.8	0.4	5.5	1.4	1	2
700	1.2	1.0	2.2	0.5	0.7	0.2	0.1	3.7	0.5	1	2
Mean	3.6	1.8	6.2	1.4	2.2	1.1	0.5	5.4	2.3		
Std deviation	1.4	0.8	3.0	0.7	0.9	0.6	0.4	1.4	1.1		
CV[1]	0.39	0.44	0.48	0.50	0.41	0.55	0.80	0.26	0.48		
Min	1.2	0.3	2.2	0.5	0.7	0.2	0.1	3.0	0.5		
Max	7.4	4.4	19.3	3.5	4.7	2.8	2.1	9.4	6.3		
Other samples											
Conc.	2.9	3.1	3.8	1.5	2.4	0.8	0.2	7.3	2.2		
Canned	4.7	1.9	7.9	3.2	3.1	0.8	0.2	6.8	2.4		
Fresh	2.8	3.4	9.5	2.1	1.5	0.3	0.1	7.5	2.9		

1. CV: Coefficient of variation

Table IV.4. Summary of phenolic content of pineapple (mg/100 mL single-strength juice, or mg/100 g fruit)

Compound	Value	Reference	Comment
Tyrosine	5.8	1	juice, average of several reports
	4.5	2	juice, average of 1st and 2nd years
	1.1	3	juice
Tryptophan	n.d.[a]	1	juice
	n.d.	2	juice
	n.d.	4	juice
	0.3	3	juice
Serotonin	2.4	5	canned juice
	1.2	5	fresh juice
	1.7	6	fruit
	3.2	6	fruit pulp edge
	0.9	6	fruit core center
	0.3	6	fruit peel
DMHF	0.07	7	fruit
	1.4	8	fresh juice
	2.3	8	canned juice
DMHF glucoside	0.05	7	fruit
p-Coumaric acid	2.8	9	juice

a. not detected

1. Hodgson and Hodgson 1993
2. Elkins et al. 1997
3. Dizy et al. 1992
4. Fontana et al. 1993
5. Bruce 1960
6. Feldman and Lee 1985
7. Wu et al. 1991
8. Lee and Nagy 1987
9. Fernández de Simón et al. 1992

Aromatic amino acids

Two of the major peaks were free aromatic amino acids, tyrosine (peak 1) and tryptophan (peak 5). Although tyrosine is commonly reported in pineapple juice, there is disagreement with respect to tryptophan (see Table IV.3b). With the exception of Dizy et al. (1992), o thers ha ve not reported the existence of tryptophan (Fontana et al. 1993; Hodgson and Hodgson 1993; Elkins et al. 1997). The average concentration of tyrosine for all the concentrate samples in this investigation was 3.6, which is close to the reported average (4.5) for 1st and 2nd year samples by Elkins et al. (1997). Hodgson and Hodgson (1993) summarized a higher average value (5.8) and Dizy et al. (1992) reported a much lower average value (1.1). Considering that tyrosine in this investigation has a range of 1.2-7.8, all of the reported values are in general agreement. However, it is different for tryptophan. Tryptophan had an average concentration of 2.2 in this investigation, which is much higher than that reported by Dizy et al. (1992) at 0.3. The difference might have been caused by difference in methodology and/or sample source variation. We measured tryptophan directly by HPLC whereas other investigators used methods employing derivatization.

Serotonin

The identity of serotonin was confirmed by its retention on cation exchange resin such as CM Sephadex. The average serotonin concentration value for this concentrate database is 1.8, which is in agreement with the average values reported for fresh (1.2) and canned juices (2.4) in the literature (see Table IV.3b). The existence of serotonin in pineapple juice was first reported by Bruce (1960). Feldman and Lee (1985) investigated

serotonin content in whole pineapple fruit and also its distribution in the fruit. The reported value for whole fruit is 1.7 mg/100 g fruit, which is in agreement with this investigation assuming that most of the serotonin is extracted in juice processing. The pulp edge has the highest concentration (3.15 mg/100 g), core center next (0.87 mg/100 g), and peel least (0.27 mg/100 g). Serotonin can also be found in nuts, other fruits such as plantain, banana, Kiwi fruit, plums and tomatoes, and vegetables such as eggplant and broccoli. Nuts usually have a much higher value than fruits and vegetables, and vegetables are much lower values than fruits. Pineapple has a high serotonin concentration in the fruit category (Feldman and Lee 1985). Ingestion of food rich in serotonin will result in an increase in urinary 5-hydroxyindoleacetic acid excretion, which causes misdiagnosis of malignant carcinoid tumors (Bruce 1960; Feldman and Lee 1985). Serotonin is also a phylogenetically primitive neurotransmitter, which may play a central role in the relationship between food and brain organization (Blundell 1992). Although a high concentration of serotonin has been found in pineapple juice concentrate, as to the authors' knowledge, no research has been conducted to determine whether the serotonin in juice is absorbed and transported to brain.

DMHF and its glucoside

The neutral characteristic of DMHF and its glucoside was also confirmed by not being retained by either anion exchange resin BioRex-5 or anion exchange resin CM Sephadex. DMHF was first reported in pineapple in 1965, and it was characterized as a remarkably intense aroma (described as "burnt pineapple" or "fruity caramel") and a high degree of instability in air (Rodin et al. 1965; Silverstein 1971). It was also found in beef

broth (Tonsbeek et al. 1968; Silverstein 1971) and maple syrup (Underwood et al. 1968; Silverstein 1971). In both of the latter cases, "cooking" is required to generate this compound. But in pineapple its genesis must be different because of its existence in fresh pineapple juice. The glucosidic form of DMHF was first reported in strawberry (Mayerl et al. 1989) and then in pineapple (Wu et al. 1990). The average value for DMHF in this database is 1.4, which is much higher than that reported by Wu et al. (1991) at 0.07 for fruit. A study by Lee and Nagy (1987) gave similar values for DMHF, which is 1.4 for fresh pineapple juice and 2.3 for canned juice (Table IV.3b). The average value for glucosidic DMHF in this database is 6.2, which is also much higher than that reported by Wu et al. (1991) at 0.05 for fruit.

Sinapyl derivatives

We were the first to identify these sinapyl compounds (peaks 6, 8 and 9) in pineapple (Wen et al. 1999). To our knowledge, their presence has not been reported in any other natural products. They are the major compounds, compromising about 36% of the total measured compounds on weight base and 46% of the total peak area in the pineapple juice concentrate data base (Table IV.3). Their presence in comparable amounts in fresh and canned pineapple juice suggests that processing is not required for their formation. Their structures suggest that they may have antioxidant properties. Their antioxidant activities were measured under both aqueous and organic solvent systems (Table IV.5). In the aqueous system, they have slightly higher antioxidant activity than the Trolox standard, while in the organic system, they have much higher activity than Trolox. The antioxidant activity is possibly caused by its substructure of S-allyl-L-cysteine, which is

the major antioxidant in garlic (Imai et al. 1994; Lin 1994). Since their structure also contains a conjugated benzyl group, sinapyl derivatives are expected to have higher antioxidant activity than S-allyl-L-cysteine.

Peak 7

This peak has almost identical retention time and very similar UV spectra as *p*-coumaric acid (see Fig. IV.3 for a spectral comparison). This is possibly why *p*-coumaric acid was reported in fruit (Macheix et al. 1990), juice (Fernández de Simón et al. 1992) and shell fibers (Larrauri et al. 1997). The exact chemical structure of this compound was not determined in this investigation. This peak is simply reported as a *p*-coumaric acid-like compound and quantified as *p*-coumaric acid. The average peak 7 value in this database is 0.5 (estimated as *p*-coumaric acid) with a range of 0.1-2.1, which is lower than that reported for juice and nectar by Fernández de Simón et al. (1992) at a range of 1.1-4.4 (2 juices and 2 nectars).

Effect of year and variety on each peak

Only c.v. Cayenne has collection covering all the 3 years, so only this variety was investigated for the effect of year on each individual peak concentration, and the results are summarized in Table IV.6. While peak 8 has the lowest variation coefficient (Table IV.3), it is the only peak that is significantly affected by year (probability ≤ 0.05). For the effect of variety on each individual peak, only the first 3 varieties are analyzed because of the limitation of sample size for other varieties. It was found that peaks 1, 4, 6, 7 and 9 are affected by variety at 0.05 significant level (Table IV.6).

Table IV.5. Antioxidant activity of sinapyl derivatives

Sample	TEAC[a]	
	Water-based	MeOH-based
S-sinapyl glutathione	1.22±0.03	3.25±0.10
N-γ-L-glutamyl-S-sinapyl-L-cysteine	1.56±0.20	4.53±0.20

a. TEAC: Trolox equivalent of antioxidant capacity

Pattern recognition analysis

Pattern recognition methods have been used to differentiate between different varieties, different geographical origins and to detect adulteration (Pilando and Wrolstad 1992; Dizy et al. 1992; Nagy and Wade 1995; Aires-De-Sousa 1996). Principal component analysis was conducted on all 54 samples to examine the relationships among the variables. Canonical variable analysis was conducted on the 3 major varieties (variety 1, 2 and 3) to examine if they can be separated. The scores for the 3 major varieties from the first 2 principal components are plotted in Fig. IV.4a (see Table IV.7 for the loading coefficients). Variety 2 and variety 3 are not separable while variety 1 has some separation from variety 2 and 3. The first principal component, which explains 48.5% of the total variance, is mainly related to aromatic amino acids and two of the sinapyl derivatives. The second principal component, which explains 21.9% of the total variance, is correlated to DMHF and its glucoside and peak 7. The scores of the first 2 canonical variables for the samples are plotted in Fig. IV.4b (see Table IV.7 for coefficients). Variety 1 can be completely separated from variety 2 and variety 3, while variety 2 and variety 3 have limited separation. The first canonical variable is highly correlated to tyrosine, 2 of the sinapyl derivatives and peak 7, while the second canonical variable is related to DMHF glucoside and tryptophan.

Figure IV.3. UV spectrum of peak 7.

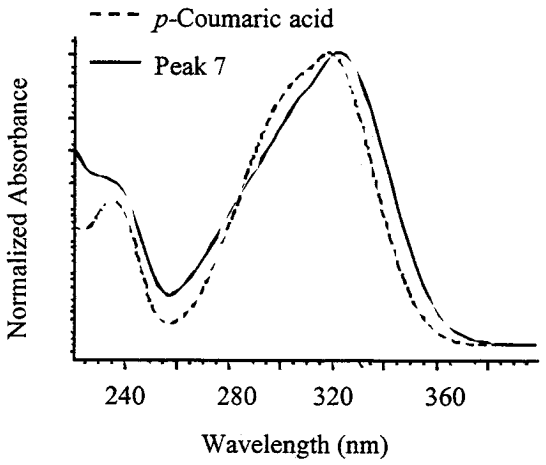

Table IV.6. Analysis of variance for effect of year and variety on phenolic concentration

Peak No.	1	2	3	4	5	6	7	8	9
Prob. (year)	0.18	0.60	0.74	0.93	0.26	0.49	0.21	0.04	0.53
Prob. (variety)	0.00	0.25	0.78	0.03	0.23	0.00	0.00	0.68	0.00

CONCLUSIONS

A total of 9 major phenolic compounds were characterized and quantified for a large set (n = 54) of authentic pineapple juice concentrate samples. These 9 peaks were present in all concentrate samples, and they were also present in fresh and canned pineapple juices. None of the pineapple phenolics reported to be present by previous workers are represented by these 9 major compounds which represent approximately 70% of the total peak area. Three sinapyl derivatives, which compromise 32% of the total peak area, are unique to pineapple juice, and they may serve as marker compounds for pineapple juice.

Figure IV.4. Cluster display of pattern recognition analysis of pineapple concentrate samples. a. 1st *vs* 2nd principal component scores for 3 major varieties; b. 1st *vs* 2nd canonical variables for the 3 major varieties. See Table IV.1 for the variety code. * Variety 1, Δ Variety 2, + Variety 3.

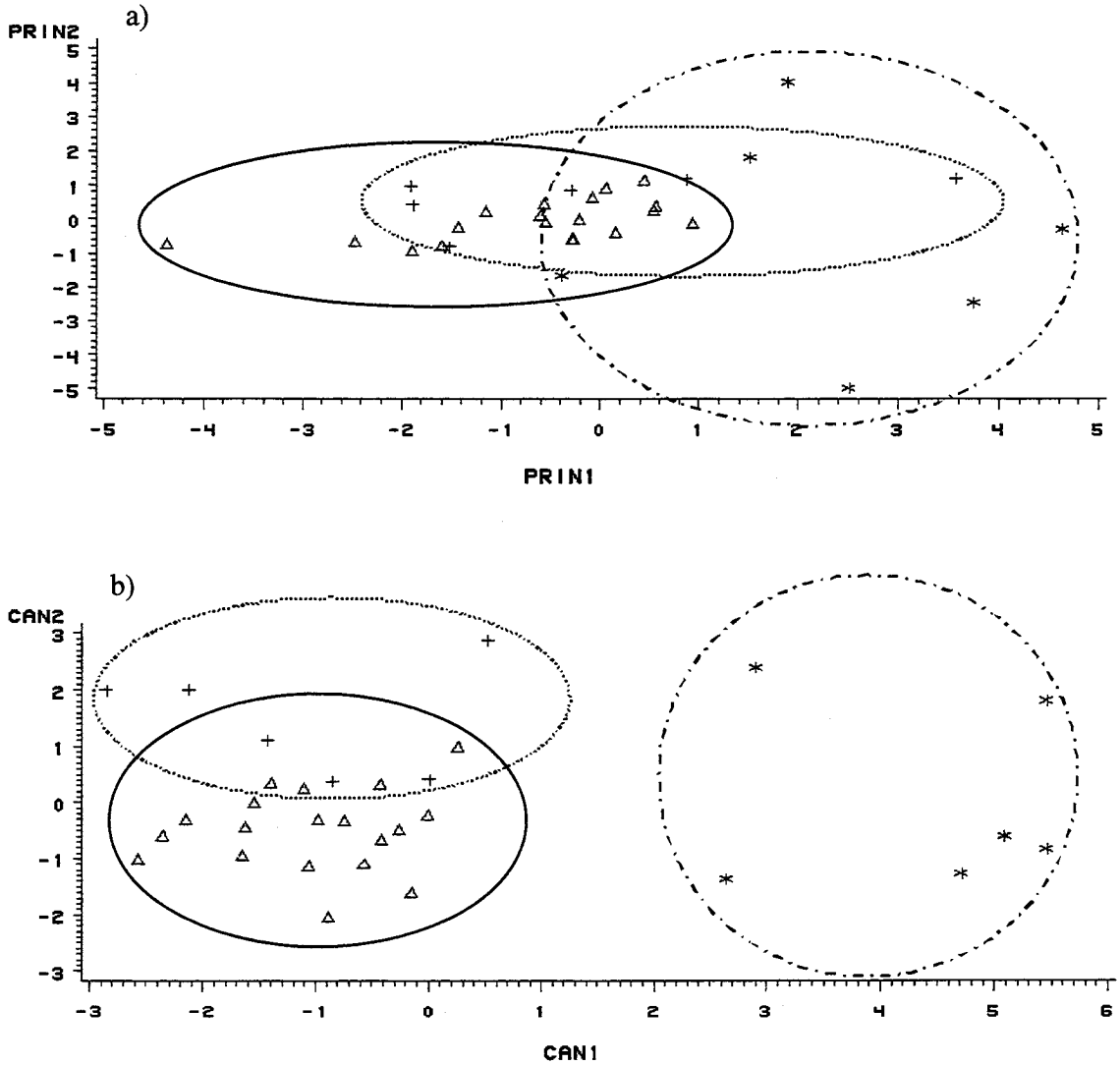

Table IV.7. Coefficients for multiple variable analysis (PCA and CVA)

	Peak No.								
	1	2	3	4	5	6	7	8	9
PCA									
PRIN1	0.4266	0.2950	0.0272	0.3199	0.4168	0.4090	0.2510	0.1579	0.4458
PRIN2	-0.1931	0.2637	0.4752	0.4586	0.0852	-0.3069	-0.4957	0.3308	0.0160
CVA									
CAN1	0.7097	-0.3326	0.1377	0.1761	0.0787	0.7681	0.6381	0.0910	0.7245
CAN2	-0.1128	0.0554	0.0714	0.7212	0.4969	0.0151	-0.2513	-0.2342	0.2598

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ACKNOWLEDGMENTS

This research was supported by a grant from the Washington-Oregon Tree Fruit Commission. Pineapple juice concentrate samples were supplied by the National Food Processors Association (NFPA). The antioxidant activities were measured by I. Vadim of Linus Pauling Institute at Oregon State University. We thank R. W. Durst for help with the HPLC analyses and C. Pereira for statistical advice. This is technical paper no. 11768 from the Oregon Agricultural Experiment Station.

CHAPTER V

ISOLATION AND CHARACTERIZATION OF ENZYMATIC BROWNING INHIBITORS FROM PINEAPPLE JUICE

Ling Wen and Ronald E. Wrolstad

Department of Food Science & Technology, Oregon State University
Corvallis, OR 97331-6602

To be submitted to Journal of Food Science

ABSTRACT

The major enzymatic browning inhibitors of different pineapple juices (fresh, canned and frozen concentrate) were characterized. The inhibition activity in fresh juice was from a high molecular weight fraction and possibly a protease enzyme. In canned juice, added ascorbic acid was a main inhibitor. In juice concentrate, a very polar organic acid (other than ascorbic, citric, malic or oxalic acid) possessed the major inhibitory activity.

KEY WORDS: apple, enzymatic browning inhibition, pineapple juice

INTRODUCTION

Fruits and vegetables are very important in our diet to maintain health, and the convenience of fresh-cut fruits and vegetables has greatly promoted their consumption. However, the choice of fresh-cut products is still very limited, and one of the main obstacles is the immediate development of enzymatic browning on the cut and peeled surfaces (Sapers and others 1995). Sulfites are effective anti-browning agents. However, the concerns over the allergenic reactions in an ever-expanding asthmatic population promoted the Food and Drug Administration (FDA) to limit the use of sulfites in fresh fruit and vegetable products.

The limitation of use of sulfites has stimulated active research for sulfite alternatives as there is a great interest in the new product group of fresh-cut fruits and vegetables. An approach our laboratory favors is to find natural browning inhibitors to serve as

alternatives to synthetic chemicals. Our laboratory previously demonstrated that pineapple juice could inhibit the enzymatic browning in apple (Lozano-de-Gonzalez and others 1993), but we had very limited information as to the nature of the inhibitor(s).

The objective of this study is to characterize the major enzymatic browning inhibitor(s) in pineapple juice. Three different types of pineapple juices have been investigated, and they are: frozen pineapple juice concentrate (CONC), fresh pineapple juice (FRESH) and canned pineapple juice (CAN). The enzymatic browning inhibition activities of these juices were measured, then their fractionation behaviors were investigated and compared, and finally the identified active fraction was further fractionated by HPLC methodology.

MATERIALS AND METHODS

Source

Frozen pineapple juice concentrate and canned pineapple juice were supplied by Dole Food Co., Inc. (Westlake Village, CA). Fresh pineapple (c.v. Smooth Cayenne, Maui, Hawaii) and Red Delicious apples (grown in Washington) were purchased from a local supermarket.

Juice preparation

The pineapple juice concentrate was diluted 4 times with deionized water, then ultracentrifuged at 23000 g and the supernatant was collected. Fresh pineapple was cut into cubes and blended into puree, then ultracentrifuged and the supernatant was

collected. There is no need of ultracentrifugation for canned juice. All the samples were subjected to 0.45 μm HA membrane filtration (Millipore Corp., Bedford, MA).

Pineapple model solution (MODEL)

A "model" pineapple juice solution was prepared, which was composed of naturally occurring sugars and acids as reported by Pilando and Wrolstad for pineapple juice concentrate (1992). The concentration of individual compounds is (per100 mL): citric acid, 0.69g; malic acid, 0.28g; quinic acid, 0.0118g; fumaric acid, 32.0 mg; shikimic acid, 0.16 mg; ascorbic acid, 3 mg; sucrose, 6.76g; glucose, 2.79g; fructose, 3.25g. The control solution was adjusted to pH 3.5 with 10% KOH.

Extraction of apple PPO

Apple PPO was isolated following a slightly modified procedure reported by Marquès and others (1994). Apple flesh (200 g) was blended with 200 mL cold 0.1 M Na-Pi buffer (pH 6.5) with added sorbitol (0.1 M) and ascorbic acid (0.02 M). The puree was filtered through 3-layers of cheesecloth and ultracentrifuged at 6000 g at 4 ^{0}C for 10 min. The thylakoid pellet was collected and washed three times with Na-Pi buffer. PPO was extracted from the pellet with 1.5 % Triton X-110 (Sigma, St. Louis) in Na-Pi buffer, and then separated from Triton X-110 by phase separation. The enzyme was finally filtered through glass wool, and kept frozen until use.

Characterization of apple PPO

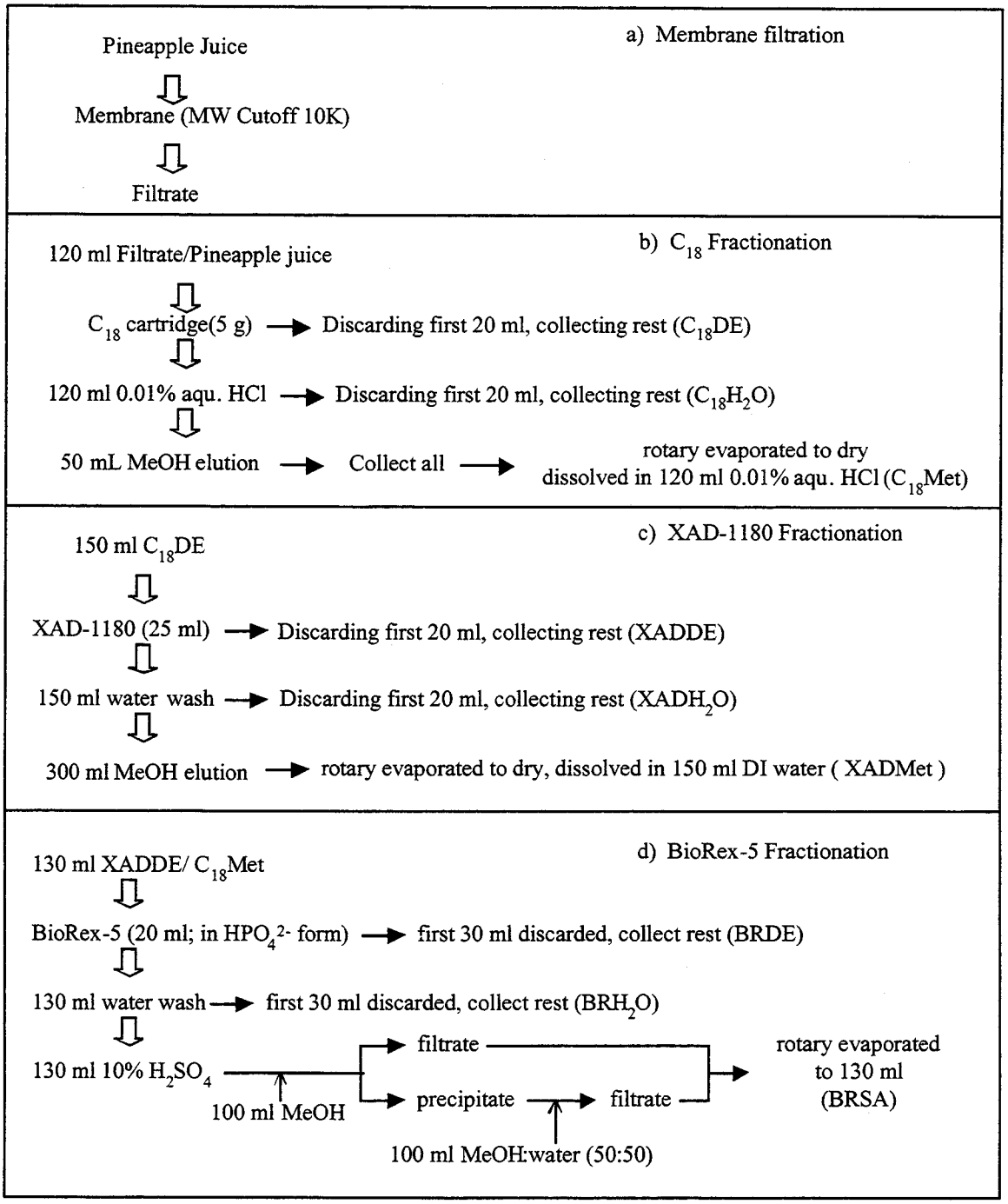
Dependence of PPO activity on pH and substrate concentration were conducted in McIlvaine's buffer (0.1 M Phosphate, 0.1 M citrate, pH adjusted to the required by adding

KOH) with two substrates, 4-methylcatechol and catechol. The enzyme reaction was conducted in a Shimadzu UV160U spectrophotometer with detection wavelength set at 412 nm and temperature maintained at 25 ± 0.1 ^{0}C. Temperature dependence was not tested.

Juice fractionation

The fractionation (Fig V.1) was conducted with a combination of C_{18} cartridge (5 g resin) from Alltech Associates (Deerfield, IL), XAD-1180 (Alltech Associates) and BioRex-5 anion exchange resin (Chloride form, Bio-Rad Laboratories, Richmond, CA). Before sample application, the C_{18} cartridge was cleaned with 50 mL MeOH, followed by 20 mL deionized water. The XAD-1180 resins was mixed with 3 volumes of MeOH and the slurry was packed into a 1.5 cm id column with a final wet volume of 25 mL. The XAD-1180 column was cleaned with 250 mL MeOH, followed with 100 mL deionized water. The BioRex-5 resin was mixed with deionized water, kept overnight, and then the slurry was packed into a 1.5 cm id column with a final wet volume of 20 mL. Before use, the BioRex-5 resin (20 mL) was converted to the phosphate form by eluting sequentially with 400 mL 1M KOH, 100 mL K_2HPO_4 and 100 mL deionized water. The isolated active fraction was further fractionated by semi-preparative C_{18} HPLC (reverse phase) and analytical amino column HPLC (normal phase).

Figure V.1. Outline of fractionation of pineapple juice



Semi-preparative C_{18} HPLC separation

A Dynamax SD-300 pump was used with a semi-preparative Microsorb C_{18} column (25 cm x 21.4 mm, 5 mm) from Rainin Instrument Co. (Woburn, MA) with a manual injection loop of 1 mL. The end of the column was connected to a flow divider, which split 1 mL/min flow to a HP 1040A Diode Array Detector (DAD) with detection at 214 nm and the rest of flow (19 mL/min) to an outlet for a Gilson Model 203 Microfraction collector with 0.5 min for each fraction. An isocratic solvent system was used, and it was 27.2 g KH_2PO_4 (pH 2.4). Each injection was 1 mL. A total of 80 mL was collected for each fraction, which was rotovaporated to 20 mL and then 40 mL MeOH was added and filtered. Another 40 mL MeOH was added to the filtrate and then filtered again. The pH of final filtrate was adjusted to 4.5 with 10% KOH, and then rotovaporated at 45 ^{0}C to 5 mL, and pH was adjusted again to 4.5 with 10% KOH.

Amino column HPLC separation

An Alltech Econosphere NH_2 column (25 cm x 4.6 mm, 5 mm) was used with a HP 1040A DAD set at 214 nm. Flowrate: 1.0 mL/min; Injection: 50 μL. An isocratic solvent -- acetonitrile : 0.05 M KH_2PO_4 buffer (pH 2.4) (75: 25). The above mentioned Gilson microfraction collector was used with 0.5 min for each fraction. Injection volume : 25 μL. A total of 7.5 mL was collected for each fraction, which was then rotovaporated to 0.5 mL.

Measurement of browning inhibition by puree assay

The method of Amiot and others (1995) was used with some modification. One apple was peeled and cored using a manual peel/corer, and divided into four portions. For each apple, four solutions were tested. Water (positive control), NaF-thiourea (negative control), and two different sample solutions. Individual portions were blended with one of the respective solutions, and kept at room temperature for 30 min with occasional agitation. Color was measured as CIE LC*h* values by HunterLab ColorQUEST system. The browning inhibition was expressed as:

$$Inhibition\% = \frac{C^*_0 - C^*_{sample}}{C^*_0 - C^*_1} \times 100$$

whereas 0 = negative control; 1 = positive control

Measurement of browning inhibition by PPO enzyme assay

Apple PPO (20 µl) and test fraction (0.5 mL) were added to 2.5 mL substrate (2.4 mM 4-methylcatechol in 0.1 M citrate-phosphate buffer at pH 4.5) with temperature maintained at 25 ± 0.1 °C. $A_{412\ nm}$ change was recorded for the first minute. The inhibition is expressed as:

$$Inhibition\% = \frac{\Delta A_{412}/\min_{(sample)} - \Delta A_{412}/\min_{(water)}}{\Delta A_{412}/\min_{(water)}} \times 100$$

Measurement of browning inhibition by slice assay

Red delicious apple was peeled by a manual peeler, and cut into small slices (~ 2.0 x 2.0 x0 .3 cm). Four slices were dipped into each solution for 5 min, wiped with Kimberly

tissue, and transferred to 15 x 10 cm sealable plastic bag, vacuum-sealed and refrigearted (5 ^{0}C). One apple was used for one comparison study. Browning was determined by observation with a scale of 1-9, with 9 representing most serious browning and 1 being no observed browning. A value of less than 5 means some browning but still acceptable.

RESULTS AND DISCUSSION

Apple PPO characterization and assay condition selection

The dependence of apple PPO activity on pH and substrate concentration for 4-methylcatechol and catechol is shown in Fig V.2. 4-Methylcatechol has much broader pH range of stable activity than catechol, and its stable pH range is also compatible with the pH of apple (about 3.5), so 4-methylcatechol is preferred over catechol for this experiment. 4-Methylcatechol has a much lower apparent Km (4 mM) than catechol (30 mM), and these values agree with that reported by Richard-Forget (see Nicolas and others 1994) (4.7-4.9 for 4-methylcatechol and 20.3 for catechol). Since the inhibitor concentration is usually low, it is very insensitive to detect the inhibition fraction using PPO assay at the concentration of maximum activity, so a lower 4-methylcatechol concentration of 2.4 mM is chosen instead.

Enzymatic browning inhibition of different pineapple juices

Table V. 1 shows that all the pineapple juices have enzymatic browning inhibition activity comparing to the water control. Water, instead of the pineapple model solution,

Figure V.2. Dependence of PPO activity on pH and substrate concentration.
a. Dependence on pH
b. Dependence on concentration
Δ -- 4-methylcatechol ; □ -- catechol.

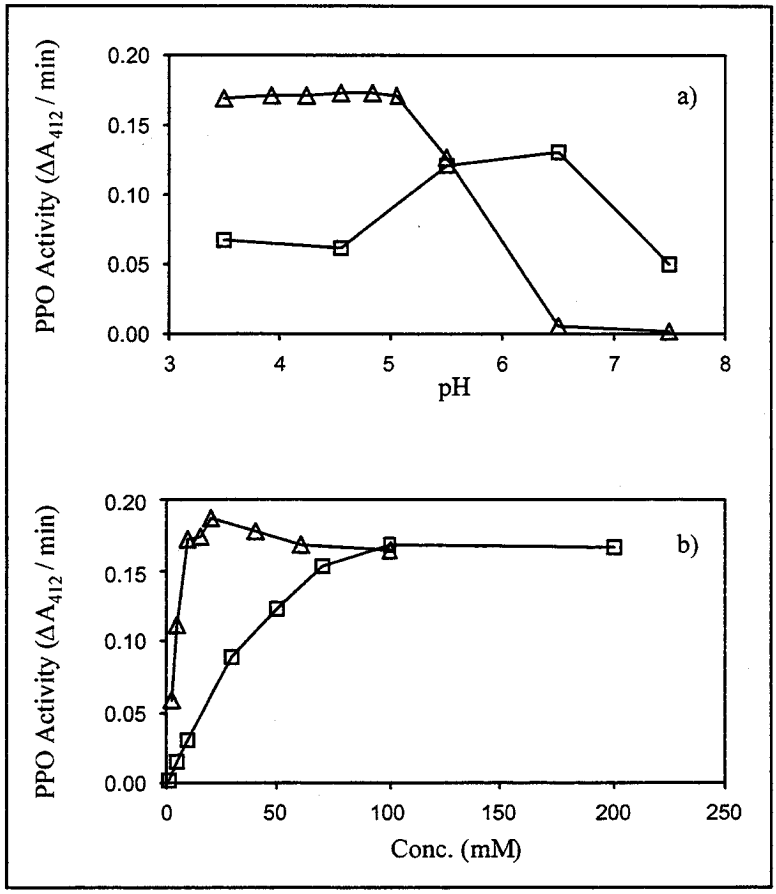


Table V. 1. Effect of enzymatic browning inhibition of different pineapple juices Inhibition assay: slice assay.

Time (hour)	Water	CONC	CAN	FRESH
0	7	1	1	1
0.5	7	2	2	2
3	8	7	6	5

MODEL: pineapple model solution
CONC: frozen pineapple juice concentrate
CAN: canned pineapple juice
FRESH: freshly made pineapple juice

was chosen as the control because the answer of interest was if pineapple juice had enzymatic browning inhibition activity. Among the different pineapple juices, fresh pineapple juice has the highest inhibition activity, and canned pineapple juice has higher activity than frozen juice concentrate.

Fractionation

Fig V.3 is the result of fractionation of pineapple juice concentrate, the enzymatic browning inhibition being measured by the puree assay. The puree assay method was chosen because it gave less variable results than the slice assay and it was more consistent with visual observations of browning than the PPO assay. C_{18} resin was used to separate polar and non-polar fractions. XAD-1180 was used to separate moderately non-polar components from polar components. BioRex-5 was used to separate organic acids from other components. This fractionation approach was satisfactory as the major browning inhibition component was always retained in one of the fractions at each fractionation step. The results show that the major browning inhibitor was a very polar negative-charged ion, possibly an organic acid. Some of the fractions promoted browning, which could be caused by experimental error or by matrices in the fractions. This paper did not attempt to explain it. There is some consideration with respect to the BioRex-5 resin used in fractionation. The BioRex-5 was specially treated by phosphate salt, which is intended to replace the Cl^- counter-ion of BioRex-5 with phosphate. Otherwise, Cl^- would be eluted by organic acids and cause the increased enzymatic browning inhibition of the direct eluate from BioRex-5 resin. In this study, when the BioRex-5 resin was not treated with phosphate, a 20% inhibition increase was observed for the pineapple model solution. In Lozano-de-Gonzalez and others paper (1993), the acclaimed unknown neutral

component, which has browning inhibition in addition to ascorbic acid, is very possibly caused by the eluted Cl^- counter-ion.

Fig V.4 gives the results for the fractionation of different pineapple juices and the model solution, along with their respective enzymatic browning inhibition as measured by the PPO enzyme assay. The PPO enzyme assay was chosen because it is more repeatable than the puree assay. Otherwise it is very difficult to compare the inhibition results of different fractions by either the puree assay or the slice assay because of the natural variability in experimental materials used for repeated assays. The pineapple model solution was used as a control to reflect the contribution of known sugars and organic acids (including ascorbic acid) in pineapple juice to browning inhibition, and it did not show any observable inhibition for each fraction. All the three pineapple juices showed similar patterns except for the membrane filtration step. The membrane filtration reduces the inhibition activity of fresh pineapple juice to the same level as frozen pineapple juice concentrate, which is not affected by membrane filtration. This reduction is caused by high molecular weight material and could very possibly be native protease enzymes. McEvily et al. (1992) reported that protease enzymes could inhibit PPO by protein hydrolysis. The inhibition activity of canned pineapple juice can be caused by the high content of added ascorbic acid, and possibly the metal ions produced by the reaction between pineapple juice and the metal can.

Figure V.3. Enzymatic inhibition result of preliminary fractionation of pineapple juice. Sample: Frozen pineapple juice concentrate; Inhibition assay: puree assay.

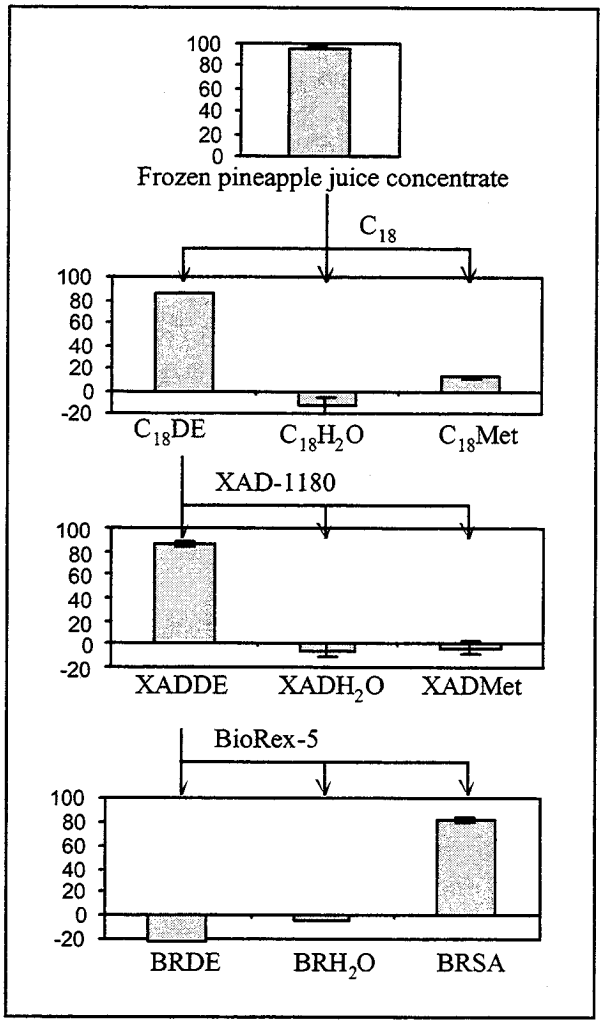
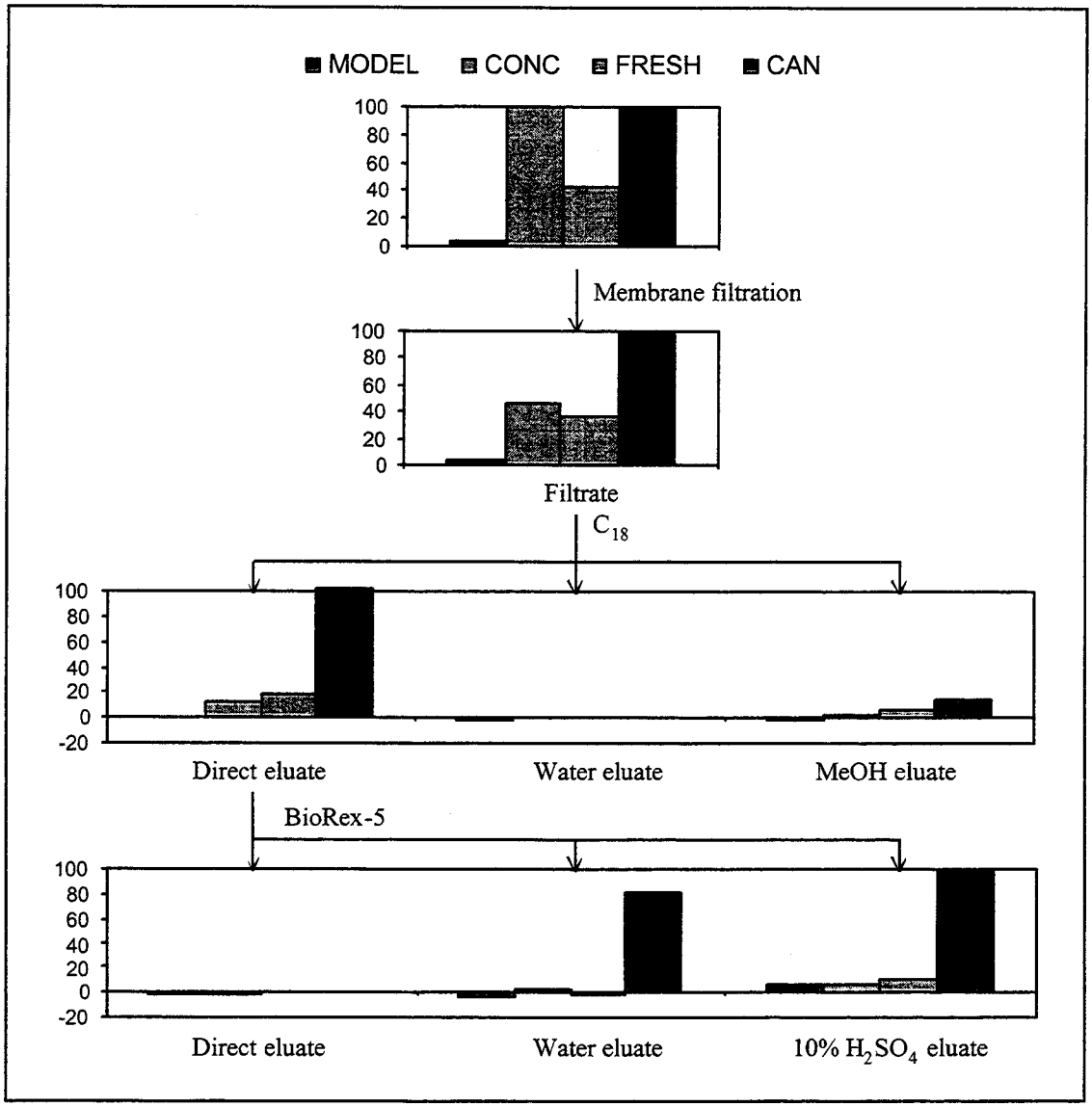


Figure V.4. Enzymatic inhibition result of preliminary fractionation of pineapple juice. Sample: Different pineapple juices; Inhibition assay: PPO assay.



Since the enzymatic browning inhibition activity of fresh and canned pineapple juices mainly came from protease and added ascorbic acid, the active fraction of frozen pineapple juice concentrate was chosen as the desired material for further characterization of browning inhibitor(s) by HPLC methodology.

HPLC fractionation

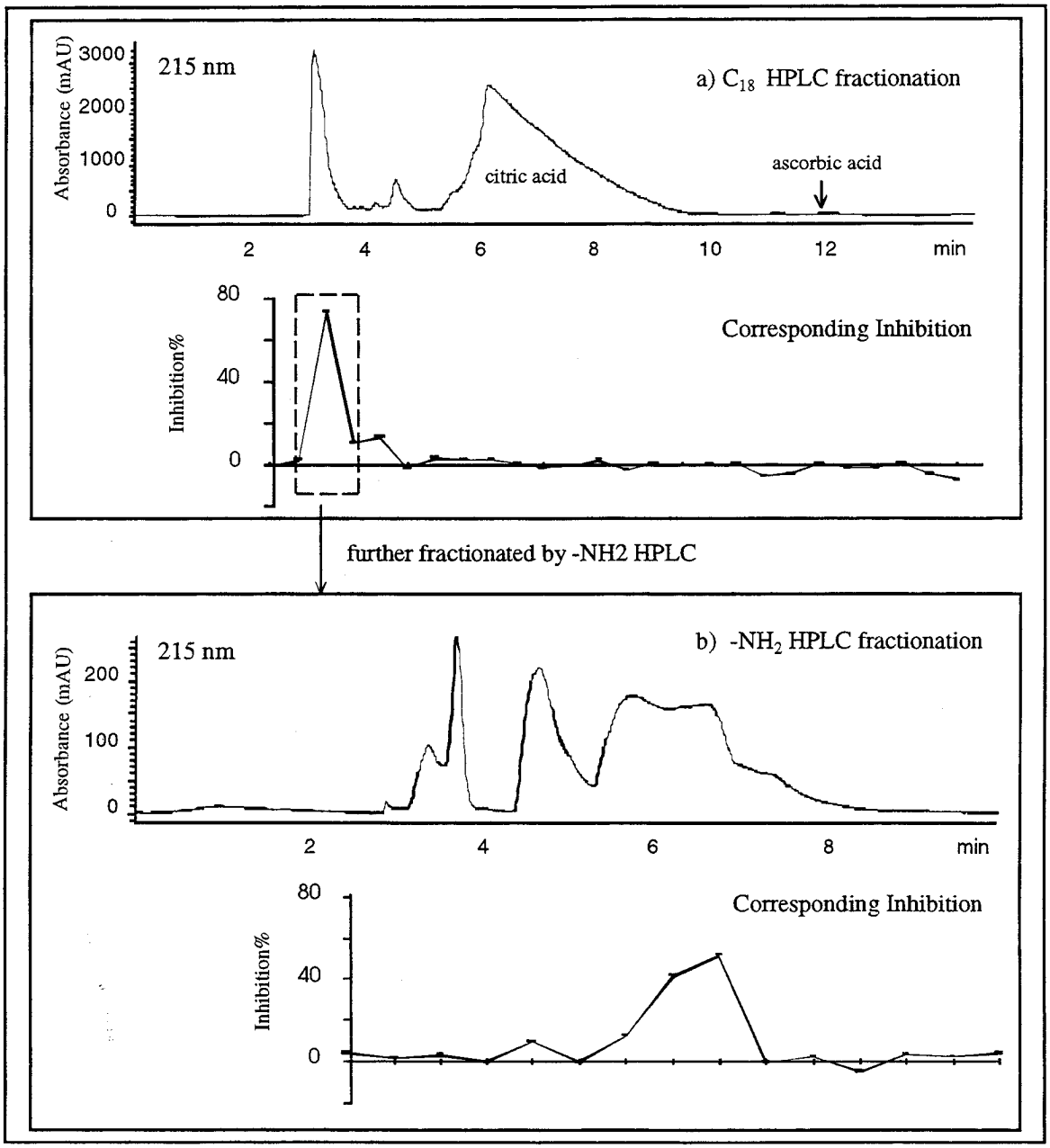
The results are shown in Fig V.5. The inhibition was measured by the PPO enzyme assay because of the volume limitation of each fraction. The active fraction was not retained by the C_{18} column, eluting within 3 min. This active fraction cannot be any of the reported organic acids in pineapple juice (citric, ascorbic, malic, quinic, fumaric or shikimic acids) since they all have longer retention times on this column (data not shown). Further fractionation of this non-retained fraction was achieved on an amino column. The separation may be achieved by two modes, ion-exchange and hydrogen-binding. According to its behavior, this active inhibitor is possibly a very polar organic acid. Because of the low concentration, high polarity and the high salt content in the mobile phase, it is very difficult to recover the active inhibitor, so no further effort was made to determine its identity. Son and others (2000) reported that the antibrowning activity of rhubarb juice is caused by oxalic acid, a very polar organic acid. Son and others (2001) also compared the enzymatic browning inhibition activity of oxalic acid with 12 other natural carboxylic acids, and they found that both oxalic acid and oxalacetic acid have strong antibrowning activity while others have medium or very weak antibrowning activity. However, the unknown inhibitor in this investigation can not be either oxalic acid or oxalacetic acid because these two compounds would have different elution behaviors on the amino column.

CONCLUSIONS

The pineapple juice fraction having the greatest apple PPO inhibiting activity is a "polar, negatively charged" fraction. In addition to ascorbic acid, this fraction contained an unidentified polar organic acid with high inhibiting activity. The compound is none of the commonly reported organic acids in pineapple juice.

Figure V.5. HPLC fractionation of enzymatic browning inhibitor(s).

a. C_{18} HPLC fractionation.
b. Amino column HPLC fractionation.



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ACKNOWLEDGMENT

This research was financially supported by Washington-Oregon Tree Fruit Commission. Frozen pineapple juice was supplied by Dole Food Company. We thank R.W. Durst for help with HPLC analyses. This is technical paper no. from the Oregon Agricultural Experiment Station.

CHAPTER VI. SUMMARY

Nine major peaks accounting for 70% of total peak area in the phenolic profile of pineapple juice have been characterized, and none of these peaks are represented by the pineapple phenolics reported to be present by previous workers. Of these nine peaks, three are newly identified sinapyl derivatives, which compromise 32% of the total peak area. Their structures were elucidated from UV spectra, acid hydrolysis and subsequent amino acid analysis, mass spectrometry, and two-dimensional NMR Spectroscopy. These derivatives are unique to pineapple juice, and they may serve as marker compounds for pineapple juice.

A phenolic compositional database is established for commercially produced pineapple juice concentrates (n = 54), which have been collected over 3 years from significant growing regions around the world.

The major enzymatic browning inhibitor in frozen pineapple juice is a very polar, possibly organic acid, which is none of the commonly reported organic acids in pineapple juice such citric acid and ascorbic acid. The major inhibitor is protease in fresh pineapple juice and is ascorbic acid in canned pineapple juice.

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APPENDIX

NATURAL ANTIBROWNING AND ANTIOXIDANT COMPOSITIONS AND METHODS FOR MAKING THE SAME

Ronald E. Wrolstad and Ling Wen

Department of Food Science and Technology , Oregon State University

Patent No.: US 6,224,926 B1
Date of Patent: May 1, 2001

Application No.: 09/378,986
Filed: August 23, 1999

ABSTRACT

The present invention, provides natural-source compositions having consistent, effective antibrowning and antioxidant characteristics. Additionally, the present invention provides methods for making the antibrowning/antioxidant compositions of the present invention. More specifically, the present invention provides natural-source, browning and oxidizing inhibitor compositions comprising S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapyl glutathione, or various mixtures thereof. Methods for making such natural, antibrowning/antioxidant compositions from pineapple juice and/or from pineapple processing plant waste streams include efficient and effective separation of the present invention compositions from unwanted sugars, acids, phenolic compounds, and other undesirable compounds present in pineapple juice and/or pineapple processing plant waste streams.

FIELD OF THE INVENTION

This invention relates to natural antibrowning, antioxidant compositions for foods, and methods for making the same.

BACKGROUND AND SUMMARY OF THE INVENTION

The food industry is concerned with how quickly most fruits (and various fruit products) become discolored upon exposure to air. The discoloration upon exposure to air is caused by a chemical reaction known as oxidation. Oxidation (typically indicated by discoloration) of fruits involves an enzyme-catalyzed oxidation of phenolic compounds present in the fruit. Browning of fruits is also a concern of the food industry. Browning of a fruits typically occurs following a mechanical injury to the fruit, such as during the harvesting or processing of such foods.

Bisulfite compounds are currently used to inhibit the enzymatic oxidation and browning in "fresh-cut" and processed fruits. Since a segment of the population is hypersensitive to sulfites, however, food processors prefer to avoid using sulfite compounds. Further, concern over labeling requirements for sulfur dioxide (due to allergic reactions by many users) also causes food processors to avoid its usage. It is particularly desirable in the food industry that an oxidation/browning inhibitor composition be derived from a natural source, rather than a synthetic chemical. That is, main-line food processing companies are seeking effective, natural alternatives to synthetic food additives. Additionally, the FDA typically requires less extensive testing information for a preparation derived from a natural source than for a preparation derived from a synthetic chemical. This is particularly true if the "natural source" is a common foodstuff.

Other currently available oxidation and browning inhibitors include, for example, 4-hexyl resorcinol, sulfurdioxide metal chelators such as citric acid and phosphates in combination with ascorbic acid. Oxidation and browning inhibitors such as citric acid

and phosphates in combination with ascorbic acid, however, are not sufficiently effective. The use of inhibitor 4-hexyl resorcinol is limited in the United States to use with shrimp. Additionally, even if 4-hexyl resorcinol is approved for use with fruits and fruit products, many food processors will likely be reluctant to use it because it is derived from a synthetic chemical rather than from a natural source.

Pineapple juice has also been shown to inhibit browning and oxidation of fresh fruit (P.G. Lozano-de-Gonzalez, D.M. Barrett, R.E. Wrolstad, and R.W. Durst, Enzymatic Browning Inhibited in Fresh and Dried Apple Rings by Pineapple Juice, J. Food Sci. Vol. 58, pp. 399-404 (1993)). The antibrowning/antioxidant effectiveness of pineapple juice is, however, unacceptably variable for use in the food industry. That is, the effectiveness of pineapple juice for such purposes varies from one type of pineapple to another, and from one pineapple to another within a particular pineapple type. The effectiveness of the pineapple juice as an antibrowning/antioxidizing agent also varies depending upon where the pineapple was grown.

The present invention provides natural-source compositions having effective and consistent antibrowning and antioxidant characteristics. Additionally, the present invention provides methods for making the antibrowning/antioxidant compositions of the present invention. More specifically, the present invention provides natural browning and oxidizing inhibitor compositions comprising S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapyl glutathione, or various mixtures thereof.

Methods for making such natural, antibrowning/antioxidant compositions from pineapple juice and/or from pineapple processing plant waste streams are also provided. The present invention provides methods for making antibrowning/antioxidant compositions that are efficiently and effectively separated from sugars, acids, and other

phenolic compounds present in pineapple juice and/or pineapple processing plant waste streams.

More specifically, one method of the invention generally includes centrifuging and filtering a quantity of pineapple juice to obtain a filtrate. The filtrate is applied to a resin column. The resin column is washed with acidified water to remove undesirable constituents, such as sugars, acids, and other polar compounds. The constituents of the antibrowning/antioxidant compositions are eluted from the column using a suitable media such as an alcohol. The eluate constituents are evaporated to dryness and re-dissolved in a liquid, such as water. The re-dissolved constituents are applied to an anion-exchange resin column. The anion-exchange resin column is washed to remove undesirable, neutral phenolic compounds. The anion-exchange resin column is then treated with an acidic liquid, such as sulfuric acid. The acidic liquid elutes the remaining constituents from the column to produce an eluate. The eluate is neutralized to form one of the present invention antibrowning/antioxidant compositions. One or more of the individual constituents of the neutralized eluate may be isolated to make alternative embodiments of the antibrowning/antioxidant compositions of the present invention.

The foregoing and other features and advantages of the present invention will become more apparent from the following detailed description, drawings, and examples of the browning, oxidation inhibiting compositions and methods for preparing the same.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a HPLC chromatogram of an antibrowning/antioxidant composition of the present invention.

FIG. 2 is a semi-preparative HPLC chromatogram of another composition of the present invention.

FIG. 3 illustrates an acid hydrolysis pattern of another composition of the present invention.

FIG. 4 is the identification of compounds as identified in an ultra-violet spectrum of another composition of the present invention.

DETAILED DESCRIPTION

The present invention provides natural antibrowning and antioxidant compositions for use with fruits and fruit products.

As used herein, "antibrowning" means the reduction of or substantial inhibition of browning of fruits exposed to mechanical injury. Also as used herein, "antioxidizing" means the reduction of or substantial inhibition of oxidation (i.e., the ability to trap free-radicals) of fruits exposed to air or other oxygen sources.

As used herein, an "isolated" constituent or component means a constituent of pineapple juice that has been substantially separated from other constituents in pineapple juice. The terms "substantially separated," "purified" or "purified component" do not mean absolute purity or separation. Rather, the substantial separation, purification or purity of a component mean more concentrated than when in a prior solution from which

the component was separated. Thus, for example, a purified phenolic compound is a phenolic compound wherein the concentration of the phenolic compound is greater than the concentration of the phenolic compound when in its natural environment, such as in pineapple juice.

Also as used herein, a "natural" antibrowning/antioxidizing composition is a composition isolated from a natural source rather than a synthetic source. Natural source food preparations are much more readily received by the consumers and, thus, the food processors. That is, a significant portion of the consuming public has the perception that food additives or food preparatives from a natural source are better for health reasons than synthetic additives and preparatives.

Antibrowning and antioxidant capabilities of a composition is typically determined by comparison to the antibrowning and antioxidant capabilities of TROLOX. TROLOX, an excellent antioxidant, is a water-and methanol-soluble analog of alpha-tocopherol (i.e., 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) available from Aldrich of Milwaukee, Wisconsin. TROLOX is widely used by persons skilled in the art, as a reference standard for measuring comparative antioxidant capacity. TROLOX has an activity equal to 1 in comparison to other antibrowning/antioxidant compositions. The antioxidant capacity (AOC) of a composition is referred to as a TEAC value, i.e., TROLOX Equivalent of Antioxidant Capacity. In other words, the antioxidant capacity of a composition is typically expressed as the calculated molar amount of TROLOX with AOC corresponding to 1 mole of assayed composition.

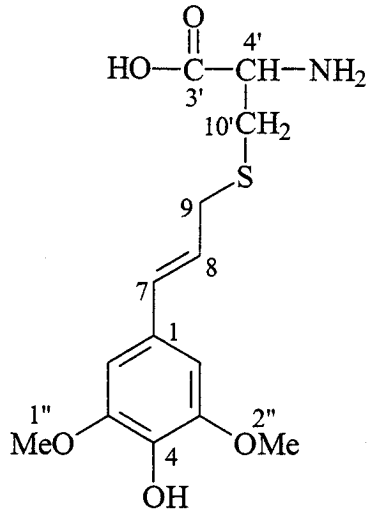
Specifically, a photo-chemiluminometer is typically used to measure the antioxidant capacity of particular compositions. Such a technique permits measurement of antioxidant capacity of water-soluble, lipid-soluble, and protein-associated compositions.

Using such techniques, superoxide radicals are generated in a photo-sensitizer (e.g., luminol) solution using ultra-violet irradiation. The antioxidant capacity is measured as inhibition of light output: $I = 1 - A/A_0$ wherein A is equal to the area under the chemiluminescense emission curve and is expressed as TROLOX Equivalent of Antioxidant Capacity (i.e., TEAC).

The antioxidant capacity of compositions is also often determined by measuring the compositions' oxygen-radical absorbance capacity (ORAC). Quantitating the ORAC of antioxidants is based on the use of beta-phycoerythrin (beta-PE) as an indicator protein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, the TROLOX as a control standard. As in the TEAC assay, results are expressed relative to the antioxidant activity of TROLOX, where 1 ORAC unit equals the net protection produced by 1 :M TROLOX.

The natural antibrowning and antioxidant compositions of the present invention comprise at least one compound having antibrowning/antioxidizing properties. The compositions of the present invention may be made from natural sources, such as pineapples, pineapple juice, or pineapple process plant waste streams. The antibrowning/antioxidizing compositions include S-sinapyl-L-cysteine, S-sinapyl glutathione, N-L-γ-glutamyl-S-sinapyl-L-cysteine, or various combinations of these sinapyl compounds (having various relative concentrations of the sinaply compounds).

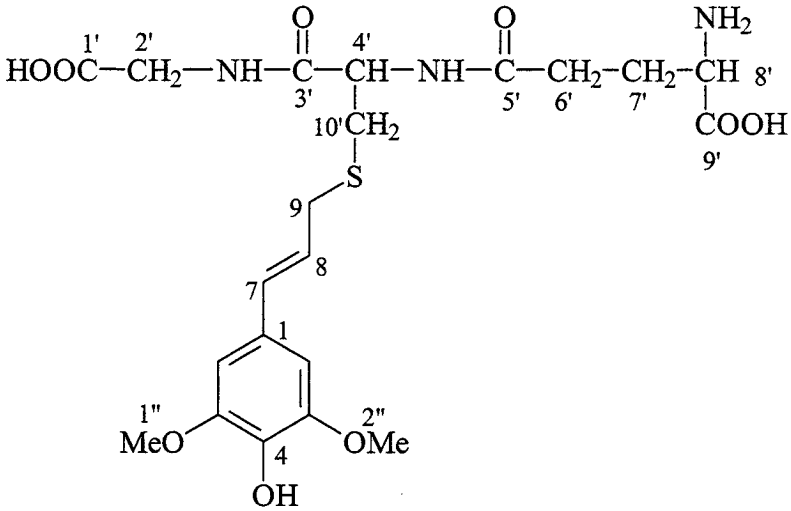
S-sinapyl-L-cysteine is typically a slightly yellow solid having a chemical structure substantially as shown in FORMULA 1.



FORMULA 1

wherein MeO and OMe represent methoxy groups.

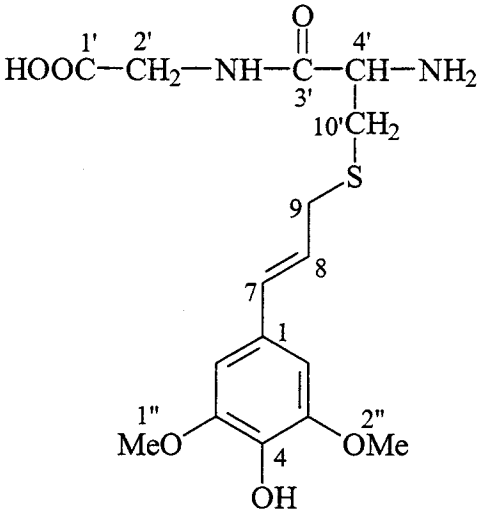
S-sinapyl glutathione is typically a slightly yellow solid having a chemical structure substantially as shown in FORMULA 2.



FORMULA 2

wherein MeO and OMe represent methoxy groups.

N-L-γ-glutamyl-S-sinapyl-L-cysteine is typically a slightly yellow solid having a chemical structure substantially as shown in FORMULA 3.



FORMULA 3

wherein MeO and OMe represent methoxy groups.

Methods for making the antibrowning, antioxidant compositions of the present invention generally comprise isolation of desirable constituents of extracted pineapple juice and/or pineapple processing waste streams. Although any type of pineapple may be used to practice the methods of the present invention, pineapples found to be useful in practicing the methods include: champaka, cayenne, champaka-smooth cayenne, cayena Lisa, cayenne F-200, and smooth cayenne. Likewise, waste streams from the processing of such pineapples may also be used to make the compositions of the present invention.

Suitable pineapples may first be washed with water or chlorinated water to eliminate extraneous matter and particulates. The pineapples (or pineapple waste stream solutions) may be stored at about 4°C until the pineapples (or waste stream solutions) are to be treated. Alternatively, the pineapples (or waste stream solutions) may be treated immediately following the wash. The pineapple skins and tops may be removed. The pineapples are then preferably cut into smaller pieces, ground, or The pineapple pieces or puree may then be extracted to obtain a pineapple juice solution. Typically, the pineapple

pieces or puree are pressed using a conventional juice hydraulic press. The pineapple juice concentrate is diluted with deionized water. Dilution of the pineapple concentrate is preferable as the concentrate is typically too viscous for efficient subsequent filtration and resin treatments. Single strength values are acceptable (about 10° brix to about 15° brix has been found to be useful), but a higher range of from about 10° brix to about 25° brix will work. The diluted pineapple juice is ultracentrifuged to remove particulates, e.g., high molecular weight proteins and poly-saccharides. The resulting supernatant is collected and filtered to remove remaining or residual particulates.

The filtrate is then passed through a suitable resin, such as a methanol activated C18 resin. The C18 resin is a silica-based resin having long carbon chains (i.e., 18 carbon atoms) covalently bonded to silica. (Such resins are available from Alltech Associates of Deerfield, Illinois). Other suitable silica resins, however, may be used to practice the methods of the present invention. The C18 resin is then preferably washed with an acidic solution, e.g., 0.01% aqueous HCl to remove undesirable compounds, such as sugars, acids, and other polar compounds.

The constituents of the antibrowning/antioxidizing compositions are then eluted from the C 18 r esin us ing a s uitable m edia, such as methylalcohol. The resulting eluate is preferably stored at about -10°C for a period of about 12 hours. Although storage at about -10°C is not critical, low-temperature storage seems to hasten precipitation of un-wanted materials. The temperature and time period the eluate is stored is not critical, but a temperature within a range of from about -15°C to about 5°C has been found to be useful. The eluate is then passed through a filter media, such as a 0.45 :M membrane filter to remove precipitate. Without being tied to any particular theory, the inventor believes the precipitate comprises polysaccharides or proteins. The filtrate is collected

and is preferably evaporated to dryness using a rotary evaporator at about 35°C. Other suitable evaporation methods may be used.

The evaporate is re-dissolved in water or an acidic solution. Re-dissolution in an aqueous acidic solution is preferred for dissolving sinapyl compounds for application to resin. The compounds should be in an aqueous solution for later application to an anion-exchange resin. The sinapyl compounds with their carboxylic acid (anions) will exchange with anions on the resin. It has been found that a more effective evaporation takes place if the sinapyl compounds are re-dissolved in a neutral or acidic environment.

The solution including the re-dissolved constituents is applied to an anion-exchange resin (e.g., a BIOREX-5 anion-exchange column available from Bio-Rad Laboratories, of Richmond, California). The anion-exchange resin is washed with water to remove neutral phenolic compounds. The composition constituents are then eluted from the anion-exchange resin, preferably using an acidic solution, e.g., a hydrochloric acid solution having a pH of about 1 or less. The eluate is then adjusted to a pH of from about 3 to about 5, and preferably to a pH of about 3.5. The pH of the eluate is adjusted with a basic solution, such as sodium hydroxide or other alkalizing agents such as potassium hydroxide. The eluate is then preferably filtered, preferably through a 0.45 :m membrane. The resulting antibrowning/antioxidation composition may then be used and/or individual components of the composition or combinations of components of the composition may be isolated to form alternative embodiments of the composition of the present invention. One or more of the sinapyl components of the composition may be isolated or separated using any of a variety of suitable methods, such as by high-performance liquid chromatography (HPLC) or by electrophoresis.

Although the method set forth above produces consistent and effective antibrowning, antioxidant compositions, if desired, the concentration of the antibrowning, antioxidation components of the resulting compositions may be determined using analytical HPLC. The resulting compositions may have effective antibrowning, antioxidant characteristics over a broad pH range (i.e., within the range of food product pH values). Food products are typically at a pH range of from about 3 to about 8.

Examples

The National Food Processors Association (of Washington, D.C.) and Dole Food Company, Inc. (of Westlake Village, CA), provided fifty-four pineapple juice concentrate samples. The pineapple juice samples were stored at about -20°C until use. The pineapple samples were mainly three varieties: Cayenne (from Bukidnon, Philippines), Smooth Cayenne (from Thailand and Philippines), and Champaka (from Maui, Hawaii).

About 400 g of pineapple juice concentrate was diluted four fold with deionized water. The diluted pineapple juice was ultracentrifuged at about 23,000 *g* for about 20 minutes. The supernatant was collected and filtered through Whatman #1 filter paper with a CELITE diatomaceous earth filtration aid (available from Celite Corp. of California). About 100 mL of the filtrate was passed through a methylalcohol activated C18 resin having about 5 g resin (C18 resin available from Alltech Associates of Deerfield, Illinois). The activated C18 resin was then washed with about 100 mL of about 0.01% aqueous HCl and then eluted with about 50 mL of methylalcohol. The about 50 mL of methylalcohol eluate was combined with the about 100 mL of about 0.01% HCl eluate and the eluate mixture was stored for about 12 hours at about -10°C. The eluate mixture was then filtered using a 0.45 μm Millipore HA membrane (available from

Millipore) to remove precipitate. The filtrate was rotary evaporated substantially to dryness at about 35°C and re-dissolved in about 10 mL of about 0.01% aqueous HCl:Methylalcohol (at a ratio of about 70:30). The aqueous HCI:Methylalcohol solution was filtered with a 0.45 :m Millipore HA membrane prior to semi-preparative HPLC isolation.

Isolation of Sinapyl Compounds Using Semi-Preparative HPLC

Two Dynamax SD-300 pumps (available from Dynamax Corp. of Houston, Texas) were used with a semi-preparative Microsorb C18 column (25 cm x 21.4 mm, 5 :m) (available from Rainin Instrument Co. of Woburn, Massachusetts). The end of the C18 column was connected to a flow divider that split a 1 mL/min flow to a HP 1040A Diode Array Detector (DAD) (available from Hewlett Packard of Corvallis, Oregon) having a detection at 280 nm. The remainder of flow, at 19 mL/min, was directed to an outlet for manual peak collection. The following gradient employing solvent A (methanol) and solvent B (0.07 M K-PO_4 buffer, pH 2.4) was used: 7 minutes from 35% to 45% solvent A, then 3 minutes from 45% to 48% solvent A and holding for 5 minutes. Three sinapyl compounds were isolated and identified (i.e., compound 1, S-sinapyl-L-cysteine, compound 2, S-sinapyl glutathione, and compound 3, N-L-γ-glutamyl-S-sinapyl-L-cysteine).

The peak solution purity was verified by analytical HPLC as shown in Fig. 1 (discussed below). Each peak solution collected from the preparative HPLC (i.e., each of the three isolated sinapyl compounds) was rotary evaporated at about 35°C for about 10 minutes to remove methylalcohol. The isolated solutions were then extracted with a 5 g

C18 resin (available from Alltech Associates, Inc. of Deerfield, Illinois). The methylalcohol eluate from the C18 resin was rotary evaporated to dryness and stored at about -15°C until analysis. As shown on the chromatograph of Fig. 1, peaks 1, 2 and 3 were obtained. The three peak solutions (i.e., compound 1, S-sinapyl-L-cysteine, compound 2, S-sinapyl glutathione, and compound 3, N-L-γ-glutamyl-S-sinapyl-L-cysteine) were further analyzed by analytical HPLC (as discussed below).

Analysis of Isolated Compounds 1, 2, and 3 by Analytical HPLC

A Supelco LC-18 column (25 cm x 4.6 mm, 5 :m) was used with a HP 1040A DAD set at 280 nm. The flowrate was set at about 1.0 mL/min, the injection was set at about 50 :L. The following gradient employing methanol (solvent A), acetonitrile (solvent B) and solvent C (0.07 M K-PO_4 buffer, pH 2.4) was used: 10 minutes from 10% solvent A/0% solvent B to 22% solvent A/0% solvent B, then 25 minutes from 22% solvent A/0% solvent B to 22% solvent A/25% solvent B, the remainder being solvent C. Approximately 5 mg of compound 1, 50 mg of compound 2, and 50 mg of compound 3 were isolated and identified. Compound 1 was identified as S-sinapyl-L-cysteine; compound 2 was identified as S-sinapyl glutathione, and compound 3 was identified as N-L-γ-glutamyl-S-sinapyl-L-cysteine.

Structural Analysis of Isolated Sinapyl Compounds 2 and 3 by Acid hydrolysis

About 0.5 mg each of compound 2 and compound 3 were acid hydrolyzed in a TEFLON-lined, screw-cap test tube with about 5 mL of about 2 N HCl at about 100°C for about 30 minutes in the dark (about 10 minutes N_2 flushing before hydrolysis). The

solution was cooled in ice-bath and extracted with C18 Sep-Pak (360 mg resin) (available from W aters As sociates of Milford, Massachusetts). The methylalcohol eluate of the Sep-Pak was rotary evaporated to dryness and re-dissolved in an about 0.01% aqueous HCl solution and analyzed by HPLC using the same conditions as described above. Sinapyl compounds 2 and 3 of the present invention were identified. The acid hydrolysis pattern of the sinapyl compounds was thus determined, as shown in Fig. 3.

Structural Analysis of Sinapyl Compounds by Amino Acid Analysis

About 200 μL of the methylalcohol eluate from the isolation step above was transferred to a 1.5-mL TEFLON-lined screw-cap reaction tube. The eluate was flushed dry with N_2, dissolved in about 120 :L of an about 6 N HCl solution and flushed with N_2 for about one minute and the tube was capped. Hydrolysis was performed in a heating block at about 95°C for about 3 hours. An amino acid analysis was performed on the hydrolysate using a PITC derivatization as described b y H agen e t a l. in *F ood Chem.* 46:319-323, 1993. The identity of hydrolyzed amino acids were confirmed by spiking sample with the standard PITC derivatives.

Structural Analysis of Sinapyl Compounds by Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) were performed using a Perkin Elmer Sciex API III+ triple quadrupole ionspray mass spectrometer (available from Perkin Elmer of Ontario, Canada) by pneumatically assisted electrospray to determine the molecular weights of the sinapyl compounds. The operating conditions were standard, as known to persons skilled in the art. Tandem MS/MS were recorded on the same

instrument using Ar-N_2 (9:1) as a target gas mixture. The collision energy was at about 15 to about 25 V. Operating conditions were again standard, as known to persons skilled in the art. A fast atom bombardment mass spectrometry sample was prepared in a matrix of about a 0.1 N toluenesulfonic acid solution in 1:1 glycerol/3-nitrobenzyl alcohol solution. The positive spectra were recorded on a Kratos M550TC instrument (available from Kratos Analytical, Ltd. of Manchester, England) at a scan speed of about 10 sec/decade us ing a r esolution of 1100 with a gun producing a 7 KV beam of Xenon atoms.

Structural Analysis of Sinapyl Compounds by Nuclear Magnetic Resonance (NMR)

NMR was used to confirm the glutathione structure in peak 1 of Figs. 1 and 2 and to confirm the sinapyl structure of compounds 1, 2, and 3 of the present invention. Referring to Table 1, ^1H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded at about 25°C using a Bruker DRX 600 NMR Spectrometer (available from Bruker Instruments, Inc. of Billierica, Massachusetts). A mixed solvent comprising D_2O:CD_3OD:CF_3COOD at about 60:30:10 by volume and a residual D_2O resonance was used as internal chemical shift reference. A ^1H-^1H correlation spectroscopy (COSY) and a total correlation spectroscopy (TOCSY), a ^1H-^{13}C heteronuclear multiple bond quantum correlation (HMQC), and a heteronuclear multiple bond c orrelation (HMBC) a nalysis were performed using a standard pulse sequence, as known to persons skilled in the art. Spectral widths of about 10 ppm and 200 ppm were used in the ^1H and ^{13}C dimensions, respectively.

S-sinapyl-L-cysteine, compound 1, was identified as, a slightly yellow solid, having the following parameters: ESI-MS m/z of 314.0 (MH^+), 192.8 (MH^V - cysteine), 160.8

MS/MS of daughter ion (m/z 192.8, collision energy 25 volts) m/z (rel. int.): 192.8 (20), 161.2(100), 143.2 (15), 133.2(100), 115.0 (60), 105.2 (100), 79.0 (15), 55.0 (8). An ultraviolet spectrum showed a λ_{max} of 285 nm (mobile phase). For the ^{1}H NMR results, see Table 1.

S-sinapyl glutathione, compound 2, was identified as a slightly yellow solid having the following parameters: ESI-MS m/z 500.0 (MH^+), 308.0 (Glutathione·H^+), 192.8 (MH^+ - Glutathione). An ultraviolet spectrum was obtained that was identical to that UV spectrum from compound 1, as discussed above. For the ^{1}H and ^{13}C NMR results see Tables 1 and 2. For the ^{1}H -^{13}C HMQC results see Table 3.

N-L-γ-glutamyl-S-sinapyl-L-cysteine, compound 3, was also identified as a slightly yellow solid having the following parameters: ESI-MS m/z of 442.8 (MH^+), 250.8 (Glu-Cys·H^+), 192.8 (MH^+ - Glu-Cys). A FAB-MS m/z of about 443.1 (calculated for $C_{19}H_{27}O_8N_2S_1$: 443.1458; deviation: -3.4 ppm). An ultraviolet spectrum of N-L-γ-glutamyl-S-sinapyl-L-cysteine is the same as for compounds 1 and 2. For the ^{1}H NMR results see Table 1. The ^{1}H NMR results provide information for interpretation of the ultraviolet spectra. Such measurements give the electromagnetic information characteristics of different protons.

Results for mass spectral analysis of a sinapyl alcohol standard (available from Aldrich of Milwaukee, Wisconsin) had the following parameters: ESI-MS m/z of 193.0 (MH^+-H_2O), a MS/MS (collision energy 15 volts) m/z (rel. int.) of 192.8 (16), 161.2 (80), 143.2 (5), 133.2 (83), 115.0 (45), 105.2 (100), 79.0 (10), 55.0 (5) and an ultraviolet spectrum that was the same as other sinapyl derivatives (in the mobile phase). Sinapyl alcohol was used as a reference standard. Sinapyl alcohol's structural similarity to the

sinapyl compounds of the present invention provide appropriate reference points for interpretation of ultraviolet and mass spectral data.

Table 1 ^{1}H NMR Data for Compounds 1, 2 and 3*

H	Compound 1*	Compound 2*	Compound 3*
2, 6	6.44 s	6.35 s	6.40 s
7	6.13 d(15.6)	6.04 d (15.6)	6.08 d (15.6)
8	5.78 dt (15.6, 7.8)	5.69 dt (15.6, 7.8)	5.73 dt (15.6, 7.8)
9	3.07 d (7.8)	2.94 d (7.8)	3.00 d (7.8)
1', 2"	3.54 s	3.47 s	3.51 s
2'		3.58 s	
4'	3.95 dd (7.8, 4.2)	4.20 t (6.6)	4.26 dd (8.4, 4.8)
6'		2.18 t (7.2)	2.21 t (7.2)
7'		1.86 dt (7.2, 7.2),	1.90 dt (7.2, 7.2),
		1.80 dt (7.2, 7.2)	1.84 dt (7.2, 7.2)
8'		3.69 t (6.6)	3.74 t (6.6)
10'	2.86 dd (15.0, 4.2),	2.60 dd (13.8, 4.8),	2.69 dd (13.8, 4.8),
	2.71 dd (15.0, 7.8)	2.44 dd (13.8, 8.4)	2.52 dd (13.8, 8.4)

Coupling constant (J in Hz) given in parentheses
Chemical shifts assigned by comparison to chemical shift pattern of compounds.
*Compound 1 is S-sinapyl-L-cysteine; compound 2 is S-sinapyl glutathione; compound 3 is N-L-γ-glutamyl-S-sinapyl-L-cysteine.

Table 2 ^{13}C NMR Spectral Data for Compound 2*

C	ppm
1	128.7
2	104.0
3	147.7
4	134.3
5	147.7
6	104.0
7	132.6
8	123.6
9	34.0
1", 2"	56.3
1'	172.6
2'	41.0
3'	172.7
4'	53.2
5'	174.0
6'	31.0
7'	25.5
8'	52.2
9'	171.2
10'	31.9

1' and 3' are overlapping.
*Compound 2 is S-sinapyl-L-cysteine.

The ^{13}C NMR data list in Table 2 aids in the determination of the carbon skeleton of the c omponent c ompounds o f the present invention compositions. That is, such data (along with the HMBC data shown in Table 3 discussed below) aids in final determination of the structure of the component compounds of the present inventions compositions.

Table 3 Cross-Peaks in HMBC Spectra of Compound 2*

	C	
H	3-bond	2-bond
2, 6	6, 2, 4, 7	3, 5
7	2, 6, 9	1
8	1	9
9	7, 10'	8
1", 2"	3, 5	
2'	3'	1' (?)
4'	5'	3', 10'
6'	8'	5', 7'
7'	5', 9'	6', 8'
8'	6'	7', 9'
10'	9, 3'	4'

*Compound 2 is S-sinapyl-L-cysteine.

The heteronuclear multiple bond correlation (HMBC) data shown in Table 3 is the resulting data of an NMR technique useful for identifying the neighboring carbon to a proton of component compounds of the present invention compositions.

Antioxidant Properties of the Present Invention Compositions

Sample Preparation for Measurement of Antioxidant Properties

About 4.2 ml of methanol and about 10 liters of about 1N HCl were added to about 2.1 mg of S-sinapyl glutathione (having a molecular weight of about 499). The mixture was vortexed until the S-sinapyl compound was completely dissolved (1 mM final).

About 4.98 ml of methanol and about 10 liters of about 1N HCl were added to about 2.2 mg of N-L-γ-glutamyl-S-sinapyl-L-cysteine (having a molecular weight of about 442). The mixture was vortexed until the sinapyl compound was completely dissolved (1 mM final).

Prior to the ACW analysis, the 1 mM solutions of N-L-γ-glutamyl-S-sinapyl-L-cysteine and S-sinapyl glutathione were diluted about 1:100 with methanol (about 0.01 mM final) and then about 10 liters of sample (at about 0.1 nmol) were assayed. Prior to ACL analysis, the 1 mM solutions of N-L-γ-glutamyl-S-sinapyl-L-cysteine and S-sinapyl glutathione were diluted about 1:10 with methanol (about 0.1 mM final) and then about 10 liters of sample (at about 1 nmol) were assayed.

Antioxidant Capacity Water Soluble Assay (Water Based PCL Assay)

Assayed compounds compete with photo-sensitizer (luminol) for interaction with superoxide radicals produced by UV irradiation, resulting in a delay of the propagating phase of kinetic curve of chemiluminescence. The delay was expressed as a lag-time in seconds. TROLOX, an excellent antioxidant, a water-and methanol-soluble analog of alpha-tocopherol (i.e., 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), was used as a reference antioxidant (TROLOX is available from Aldrich of Milwaukee, Wisconsin). A calibration curve for TROLOX was generated by plotting a shift in lag-time (lag sample versus lag control) against the amount of TROLOX taken for the assay. The results are expressed as a molar ratio of (antioxidant capacity) AOC of the assayed compound of the present invention to the AOC of TROLOX. In the water-based PCL assay, TROLOX mimics the AOC of ascorbic acid, a known antioxidant. Comparison of present compositions to TROLOX are discussed below and tabulated in Table 4.

Antioxidant Capacity Lipid Soluble Assay (MeOH Based PCL Assay)

Assayed compounds compete with photo-sensitizer (luminol) for interaction with superoxide radicals produced by UV irradiation resulting in an inhibition of the intensity

of chemiluminescent signal. The measured parameter was an area under the kinetic curve of the light output (A). Inhibition (I) of chemiluminescence was calculated as $I=1-A/A_0$, wherein A_0 is the area under the control (the non-antioxidant present) curve. A calibration curve was generated from a plot of the inhibition versus the amount of TROLOX. The results (mean) are expressed as a molar ratio of the AOC of the assayed compound of the present invention to the AOC of TROLOX. In the MeOH-based PCL assay, TROLOX mimics the AOC of alpha-tocopherol. Comparison of present compositions to TROLOX are discussed below and tabulated in Table 4.

Antioxidant Effectiveness of the Present Invention Sinapyl Compositions

The antioxidant capacity (AOC) of two of the three sinapyl derivatives (S-sinapyl glutathione, and N-L-γ-glutamyl-S-sinapyl-L-cysteine) was measured in water-soluable and methanol-soluble photo-chemiluminescent assays. AOCs of the sinapyl compositions of the present invention were expressed in AOCs of TROLOX (i.e., the standard or reference antioxidant).

As shown in Table 4, both S-sinapyl glutathione, and N-L-γ-glutamyl-S-sinapyl-L-cysteine demonstrated antioxidant capacities comparable to that of TROLOX, both in water and in methanol soluble assays. There also appears to be positive health benefits of such antioxidant compositions of the present invention.

Table 4 Antioxidant activity of sinapyl compounds

	AOC (mol of sample necessary to match AOC of 1 mol of TROLOX)	
Sample	Water-Based PLC Assay*	MeOH-Based PLC Assay*
S-sinapyl glutathione	1.22±0.03	3.25±0.10
N-L-γ-glutamyl-S-sinapyl-L-cysteine	1.56±0.20	4.53±0.20

*Data represent mean ±SD from triplicate measurements.

Results of Isolation and Identification of the Present Invention Compositions

S-sinapyl-L-cysteine, S-sinapyl glutathione, and N-L-γ-glutamyl-S-sinapyl-L-cysteine were extracted from pineapple juice or pineapple process waste streams and analyzed as discussed above. Figure 1 shows the analytical HPLC chromatogram of pineapple juice with peaks 1, 2 and 3 being the three phenolic compounds (i.e., S-sinapyl-L-cysteine, S-sinapyl glutathione, N-L-γ-glutamyl-S-sinapyl-L-cysteine). Figure 2 shows a semi-preparative HPLC chromatogram of pineapple juice after a C18 resin fractionation that resulted in isolation of the three compounds (i.e., S-sinapyl-L-cysteine, S-sinapyl glutathione, N-L-γ-glutamyl-S-sinapyl-L-cysteine) (labeled peaks 1, 2 and 3) without impurities.

Figure 3 shows a resulting acid hydrolysis pattern of compounds 2 and 3 (i.e., S-sinapyl glutathione, N-L-γ-glutamyl-S-sinapyl-L-cysteine, respectively). The acid hydrolysis of compound 2 generated compounds 1, 3, and an additional compound having an identical ultraviolet spectrum. Acid hydrolysis of compound 3 (i.e., N-L-γ-glutamyl-S-sinapyl-L-cysteine) generated only compound 1 (i.e., S-sinapyl-L-cysteine).

Proton NMR spectra of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione all exhibit resonances with chemical shifts at 6.4, 6.0, 5.5, 3.5

and 3.0 ppm. The 6.4 ppm chemical shift is the characteristic resonance of aromatic protons, while 6.0 and 5.5 ppm shifts are traits of double-bond protons. The final structure was established using ^{13}C NMR (see Table 2), HMQC and HMBC (see Table 3). The same ultraviolet spectrum (resulting structure identification shown in Fig. 4) and fragmentation pattern as a sinapyl alcohol standard further confirmed the sinapyl core structure of the sinapyl compounds of the present compositions. The two protons at the C-2 and C-6 positions undergo deuterium exchange under very acidic condition when kept at room temperature for over two days. This exchange was followed by ^{1}H NMR resulting in the almost complete loss of proton chemical shifts at 6.4 ppm. The deuterium exchange was further confirmed by 2 mass units increase in the fragment ion corresponding to the sinapyl moiety [m/z (without deuterium exchange): 193] in the ESI-MS. This deuterium exchange can be used to establish the fragmentation pathway during MS/MS by comparing the fragmentation pattern of the compound with that of the deuterium-exchanged compound.

Whereas the invention has been described with reference to particular compositions and methods, it will be understood that the invention is not limited to those particular compositions and methods. On the contrary, the invention is intended to encompass all modifications, alternatives, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

CLAIMS AS ALLOWED

1. An antibrowning and antioxidant composition comprising:

a sinapyl compound that has been isolated from a natural source.

2. The composition of claim 1, wherein the natural source comprises pineapple juice.

3. The composition of claim 1, wherein the sinapyl compound isolated from a natural source further comprises a compound selected from a group consisting essentially of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapyl glutathione, and mixtures thereof.

4. The composition of claim 1, wherein the natural source comprises pineapple juice and the sinapyl compound isolated from the natural source comprises a compound selected from a group consisting essentially of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapyl glutathione, and mixtures thereof.

5. An antibrowning and antioxidant food preparative composition for use with fruit and fruit products, comprising:

a mixture of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione, isolated from a natural source.

6. The composition of claim 5, wherein the mixture of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione is isolated from pineapple juice.

7. An antibrowning and antioxidant food preparative composition consisting essentially of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione.

8. The composition of claim 7, wherein the S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione are isolated from pineapple juice.

9. A method of making an antibrowning and antioxidant composition comprising:

extracting juice from a pineapple; and

isolating a sinapyl compound from the juice.

10. The method of claim 9, wherein isolating a sinapyl compound from the juice further comprises isolating S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, or S-sinapyl glutathione from the juice.

11. The method of claim 9, wherein isolating a sinapyl compound further comprises:

applying juice extracted from a pineapple to a resin column; and

eluting a first eluate including the sinapyl compound from the resin.

12. The method of claim 11, further comprising:

applying the first eluate to an anion-exchange resin.

13. The method of claim 11, further comprising:

applying the first eluate to an anion-exchange resin;

eluting neutral phenolic compounds from the anion-exchange resin to remove the neutral phenolic compounds from the first eluate; and

eluting a second eluate including the sinapyl compound from the anion-exchange resin.

14. The method of claim 13, wherein the second eluate is eluted from the anion-exchange resin by application of an acidic liquid to the anion-exchange resin.

15. The method of claim 14, further comprising neutralizing the second eluate to a pH value of from about 3 to about 8.

16. A method of preparing fruit or fruit products to substantially inhibit browning and oxidation thereof, the method comprising:

applying a composition consisting of sinapyl compounds to the fruit or fruit products.

17. The method of claim 16, wherein the sinapyl compounds are selected from a group consisting essentially of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapyl glutathione, and mixtures thereof.

18. The method of claim 16, further comprising isolating sinapyl compounds from pineapple juice prior to application of the sinapyl compounds to the fruit or fruit product.

19. The method of claim 16, further comprising:

isolating S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapyl glutathione, and mixtures thereof from pineapple juice prior to application of such sinapyl compounds to the fruit or fruit product.

20. A method of preparing fruit or fruit products to substantially inhibit browning and oxidation thereof, the method comprising:

applying a composition consisting essentially of sinapyl compounds to the fruit or fruit products, wherein the sinapyl compounds are selected from a group consisting of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, S-sinapyl glutathione, and mixtures thereof.

21. The method of claim 20, further comprising isolating sinapyl compounds from pineapple juice prior to application of the sinapyl compounds to the fruit or fruit product.

22. An antibrowning and antioxidant food preparative composition for use with fruit and fruit products, comprising:

a mixture of S-sinapyl-L-cysteine, N-L-γ-glutamyl-S-sinapyl-L-cysteine, and S-sinapyl glutathione, extracted from a natural source, wherein the mixture provides consistent antibrowing and antioxidation regardless of the composition of natural source from which it is extracted.