AN ABSTRACT OF THE THESIS OF

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Title: Development of Analytical Methodology for Verification of Authenticity of Apple Juice.

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Dr. Ronald E. Wrolstad

Apples growing in New Zealand, Argentina, Mexico, Michigan and Washington were used to provide the compositional data and to develop the analytical procedures for the use in verification of authenticity of apple juices.

High Performance Liquid Chromatography (HPLC) techniques were applied for glucose, fructose, sucrose, sorbitol, malic, citric, quinic, shikimic, fumaric, chlorogenic acid, and hydroxymethylfurfural (HMF). L-malic acid was determined by an enzymic method. Sep-Pak C18 cartridge, anion exchange resin and millipore filter were used for sample preparation of HPLC.

Influence of postharvest storage on sucrose and sorbitol content was observed but it had no effect on the glucose/fructose ratios. Quinic was the next most abundant acid in all the apple varieties examined. Paired t-test showed good agreement ($p<0.01$) between HPLC and enzymic determination for malic acid. Significantly high variability in chlorogenic acid content limits its utility. The influence of processing procedures on the phenolic compound was observed by comparision with commercial, self-pressed

and gelatin treated apple juices.

All apple juices showed two characteristic UV absorption maxima at around 280nm and 320nm, the ratio of the two maxima being relatively constant. First, second derivative spectra and fluorescence excitation and emission maxima were measured. As the order of derivative increased, the spectra became more complex and the resolution was enhanced.

For the stable carbon isotope ratio analysis, juice, pulp, seeds and sugars, acids, and phenolics were fractionated using ion exchange resins and polyclar AT. Mass spectrometric measurements of $^{13}C/^{12}C$ ratio from whole juices showed low variability as compared to other fractions. Organic acids showed more negative values than the sugars. Phenolic fraction was most enriched in ^{12}C when compared to the juice by as much as 4.6 ppt.

The evidence suggests that postharvest storage and processing conditions played an important role in the variability of apple juice components. There are no marked differences in del ^{13}C values with regard to apple variety or geographic origins.

Development of Analytical Methodology for verification of Authenticity of Apple Juice.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DEVELOPMENT OF ANALYTICAL METHODOLOGY FOR VERIFICATION OF AUTHENTICITY OF APPLE JUICE

INTRODUCTION

The consumption of apple juice whether as single strength juice or diluted in beverages, has increased greatly throughout the world. This is partly due to improvements in methods of processing, storage and also to steady rise in the standard of living. Together with this increase in demand for apple juice, there has been increases in the marketing of products purporting to be, or to contain, apple juice. Some of these products contain little or no apple juice and it is therefore necessary to develop techniques to detect such adulteration.

Throughout the history of the apple juice industry, apple juices have been vulnerable to the practice of adulteration, and this has taken many different forms (1-3). In earliest times, direct dilution of the juice with appropriate amounts of sugar and acid was common, often with added coloring matter to improve the appearance.

The wide spread interest in the adulteration and characterization of apple juices has prompted numerous investigations using many detailed analyses, such as stable carbon isotope analyses (4,5), organic acids (3,6,7), sugars (1,3,8), phenolics (2), minerals (2,9-11), amino acids (3,12,13),

microbiological assay (14) and spectral analyses (15,16). These tests, however, have not been combined in a comprehensive manner to apply to the apple juice adulteration and fraud.

From the literature, it is clear that very wide natural variations exist in both the concentration and nature of the components. This is attributed to many factors including seasonal variations, environmental and growing conditions, varietal differences and state of maturity etc. (17). The composition of commercial juice is also dependent on processing and storage conditions.

Most of the apples grown in the United States are sold fresh, whole and unprocessed, an affidavit in the New York district court stated that " at least 75 % of the apple juice concentrate used in the United States is imported, principally from South America, South Africa, Spain, West Germany, and Israel"(18).

This dissertation is divided into three parts. The first part, the literature review contains a thorough review of apple juice composition and the analytical methodology for determining of chemical components and their utilization for adulteration. The purpose of the second part of this study is to analyze the sugars, non-volatile organic acids , phenolic acids, UV-spectra and to develop and improve the analytical procedures for determining the authenticity of apple juice. The third part focuses on the variability of carbon isotope composition in foreign and domestic apple juices and their subfractions.

LITERATURE REVIEW

Apple Juice

The Processed Apples Institute, proposed standards of identity for apple juice from concentrate, frozen apple juice from concentrate and apple juice concentrate for manufacturing (19). Apple juice is the unfermented juice obtained from sound, ripe apples of the species Pyrus Malus L. with or without parts thereof. The juice may be clarified or non-clarified but must meet the specifications of Federal Register 146.103 (19). While the standard of identity was not adopted, it represents an effort to define apple juice more explicitly and will be referred to in this thesis.

Chemical Composition of Apple Juice

Much of the information has been reported on general composition of apples from many regions of the world. A detailed review was made by Smock and Newbert (20) and Hulme (21).

Apple juice contains a considerable portion of the soluble constituents of the original apple- sugars, acids, other carbohydrates and minerals. Some varieties give a low yield of high soluble solid juice, while others give a high yield of low soluble solid juice (22). The chemical composition from Argentina(23), New Zealand (24), Canada (25), and England (26) have been presented.

Table I.1 summarizes mean and range values of components of apple juice as a guide to composition. This table contains data only from non-commercial, self-pressed apple juices which have a relatively large sample size, mean and range values. The complex nature of these components has in the past limited analyses to the determination of the total amounts of substances belong to a certain class, but in recent times improved analytical techniques have made more detailed analyses possible.

Sugars in Apple Fruit and Juice :

Sugars constitute the major portion of the carbohydrate materials in apples. They also constitute most of the soluble solid materials in the flesh of the apples (20). Wrolstad and Shallenberger (27) presented data from the literature on free sugar and sorbitol content of apple fruits and juices. They include data on different varieties from many sources using different methods of analysis. In Canadian apple juice, the sugar content represents 90 % of the soluble solids; values for sucrose and sorbitol show larger variations than those for either fructose or glucose (25) which may be due to the fact that sucrose and sorbitol are the major carbohydrate translocated in apples and may function as a respiratory substrate (28).

TABLE I.1 ANALYTICAL DATA FOR APPLE JUICE.

	MEAN	RANGE	NO. OF SAMPLES	REFERENCE
SPECIFIC GRAVITY	1.0511	1.0372-1.0705	93	9
	1.0456	1.0409-1.0503	9	25
	1.0510	1.0420-1.0620	29	24
	1.0440	1.0330-1.0520	20	23
TOTAL SUGARS(g/100ml)	10.74	9.02-13.34	20	25
	10.57	6.08-16.87	93	9
	11.0	9.04-13.98	-	27[a]
	9.87	7.52-11.36	20	23
SOLUBLE SOLIDS(%)	12.74	9.80-16.90	93	9
	11.20	10.08-12.68	21	25
	13.50	10.70-15.60	6	3
	12.40	10.00-14.50	29	24
	10.04	8.18 -12.01	20	23
SUCROSE(g/100ml)	1.75	0.65 -2.40	20	25
	2.68	0.88 -5.62	93	9
	1.33	0.70 -2.70	8	2
GLUCOSE(g/100ml)	2.50	1.72 -3.93	20	25
	3.04	1.90 -4.10	8	2
	2.07	0.89 -3.99	93	9
FRUCTOSE(g/100ml)	5.48	4.29 -6.48	20	25
	6.83	5.60 -8.50	8	2
	5.79	3.00 -10.5	93	9
SORBITOL(g/100ml)	1.01	0.57 -1.67	20	25
	0.52	0.16 -1.20	93	9
	0.39	0.17 -0.63	6	3
	0.51	0.20 -1.01	-	27[a]
FRUCTOSE/GLUCOSE	2.37	1.68 -3.68	8	2
	3.03	1.67 -6.09	93	9
TITRATABLE ACIDITY (% as malic acid)	0.49	0.38 -0.58	20	25
	0.42	0.15 -0.91	93	9
	0.48	0.19 -1.01	29	24
	0.40	0.19 -0.85	20	23
pH	3.45	3.38 -3.52	9	25
	3.69	3.23 -6.54	93	9
	3.69	3.28 -4.10	29	24
	3.44	3.20 -4.00	20	23

TABLE I.1 CONTINUED.

MALIC ACID(mg/100ml)	628	545-760	20	25
	508	220-900	30	6
	1000	710-1330	6	3
CITRIC ACID(mg/100ml)	20	10-40	12	6
	7.5	5 -10	6	3
QUINIC ACID(mg/100ml)	29.9	20.4-41.5	20	25
PHOSPHORIC ACID(mg/100ml)	15.1	10.5-25.5	20	25
FUMARIC ACID(mg/100ml)	0.26	0.09-0.43	14	6
ISOCITRIC ACID(mg/100ml)	3.2	0.5-8.0	6	3
ASCORBIC ACID(mg/100ml)	0.74	0.1-4.1	29	24
POLYPHENOLICS(A_{325nm})	0.47	0.308-0.651	7	2
CHLOROGENIC ACID(PPM)	170.1	93.0-232.0	8	2
CAFFEIC ACID(PPM)	3.46	1.40-6.40	7	2
K(PPM)	1300	1140-1490	21	25
	1005	680 -1280	6	2
	1073	685 -1510	93	9
Cd(PPB)	6.22	1.10-29.10	93	9
Ca(PPM)	38.61	19.70-63.40	93	9
Fe(PPM)	1.10	0.28-3.72	93	9
Pb(PPB)	33.65	11.80-163.50	93	9
P(PPM)	125.36	29.20-289.80	93	9
Zn(PPM)	0.37	0.15 -1.06	93	9
Na(PPM)	20.80	13.50-33.30	93	9
	28.13	9.00 -49.00	23	11
	35.06	4.00 -176.00	18	10
ASH(%)	0.24	0.21 -0.26	21	25
	0.21	0.11 -0.30	93	9
	0.24	0.14 -0.33	29	24

TABLE I.1 CONTINUED.

TOTAL AMINO ACIOS(meq/100ml)	0.308	0.211-0.470	21	25
FORMOL NUMBER	3.19	1.67 -7.40	7	2
PROLINE(PPM)	5.47 3.70	1.27 -13.8 2.0- 4.4	93 6	9 3
ASPARTIC ACID(mMol/l)	0.93	0.62 -1.45	12	12
THREONINE(mMol/l)	0.05	0.02 -0.08	12	12
SERINE(mMol/l)	0.19	0.07 -0.36	12	12
ASPARAGINE(mMol/l)	5.25	1.63 -12.88	12	12
GLUTAMIC ACIO(mMol/l)	0.35	0.17 -0.62	12	12
GLUTAMINE(mMol/l)	0.05	0.01 -0.08	12	12
GLYCINE(mMol/l)	0.02	0.01 -0.03	12	12
ALANINE(mMol/l)	0.13	0.08 -0.26	12	12
VALINE(mMol/l)	0.07	0.03 -0.17	12	12
ISOLEUCINE(mMol/l)	0.06	0.02 -0.10	10	12
LEUCINE(mMol/l)	0.05	0.02 -0.11	10	12
PHENYLALANINE(mMol/l)	0.04	0.01 -0.15	10	12
r-AMINOBUTRIC ACIO(mMol/l)	0.09	0.03 -0.13	12	12
NH_3(mMol/l)	0.26	0.06 -0.28	12	12
LYSINE(mMol/l)	0.02	0.01 -0.04	10	11
HISTIOINE(mMol/l)	0.02	0.01 -0.04	10	12

a is g/100g

Most fruits contain invert pattern and occasionally glucose concentration exceeds that of fructose, while in apples fructose is present in amounts up to three times greater than glucose (21). In cider apples, the relative proportion may be even greater as glucose comprises only 9-26.5 % and fructose 73.5-91 % of the reducing sugars(20). Hexoses other than glucose and fructose are rarely found in apple and then usually in trace amounts only. Arabinose has been reported by Wali and Hassen (29). Whiting and Coggins (30) found xylose in cider apple juice to have average concentration of 0.05 %.

Sucrose is the main dissaccharide present in apples. Dissaccharides other than sucrose have rarely been reported. D-glucitol (sorbitol) is found in many fruits, notably those of the family Rosaceae (31). Mattick and Moyer (9) found 160-1200 mg sorbitol/100ml from 93 U.S.A. apple juice samples. Ash and Reynold (32) found its concentration approximated to that of sucrose. Weiss et al. (33) extensively investigated the D-sorbitol content from Rosaceae family juices with enzymic methods and reported an amount of 2.6-9.2 g/L in apple juice samples. Richmond et al. (34) described that sorbitol was often, but not always present when they examined the juice of the Rosaceae family. Data has been reported for Canadian (25) and Swiss (3) apple juice and recently Wrolstad (35) found good agreement between a literature compilation (27) and the values reported by Mattick and Moyer (9) for free sugar and sorbitol contents of apple juices. Table I.1 shows that the values for glucose, fructose and total sugars are in fair agreement, but

that sucrose and sorbitol show more variability. This may be due to increased analytical variation which often occurs in quantity minor compounds in addition to the inherent variability of these sugars.

Non-Volatile Organic Acids in Apple Fruit and Juice :

The acidic components of apple juice impart important flavor and taste properties to the juice, and play a critical role in processing and preservation. The acidity has also been used as a criterion of adulteration of one fruit with another (36).

The acid in the fruit of apple is primarily malic acid, comprising 80-90% of total acids(37); apple juice was found to contain over 90% of total organic acids as malic (25). Apple contains at least 9-12 acids (38). Hulme (21) listed 13 different acids that have been reported in apples up to the data of his review. Besides malic acid, the other acids found are citric, succinic, citramalic, shikimic, glyceric, glyoxylic, isocitric, glycolic, lactic and galacturonic acid. Morover, there are three keto acids, oxaloacetic, pyruvic and ketoglutaric acid(38). These acids represent only a minor fraction of the total organic acids present.

Other acids like ascorbic acid,oxalic acid are present in such small amounts. In most instances ascorbic acid was less than 2mg/100ml in juice(24) and could not contribute very much to the acidity of the fruit(20). It is true that malic and citric acids make the major contribution to the acidity of the apple.

In certain young apples the amount of quinic acid present was

greater than that of malic acid (21). Phillips et al.(39) found quinic acid as the acid next in importance in quantity to malic acid. In one juice, the quinic acid content was equal to 50 % of the malic acid content. Citric acid content is low, 5-10mg/100ml in Switzerland(3), 0-15mg/100ml in New Zealand(24) and 10-40mg/100ml in the United States(6). Krotkov et al.(40) found that citric acid of McIntosh apples never exceeded 5% of the total acids.

Quinic and shikimic acid, small in absolute amount, also fluctuate much more than malic acid (25). Hulme (41) has suggested that shikimic acid and quinic acid might be part of a dehydrogenase system analogous to the succinic-fumaric -malic system.

Phillips et al. (39) and Buch et al.(42) reported the presence of lactic acid in apple juice. It is possible that lactic acid found in trace amounts in fruit juice may have originated during isolation from the action of anion resins on sugars present in the juice(43). It also may have originated from the action of microorganisms(21).

Citramalic acid appears in the peel only at maturity (44). This acid is readily oxidized in vitro to acetoacetic acid which in turn, readily breaks down to acetone (44,45). Significant amounts of phosphoric acid and traces of shikimic acid were also detected(25). Ryan and Dupont (36) also reported the presence of phosphoric acid. However, as the retention time of this acid is very similar to those of succinic, fumaric, malonic, and maleic acid on both SE-30 and OV-17 stationary phase, it can be easily confused

with them unless supplementary evidence is available. Also this acid has not been detected by HPLC methods, but only reported using GLC-TMS methods. Withy et al. (24) reported the individual acids in New Zealand apple juices. The results were very similar to those reported previously for Canadian apple juice (25).

Phenolic Acids in Apple Fruit and Juice:

A measure of the phenolic substances present in the fruit has been of great interest to pomologists and fruit juice and cider makers, because of these compounds roles in the taste(bitterness, astringency), the turbidity of the juice and its browning due to polyphenol oxidase and oxygen.

The major phenolics so far indentified in the fruit of apple are almost all of a flavonoid nature(21). Phenolic acids are of widespread occurrence in plants and can exist in the cells as simple phenolic acid or as labile esters. Phenolic acids can also be linked to other compounds by glycosidic linkages (46). Neish (47) has described that they were originally by-products of aromatic amino acids.

Apples are unique from other fruits in that they contain certain polyphenolic and other compounds in trace but measurable amounts (21). Many reports (46,48,49)indicated that chlorogenic acid (3-O-caffeoyl-D-quinic acid) is the most important cinnamic acid derivative found in apples. Because chlorogenic is a prominent ortho-dihydroxy compound, it serves as a substrate of polyphenol oxidase in apple fruit(50). Bradfield et al. (51) established

through the use of paper chromatograms, the presence of chlorogenic acid in the juice of the apple. The amounts in apple juice are reported as 93µg to 232µg per 100g of self-pressed juice(2).

Isomers of chlorogenic are also prominent and often occur together in the apple (52). Trace amounts of isochlorogenic, "band 510" and neochlorogenic acid have been reported from McIntosh apples(53). Isochlorogenic is isomeric with chlorogenic in that the caffeoyl group is substituted on the 5-OH group of quinic acid (54). Scarpati and Esposito (55) identified that the neochlorogenic acid is the 5-O-caffeoyl-D-quinic acid, and band 510 is the 4-O-caffeoyl-D-quinic acid.

Williams (56) tentatively identified the presence of another quinic acid derivative, a p-coumarylquinic, in immature cider apples which he believed to be the 3-isomer (3-O-P-coumaroyl-D-quinicacid). This compound may be a immediate precursor in the biosynthesis of chlorogenic acid (57). Both mature and immature apples have the 4-ester of P-coumaroylquinate as the major 2 isomer (58).

Durkee and Poapst (59) identified caffeic acid, P-coumaric acid, phloretin, phloretic acid and traces of ferulic acid after acidic and alkaline hydrolysis of the core tissue extracts from McIntosh apples. Chlorogenic acid and phloridzin were the main phenolic constituents in the core tissues and seeds by means of paper chromatography. The phenolic acids in the apple seeds resembled closely those found in core tissues. Phloridzin, a dihydrochalkone glucoside, has been known as the principal tannin in leaves,shoots,and roots of various varieties of apples (49). Many

workers detected this compound in apple juice (60-62) and cider (63,64). Macheix et al. (65) reported chlorogenic (75 %), P-coumaryl glucose (5 %), P-coumarylquinic (15 %) and feruly1 glucose (5 %) as the main hydrocinnamic esters in apples.

More complex phenolics such as flavanols are also common in apples (49,66). It has been known that the only naturally occurring flavanols are those with (+)catechin or (-)epicatechin configurations (67). Flavones were not identified in apples except as traces in the peels (68,69). Falvonol glycosides are mostly concentrated in peel tissues. Typical values are between 0.5 and 18 mg/g of fresh peel(70,71). These are mainly glycosides of quercetin having galactose, glucose, arabinose, rhamnose or xylose as the sugar group (52). Siegelman(72) included rutin (quercetin-3-rutinoside). Teuber and Herrmann (73) observed that there is no distinctive glycoside patterns between the different varieties of apples.

Macheix and Delaporte (74) studied the distribution of the main O-diphenolic compounds in the apple. Skin contained a higher concentration of chlorogenic (19.1), catechin (45.8) and glycosides of quercetin (17.8 mg/100g of tissue) than the parenchyma (15.1, 22.6 and 3.7 mg/100g respectively).

It has been reported (75) that polymeric procyanidins also make up a large proportion of the apple juice phenolics. Schmidt and Neukom (76) described the structure of the main oligomeric apple procyanidin as a dimer of two (-) epi-catechin units, $C_{30}H_{26}O_{12}$, linked together by a C_4-C_8 bond.

Major series of procyanidins are based entirely on epicatechin units, but exceptions arise when considering the minor series of mixed catechin-epicatechin procyanidins or procyanidins of alternative stereochemical configuration. The principal phenolic constituents of apple juice and ciders are phenolic acids, catechins, phloridzin and procyanidins (60,64). However, until recently they have been very difficult to quantify in apple juices or ciders without pre-treatment (60). Lea (75) and Wilson (62) observed that apple juice contains a range of procyanidin polymers probably up to heptameric, based mostly on epicatechin.

Factors Affecting Chemical Composition

Effect of Maturity and Variety :

Sugar contents of fruits of particular species may vary considerably with the variety, soil, and climatic conditions during their life on the plant. Many factors affecting the sugar content in apples have been described (20). The main sugar transported from the leaves to the fruits is sucrose, but other sugars or their derivatives are important for translocation in some plants. A climacteric fruit, apple shows an increasing sucrose concentration up to normal harvest (21). Reducing sugars often increase steadily throughout growth and maturation in apples (21).

Most studies of acid changes in the fruits were confined to measurements of titratable acidity, generally expressed in terms of malic acid (77). The titratable acidity steadily decreases as fruit

matures. However, succinic acid remains substantially constant and citric acid decreases with increasing maturity in the juice of one variety, while in that of the other variety it remains substantially constant (78).

Acidity varies considerably from variety to variety. In a study of the juice from 31 varieties of apples over a period of 3 years, it was found that acidity varies from 0.15 to 0.91% and pH varies between 3.23 and 6.54 (9). In Argentine apple juices (23), the pH of the juice varies from 3.2 to 4.0 while the total acidity ranged between 0.185 and 0.854 % . From 21 canned apple juices, representing the 4 growing areas and different packing seasons in Canada, titratable acidity varies from 375 to 583 mg/100ml (25). The total titratable acidity of New Zealand grown apples is considerably less than those reported for the same varieties grown in most other countries (26). In the table I.1, the average values of titratable acidity from the United States, Canada, New Zealand and Argentine apple juices are in good agreement. Varietal differences may be assumed to be genetic in origin, however, differences within a variety are assumed to be due to environment (78).

The concentration of phenolics decreases as a fruit matures, but usually the amount per fruit increases (61). Mosel and Herrmann (69) observed the changes in the concentrations of catechin and hydrocinnamic acids during the development of apples. During the progressive growth of the fruit of apple varieties, the ratio of epicatechin to catechin increases considerably in favor of the

epicatechin.

Effect of Storage :

A major metabolic process taking place in harvested fruit is respiration. Respiration can be described as the oxidative breakdown of the more complex materials, normally present in cell, such as starch, sugars and organic acids into simpler molecules, such as carbon dioxide and water with the concurrent production of energy and other molecules (80). The largest quantitative change associated with storage is usually the breakdown of carbohydrate polymers, frequently a near total conversion of starch to sugars occurs (80). The total sugar increase is then followed by a gradual decline during the remainder of the life of the fruit (20). The decrease is due to the fact that after all the starch remaining in the fruit has been hydrolyzed, there is a net loss of sugar due to respiration.

Leinbach et al.(81) found that the reducing sugars and sugar:acid ratios increased markedly, during the storage of Golden Delicious apples. These changes occurred at approximately the same rate regardless of grade of fruits. Gorin (82, 83) stored Golden delicious apples in controlled atmosphere and found that the sucrose content decreased , whereas fructose and glucose remained practically constant. Change of sucrose content during the storage of apple juice has been observed (84). Kubo and Tamura (85) investigated the effect of maturity and low temperature storage on soluble carbohydrate content. Sorbitol level was found to increase

during maturation, while storage caused an increase in glucose and a decrease in sucrose and sorbitol. Fructose remained relatively stable. There is discrepancy among the data reported for sorbitol content in apple fruits. From McIntosh apple, Chen et al.(86) reported that the concentration of sorbitol and sucrose increased throughout the storage period. Sorbitol can be translocated (28) and is a respirable material (87). Many authors(85,88,89) indicated that where internal browning had occurred during storage, the affected parts showed increase in sorbitol levels compared to the sound parts of the apple flesh, but a causal connection was not established.

After apples have been harvested there is a decline in total titratable acidity (20). It is presumed that they serve as at least a partial substrate for respiration along with sugar (37,80). Hulme et al.(90) studied the changes in individual acids during storage. Malic acid decreased at a steady rate, quinic acid decreased during the second half of the storage period after increasing during the first half. Citric acid increased slightly over the storage period, and shikimic acid increased also. Kollas (91) reported similar results. The production of citric acid in air- stored fruits may be oxygen dependent. This might account for its higher content in air stored fruits (91). During the controlled atmosphere (CA) storage, the acid content is influenced by the composition of the atmosphere(38). The accumulation of succinic acid in apple in an atmosphere highly enriched with carbon dioxide has been observed (92). This was probably due to the strong suppression of the

succinate dehydrogenase which is responsible for the conversion of succinic acid to fumaric under the influence of excess carbon dioxide (93).

Phenolic acids in fruits have been implicated in causing senescent physiologic changes and alterations in disease resistance during storage (94). Apple proanthocyanidins play an important role in the stability of the juice during storage. Johnson et al. (95) found that under higher storage temperature, the polymeric material can further polymerize to produce an increase in color and sediment formation.

Effect of Processing :

Few studies have focused on the changes of sugar and acid components during the processing of apples. Sharkasi et al. (8) examined the effect of heat on sugar content of apple juice. As a result of heat treatment, reducing sugars increased, and sucrose content decreased. Appreciable changes, however, were not found in total sugars or sorbitol content. Sheu and Wiley (96) reported that the operating temperature and pressure in reverse osmosis have little effect on the sugar content in apple juice.

The conditions of juice preparation create opportunities for oxidative reactions involving polyphenols, thus the types of polyphenols present in finished juices are affected by juice making procedures (97). Different types of phenolic compounds react differently under juice making conditions. The catechin type phenolics and chlorogenic acid serve as good substrates for apple

polyphenol oxidase (72). The catechins oxidize more readily than the cinnamic acid derivatives and have a greater tendency to condense. Chlorogenic acid and related compounds are also readily oxidized, either aerobically in an alkaline pH region, or enzymatically with polyphenol oxidase (98). Van Buren et al. (97) studied the relationship between polyphenols in apple juice and preparation methods. Much of the loss of chlorogenic acid in juice was a result of the holding period associated with enzymatic depectinization of the juice. Johnson et al. (95) studied the changes in the proanthocyanidins and the total polyphenols during the various stages of processing. During milling and pressing, there was a big loss in proanthocyanidins and total polyphenols with no further significant losses taking place during the remaining processing stages. Similar results have been observed during cider processing (99). The effect of various concentrations of gelatin on apple phenolics has been examined (64,99,100). A decline in phenolic levels can be seen with increasing amounts of gelatin, those most greatly affected being the organoleptically most significant tetrameric and polymeric procyanidins, which suffer a loss of some 20% (64,99). Effect of type of gelatin on clarification of apple juice has been studied (100). The greatest reduction in contents of polyphenols was achieved with acid-hydrolysed gelatin with a low bloom number(approx.60).

With juice extraction, a comparision was made between apple juice produced by direct extraction(hot water diffusion process) and that obtained by pressing of fruits; the most siginificant gains

were those of phloridzin and particularly of the polymeric procyanidins from diffusion process(64). This is due to the effect of the increased solubility of phloridzin at the higher temperature . Similar results have been reported by others (99). Haug and Gierschner (101) observed that phloridzin concentration in apple juice produced by an extraction process were approximately double the concentration in juices obtained by pressing. The content of condensable polyphenols in extracted juices is also much higher than in pressed juices, and in one case the content in the extracted juice almost amounts to the threefold quantity to that contained in the respective pressed juice (102). Obviously, extraction methods influence the phenolic content in processed apple juices. Possmann(103) pointed out that the juices produced by direct extraction generally contain a somewhat higher quantity of sugar-free extracts, and a lower content of total acids than pressed juices. But there was no appreciable difference in the contents of different kinds of sugars. Heatherbell et al.(104) compared composition of apple juices clarified by ultrafiltration and by conventional pectinase/gelatine fining, and found them were very similar.

Methods of Analysis

Sugars :

Sugar analysis may be separated into the following categories (34): physical, chemical, colorimetric and enzymatic. Of the different techniques available, enzymatic and chromatographic(physical) procedures are the most commonly used. For many years, most analytical methods for sugar determination were based on the reduction of copper salts in alkaline solutions by reducing sugars (105) but these methods lack specificity.

Gas chromatographic methods have been extensively used in the past years for the rapid, simple analysis of a large number of micro samples (25,106,107). Sugars can be converted into volatile derivatives such as trimethylsilyl ethers (TMS). GLC is specific, gives good separation of the sugars and eliminates much of the time required for colorimetric analysis. In the GLC procedure, generally fructose appears first followed shortly after by the α - and β-glucose with sorbitol appearing between the two glucose peaks. Sucrose has a much longer retention time. The main difficulty in the separation of monosaccharides and di-saccharides by GLC of TMS derivatives was the formation of two or more anomers which result in a multiplicity of peaks (25,106,107). The use of the TMS derivatives of the oximes greatly simplified the interpretation of the GLC chromatograms because the derivative of each sugar produces a single peak (108). The GLC analysis of sugars can also be time

consuming, particularly in the preparation of an aqueous sugar samples for silylation .

High Pressure Liquid Chromatography(HPLC) is now being used for sugar analysis. It retains the specificity of GLC while sample preparation steps and analysis times are greatly reduced (109). Good separation and rapid analysis have been achieved with μ Bondapak/carbohydrate columns (2,110,111) with various ratios of acetonitrile and water. However, the glucose-sorbitol peak is not resolved sufficiently to accurately quantitate either compound. Dual column (34,111) has been used to improved the resolution. Shaw and Wilson (112) found that a radial compression silica column modified with tetraethylene pentamine was the best single column for separation of glucose from sorbitol. Recently ion-exchange column has been used successfully for sugar and sorbitol analysis in apple juice samples(9).

In the sample preparation, mini-column clean up (113), millipore filter (2), cation and anion exchange resins (9) and sep-pak C18 cartridge (34,112) procedures have been used to remove cations , anions and pigments. Baust et al. (114) suggested that the use of hydroxyl (OH) exchange resins as a component in the preparative clean up of samples for HPLC sugar analysis is inappropriate. Li and Schuhmann (115) used the total sugar analyzer (TSA) with an automated flow injection system to determine the sugar content of apple juice. Agreement between TSA and GLC was satisfactory but, sorbitol does not react with the color reagent in the TSA. Enzymatic method (82-84) and Borate complex ion exchange

chromatography with fluorimetric detector (116) have also been used for sugar analysis in apple juice.

Non-Volatile Organic Acids :

A number of chromatographic methods have been developed for determining organic acids in fruit samples. Primary difficulities in the separation and detection of acids have been due to the broad range of polarities of the acids, and the low detector sensitivity for underivatized carboxylates (117).

Earlier works (118-120) indicated the usefulness of anion exchange resins in the separation of a wide variety of acids. In general, the acids leave the column in the order of their increasing pk values. But acids with closely similar pk values cannot be separated quantitatively if one of them is present in relatively large amounts (90). In such cases, the mixed fractions can be resolved and the individual acids determined quantitatively by partition chromatography using silica gel (90,121) or silicic acid column (79). The gradient elution technique(90,122) also has been applied to the investigation of organic acids present in apple juices.

Total acidity or titratable acidity (36) has been used to express the acidity of fruit juices, however, the lack of specificity is an extreme draw back. The official chemical methods for organic acids in foods (123) are simply too time consuming for most purposes and in any case, official methods are available for only a few acids (124). For qualitative techniques, paper

chromatography (125,126) and thin-layer chromatography (127,128) have been developed.

A number of papers (117,120,129,130) have described the use of gas liquid chromatography(GLC) in the analysis of organic acids. GLC determination of organic acids requires the preparation of suitable volatile derivatives. The acids are usually converted into TMS derivatives. The TMS derivatives are prepared by treating the acids with trimethylchlorosilane (129,130) and hexamethyldisilane in pyridine (125,129,130) or bis (trimethylsilyl)acetamide (120). In most cases, it is necessary to concentrate and purify the acids before GLC analysis, and precipitation of non-volatile organic acids as their lead salts is a common method of isolation (120,129,131,132). However, difficulty has been encountered in washing sugars out of the lead salts (25). Although GLC analysis can be used with satisfaction for the determination of organic acids, sample preparation and derivatization remain a demanding requirement for analysis (133).

Liquid chromatographic techniques using reverse phase or anion-exchange columns with organic buffers afford a rapid and sensitive method for analyzing certain mixtures of organic acids in fruit juices (134). Palmer and List (124) used Aminex A-25 anion exchange resin. Turkelson and Richards (135) demonstrated the use of a strong cation exchange resin column. Aminex HPX-87, a strong cation exchange column, has been effectively used in apple juices (6,7). However, temperature elevation (80 $^{\circ}$C) is required to resolve closely eluting pairs with little or no sample preparation

required (6,7,136).

Coppola et al.(137) and Jeuring et al. (133) recently used a reverse phase column at ambient temperature for determining organic acids. Reverse phase C8, C18 and propylamine columns separated acid mixtures in relatively short times (15 min) and generally afforded sharp, symmetrical peaks at ambient temperatures. However, many acids are not completly separated on these columns (117).

Enzymatic(6,7,133) or optical rotation measurement by polarimeter (138) has been used for optically active acids,such as L-malic acid.

Phenolic Acids:

Methods of analysis for phenolic acids have improved considerably over recent years. Total phenol methods(139) have been used for years, however, but do not give information on the individual phenolics.

Paper chromatography, especially two-dimensional systems, has been used for phenolic acids. Many methods, solvent systems and identification methods for paper chromatography have been developed (16,140,141).

HPLC has more recently made it possible to identify and quantify phenolic acids. Methods using reverse phase columns and methanol-acidified water solvent systems have been developed for apple juice and cider (2,142). It was eventually found that better separation was obtained by a modification of a gradient system based on methanol and aqueous 0.1M KH_2PO_4 (62). However, analysis

of juice or cider is not possible without modifying the method (62) because large amounts of phenolic acids are eluted in a non-ionic form at similar retention times to procyanidins (60). A pH shift method (60) has been applied to improve the separation, but the stability of silica based column is affected by pH and ionic strength of the mobile phase, thus it is important to allow sufficient equilibration time for neutral conditions to be restored at the beginning of the next run.

Application and Criteria for Adulteration

Sugars:

The sugar composition of fruits has been helpful in determining the authenticity of fruit juices and concentrates. Wrolstad et al. (17) illustrated sugar analysis as a useful screening mechanism in juice adulteration and compiled sugar patterns characteristic of the individual fruits (27). Sawyer (143) reported the maximum ratio of sucrose to invert sugar and pentose equivalent values (expressed as equivalent xylose content per 100ml of juice) to determine the addition of sugar and low quality of fruit pulp. Fitelson (106) examined authentic as well as the commercial apple juices by quantitative determination of carbohydrates using GLC. A mixture with sugar solutions or foreign fruit juices introduced foreign peaks on GLC; thereby several commercial concentrates were judged to be adulterated on the basis of abnormal sugar pattern.

The fructose/glucose ratio in apple juice has been studied by

Evans(144). Apples usually contain at least twice as much fructose as glucose, but the ratio varies considerably from variety to variety and apples with a high sucrose content tend to have high fructose/glucose ratio. Stepak and Lifshitz (145) suggested that the ratio cannot be used as a criterion for purity in orange and grape fruit juices, because the concentration of glucose found was in all cases of citrus very near that of fructose, but may serve as a useful tool to detect addition of sugar to other fruit juices such as pear.

Niedman (1) reported the adulteration of apple juice concentrates with starch hydrolysate. He found abnormally high contents of sugar free extract, high glucose and presence of maltose from suspect juices. In the preparation of glucose syrup, maltose can be formed during the conversion of starch to a low solids syrup or liquor(146,147). Maltose in apple juice indicates addition of incompletly hydrolyzed glucose syrup. However, Lee et al. (148) reported the presence of maltose in some varieties of apples. Rigorous extraction technique which involves simmering the fruits in boiling 80% ethanol for several hours, was used. It is not clear whether maltose's presence is actually due to a more thorough extraction of carbohydrates or to a generation of artificial maltose by starch hydrolysis in the refluxing extraction. However, in the development of highly purified High Fructose Corn Syrup (HFCS), the starch is almost completly hydrolyzed to glucose (147,149). Amelioration of apple juice with pear juice has been permitted in Switzerland. Blumenthal and Helbling (3) used sorbitol content as

one of the criteria for determinig whether the permitted 10 % level had been exceeded. The average sorbitol content in pear juice was 13.6 g/L, but its highest value in genuine apple juice was 6.3 g/L with a maximum allowable 10 % sorbitol content in apple juice being 5 g/L. Dilution and solids adulteration of apple juice have been studied by Sharkasi et al. (8). They found the sorbitol/sucrose and sorbitol/total sugar ratios in apple juice lower than those in pear juice. Corroboration of the use of these two ratios have been claimed to detect the addition of sugar or pear juice over a 10 % level in apple juices. Sorbitol content can be a useful indication for dilution. However, the considerable variability of sucrose content due to enzymic or chemical hydrolysis, particularly in processed products (17) limits the extensive use of these ratios.

Brause and Raterman (2) chose less than 5 % fructose, and not more than 3.5 % glucose or fructose/glucose ratio of 1.6 minimum and a sucrose maximum of 3.5 % as a standard ranges for pure apple juices. Mattick and Moyer (9) presented the free sugars , sorbitol content and fructose/glucose ratio. The ratio was 3.03 before and 2.12 after inversion in average from 93 samples. They found the percent coefficient variation(% CV) for glucose, fructose and fructose/glucose ratio could be reduced by hydrolyzing all sucrose prior to analysis. From literature search, glucose/fructose ratio was 0.37 (17), the ratio changes but little during storage (144). Normal apple juice samples have glucose/fructose ratios from 0.24 to 0.64 (96). European criteria have revealed a more narrow range ,0.3-0.5 for authentic apple juices (150). There has been concern

over the possible changes of sugar composition in reverse osmosis(RO) concentration of apple juice; Sheu and Wiley (96) observed little change on glucose/fructose ratio and fructose content during the RO concentration of apple juice.

Non-Volatile Organic Acids :

Organic acids have been used as adulterants to give the specific acidity to maintain the proper brix:acid ratio. Wrolstad et al.(17) indicated that the pronounced influence of maturity and variety limits the use of acid profiles in screening fruit juices for authenticity. Acid composition in fruit juices were utilized by many investigators to characterize different juices and to prove the presence of natural unadulterated juice (121,151). Jorysh (122) described the quantitative ratio between major and minor acidic constituents using gradient elution technique to characterize the fruit juices.

The content of citric acid has been used to detect excess of pear juice in apple juice. Blumenthal and Helbling (3) observed that the highest value in apple juice of citric was 0.109 g/L, but average value in pear juice was 1.78 g/L. The standard range of citric acid for normal apple juice was 50-200mg/L in Germany (150) and in Holland (152). Bielig et al.(12) suggested the maximum 35mg/100g as limiting value for apple juice and Evans et al. (6) indicated the maximum 40mg/100g in certain European countries.

Jeuring et al.(133) described the comparision between enzymatic

and HPLC methods for rapid recognition of adulteration. The detection of synthetic L- or DL-malic acid in apple juice through quantitative determination of fumaric acid is described by Junge and Spadinger (7). Malic acid occurs in nature and in pure apple juice only L-configuration (129,153). Synthetic formulations of malic acid, unlike the malic acid found in apple juices, are likely to produce mixtures of both L- and D-malic acid. While it is possible to manufacture nearly pure L-malic acid, but L-malic acid is quite expensive, adjusting it to the normal range in an formulated juice would not be economically practical (7). Presence of fumaric acid may suggest adulteration with malic acid as synthetic malic acid often contains substantial quantities of fumaric acid: 0.4-1 % (7). Junge and Spadinger(7) suggested a concentration above 3mg fumaric acid/L as a evidence of added malic acid. Fumaric acid can also be formed from natural malic acid when fruit juice is subjected to heat as in the manufacture of concentrates (6). Evans et al. (6) found as much as 10mg/L of fumaric acid produced by extreme conditions during juice concentration. Also the difference between the total malic acid obtained by HPLC and the L-malic acid obtained by enzymatic represents D-malic acid. The amount of D-malic acid found by this method represent the synthetic malic acid in juice. Junge and Spadinger (7) and Evans et al.(6) reported that in natural apple juice, the difference does not appear at levels greater than 0.3g/L. Some enantiomeric D- and L- acids have been readily resolved by converting them to diastereo isomeric esters (154), but the applicability of this method to routine assay is hindered by

enantiomeric contamination of the derivatizing agent (155). Recent advances in the resolution of stereoisomers by HPLC on chiral stationary phase (HPLC-CSP) (156) can be a promising visual tools for detecting the synthetic DL-malic acid in apple juice in future.

Phenolic Acids:

There is interest in using total phenolic level as an adulteration indicator. Absorbance at 325nm in alcoholic solution was used to characterize the apple juice (16). Brause and Raterman(2) reported the total phenolic, chlorogenic and caffeic acid from 32 apple juice samples. They considered 0.150 at A325nm to be a minimum for authentic apple juice. Chlorogenic acid varied from 93 to 232µg/g for self-produced apple juices and from 0 to 208µg/g for commercial apple juices. Caffeic acid also showed large variation between samples. They stated the use of gelatin can cause the product to appear abnormal, but on the contrary no significant effect has been indicated by other investigators (64,99). As the majority of apple phenolics are low molecular weight compounds such as chlorogenic acid, catechin and epicatechin, these compounds are probably not affected by gelatin fining, but if oxidized and polymerized to larger molecules to form complexes with proteins, pectins and starch, they can be removed by gelatin fining.

Apple juices normally contain a minimal amount of hydroxymethylfurfural (HMF): 0 to 11mg/L in Danish market (157). HMF is a dehydration product formed when sugars are heated and is a constituent of caramel, a common food coloring agent (158). HMF

presence is easily detected by UV (158) and HPLC methods (160,161). Alfonso et al.(161) indicated that HMF was the most sensitive indicator of the presence of caramel in a sample. Several commercial caramel preparations ranged between 14000 and 18000 ppm. The ratio of chlorogenic acid to HMF could be useful in the case of dilution and fraud, because a coloring agent like caramel should be added to maintain the color specifications. But use of synthetic chlorogenic acid is not expected because of its high cost (2). Bielig et al. (150) stated that the content of HMF is not a criterion by which the adulteration of a juice can be judged. The concentration increases during the course of proper filling and subsequent storage to twice that of concentration(162); also HMF not being stable, readily undergoes ring scission (163).

Spectral Analysis :

Ultraviolet(UV) absorption spectroscopy is frequently used as a tool in aiding to identify certain organic compounds or chromophoric groups. The UV absorption from apples,Italian prunes, peaches and cherries show two absorption maxima 280nm and 322-324nm(15). Johnson et al. (15) indicated that there are two types of substances present which possess definite chromophoric differences. They observed that peach curves are very similar to the apple curves, but the UV absorption curves of cherry and Italian prunes are distinctly different from the apples. Vandercook and Rolle (16) reported that the ratio of the absorbance of 273-277nm to 326-332nm is essentially constant in apple juice.

Derivative spectrophotometry is a relatively new technique that offers enhancement of qualitative features and therefore greatly increases the finger printing utility of UV spectrophotometry(164,165). It has been used to test for identity and purity of dye (165), drugs (166,167), amino acids (168) and trace anaysis (169). Talsky et al. (170) introduced the 4th derivative spectrum to find out the difference between two types of beer. It was actually possible to identify the products from several different suppliers. This technique should be useful in screening fruit juices but it has not been reported to date.

Amino Acids:

The formol value, as a reflection of amino acid content, provides a quick check for simple dilution and in tandem with measurements of proline and ammonia, can help detect sophisticated dilution(171). In the German RSK-values(150), 15mg/L have been laid down as a maximum formol number whereas Brause and Raterman(2) claimed 3.75meq/L for normal and 2.50meq/L for minimum value of authenticity. Apple juices have a low proline content, normally lower than 15mg/L(150). Higher values are an indication that foreign substances have been added(172). Moyer and Mattick(9) determined proline content from apple juices. The content varies between 1.27 and 13.8ppm with mean of 5.47ppm. Blumenthal and Helbling(3) used the proline content to distinguish the pear juice solids in apple juices. The highest value found in apple juices was 4.4mg/L compared to a mean of 228mg/L in pear juices. It is

obvious that using these ratios, the control of falsification is much more powerful. Burroughs (173) examined the free amino acids of apple juices by paper chromatography. Asparagine, aspartic and glutamic acids were the principal amino acids. Fernandez et al.(174) found that apple contains only a minimal amount of amino acids, while figs, nectarines and cantaloupes have significantly greater amounts. The natural apple juice contains 4-hydroxy methyl proline and Rossetti et al. (175) suggested that this amino acid could be used as index of genuine apple juice. Ooghe and Waele(13) developed analytically and statistcally valid standards for the free amino acids content of commercial German apple juices. The addition of other juices can be detected on the basis of discrepancies from those patterns. A statistical X^2-and F-test were devised, based on % of a maximum of 8 amino acids. Bielig and Hofsommer(12) described that amino acids cannot be used for evaluation of apple juice, as their wide variations in concentration.

Minerals :

Smock and Neubert (20) reported 13 minerals from apple. The exact form in which these minerals exist in the flesh of the apple is not known. K, the main mineral in apple juice, ranged from 685 to 1510 ppm (176). Brause and Raterman (2) suggested 90-110mg of K per 100ml apple juice as a normal range. Mineral profiles of apple juice are dependent on many variables (20).

Apple contains small amounts of ash compared to other fruits (177).

If the ash content is lower than 1.9g/L, one must assume that the juice has been adulterated with water or diluted more than is permitted in Germany(150). The content of Ca is rarely higher than 80g/L and clearly increases if inappropriate water is used (150).

Generally vegetable products and fruit juices contain only small quantities of Na and higher K content , but corn syrup showed inversely higher Na and lower K content (178). The Na/K ratio of juices is an important factor in connection with the evaluation of juices to decide whether they are pure or adulterated with HFCS. Since HFCS is refined by ion exchange treatment, original cations present in HFCS are replaced by Na. The Na content of apple juice vary widely, the concentration is mainly dependent on the growing area (10). Apple juices from German northern coastal regions contained 26-40mg/L, but those from the inland area contained 3-8mg/L. It clearly shows that the Na contents of apple are closely connected with the distances of the respective acreages to the sea(11). The juices produced from Hungarian apples, which can not be influenced by the sea show a low Na content of 10mg/L. Extreme values of more than 150mg/L were found in coastal areas of Italy. Often high contents of Na are attributed to the fact that certain fining agents (179) and enzyme preparations (180) separate out Na.

Soluble Solids and Acidity :

Federal regulation (19) specifies that apple juice shall not contain less than 10.5 % m/m soluble apple juice solids, determined by refractometer at 20°C, uncorrected for acidity and read as Brix

on the international sucrose scale. Degree Brix is a term used to designate the percent by weight of dissolved sugar in a solution. It is used to indicate the percent of soluble solids contained in the juices. Soluble solids may be determined with a hydrometer (181), refractometer (182) or pycnometer (181). Detailed procedures and correction tables have been described by McAllister(183). Soluble solids in apple juices may be determined by a refractometer as given in the AOAC (184).

Titratable acidity can be defined as the amount of acid that is neutralized by a base to the pH of the phenolphthalein end point(185). The acid content in apple juice determined as total titratable acidity is calculated as anhydrous malic acid and expressed as percent by weight or grams per 100mL of juice (186). The finished apple juice should contain 0.2-0.8 gram titratable acid per 100mL as malic acid (19).

Mattick and Moyer (9) obtained following values as a mean from 93 samples: pH: 3.69, total acidity: 0.417 (as % malic) and Brix :12.74. But degrees Brix is of little value for detecting dilution because of the ease of adding sugar products to disguise the adulteration (187). Brix:acid ratio has been used as an index of maturity, it varies widely among the kinds of fruits and various climates throughout the world (24). This ratio ranged from 7.9 to 22.5 in 18 apple juice samples (188).

Standard Values and Ranges :

The federal German guidelines and tolerances (RSK-values) for

sensory and chemical characteristics and specific reference values for apple juice were reported in 1977 (189). These values were revised and adjusted to the latest scientific state in 1982 by a group of experts from science and the fruit juice industry (150). These criteria have been laid down on the basis of many years of experience regarding commercial fruit juices, manufactured on an industrial scale (150). Table I.2 lists the RSK values for apple juice. Sensory evalution includes color, appearance, odor and taste tests. Chemical analysis includes relative density, Brix, pH, titratable acidity, sugars, minerals, malic acid, citric acid, proline and formol index. As an indication of rotten fruit or microbial changes during production and storage of the juice, ethanol and biogenic acid content are also included.

TABLE I.2 RSK VALUES FOR APPLE JUICE

		STANOARO VALUE	RANGE FROM	RANGE TO	MEAN VALUE
SENSORY ANALYSIS:					
COLOR ANO APPEARANCE	(point)	MIN. 3	2	4	3
OOOR	(point)	MIN. 3	3	6	4
TASTE	(point)	MIN. 5	5	10	7
CHEMICAL ANALYSIS:					
RELATIVE DENSITY 20/20 C		MIN. 1.0450	1.0450	1.0570	1.0488
BRIX,CORRECTED		MIN. 11.18	11.18	14.01	12.08
TOTAL SOLUBLE SOLID	(g/L)	MIN. 116.8	116.8	148.1	126.7
TITRATABLE ACIDITY (pH 7.0)					
AS TARTARIC ACIO	(g/L)	MIN. 5.0	5.0	8.5	6.5
AS meq	(g/L)	MIN. 66.7	66.7	113.3	86.7
ETHANOL	(g/L)	MAX. 3.0	-	-	-
VOLATILE ACIOS					
AS ACETIC ACID	(g/L)	MAX. 0.4	-	-	-
LATIC ACIO	(g/L)	MAX. 0.5	-	-	-
TOTAL SULFURIC ACID	(g/L)	MAX. 10.0	-	-	-
GLUCOSE	(g/L)	-	18	35	26
FRUCTOSE	(g/L)	-	55	80	65
GLUCOSE/FRUCTOSE		MAX. 0.5	0.3	0.5	0.4
SUCROSE	(g/L)	-	5.0	30.0	15.0
REDUCTION FREE					
EXTRACTS	(g/L)	MIN. 18	18	29	22
ASH	(g/L)	MIN. 2.1	1.9	3.5	2.55
ALKALINITY INOEX		MIN. 11	11	14	13
Na	(mg/L)	MAX. 30	-	-	-
K	(mg/L)	MIN. 1000	900	1500	1200
Ca	(mg/L)	-	30	120	59
Mg	(mg/L)	-	40	70	52
Cl	(mg/L)	MAX. 50	-	-	-
NO3	(mg/L)	MAX. 10	-	-	-
PO4	(mg/L)	MIN. 150	130	300	220
SO4	(mg/L)	MAX. 150	-	-	-
CITRIC	(mg/L)	-	50	200	100
TOTAL MALIC	(g/L)	-	-	-	-
PROLINE	(mg/L)	MAX. 15	-	-	8
FORMOL INOEX		-	2.5	10	4.5
O-SORBITOL	(g/L)	MIN. 2.5	2	7	4

The ranges show possible variations of the chemical composition of typical components of apple juice and the mean value is not identical with the arithmetic value but is the value around which most of the values of industrially manufactured apple juices are accumulating. Deviation of these values must be regared as a sign of illegal additives or treatments (150). But RSK values must not be considered as absolute . Hofsommer et al.(190) pointed out that RSK values must not be slavishly adhered to , but are valid as an aid in juice evaluation and detection of adulteration. It is less important that all, or specfic individual values, are complied with but rather the whole result of the analysis has to be judged (191). Koch (192) suggested that it might be necessary to include the tartaric acid or isocitric acid to be able to assume that grape juice or lemon juice have been added.

Difficulties in evaluating the apple juice content in apple beverage by only according to the RSK values have been described (192,193). Since the RSK values are normally applied to the respective concentrates (190), reduction-free extract, glucose/fructose ratio, sorbitol content, formol index as well as the amino acid spectrum must appear to be too low when they are judged only according to the RSK-values. Recently Dukel (152) published the list of criteria for industrially prepared apple juices in Holland. These two criteria do not include a single absolute amino acid values. Ooghe (194) pointed out that amino acid values have to be introduced to give more reliabilty to the criteria for industrially prepared apple juice.

Data Evaluation :

Evaluation of juice purity has been tested by many statistical methods: Multiple regression of several constituents (195), chi-square test (196), and non-parametric methods (197). Multivariate normal test was applied to develop an equation for the detection of apple juice adulteration with sugar solution or pear juice (8). Means, standard deviations, correlation coefficient, and covariance matrix have been calculated.

Brown and Cohen (198) described that all proposed methods suffer from the same problem: they are applicable only to samples obtained under conditions similar to those intially taken. Brown et al. (199) indicated that pure juices can mistakenly be rejected as adulterated, or adulterated juices can be accepted as pure, if the effects of differences among countries, species and climatic factors and differences from season to season in the same species are not considered.

For the multicomponent analysis, pattern recognition methods are being suggested (200). These methods offer a means of finding relationships not only between a measurement and sample composition, but also relationships among multiple measurements and sample functions and/or origins. Pattern recognition is the study of data sets in order to find regularities and similarities inherent in the data (201). These techniques have been applied and proven successful in a wide variety of chemical problems. Classification of geographic origin of wines (202-204) and orange juices (205,206)

by their chemical compositions has been done. The data from atomic absorption, GLC, Mass, HPLC, C-12NMR,IR have been used in this technique. General review of the theory and application of pattern recognition has been published by Kowalski(207).

Stable Isotope Ratio Analysis

Carbon Isotopes :

The Biologically important elements in living tissues are H, C, O, N & S. Each of these elements have more than one stable isotopes. Carbon occurs in three isotopic forms; ^{12}C, ^{13}C and ^{14}C. The first two isotopes are stable, whereas ^{14}C disintegrates radioactively over time. The three react chemically in the same way, but because their atoms have different atomic weights and are of different sizes, they react at different rates. Thus different chemical and metabolic processes change the ratios between the isotopes in characteristic ways. The natural abundances of the two stable isotopes of carbon are ^{13}C=1.108% and ^{12}C=98.892%(208), thus atmospheric carbon dioxide contains about 1.108% of the heavier carbon isotope ^{13}C and 98.892% of the lighter isotopes ^{12}C.

Fractionation of Carbon Isotopes:

Isotopic fractionation, change in isotopic ratios between materials due to the different rates at which various isotopes

undergo chemical reactions is a well established phenomenon(209,210). It is an observable effect generally described in terms of enrichment or depletion of the heavy isotopes(209). The first measurement was made by O'Nier and Gulbransen(211), who compared $^{13}C/^{12}C$ ratios of Boston clamshells, pine, and the air over Cambridge. Various aspects of $^{13}C/^{12}C$ ratio variations in natural materials have been considered by Craig(212), using a precise mass spectrometer. Isotopic variations between these reservoirs are a consequence of the differences in chemical and physical properties of different isotopic species of a molecule(213).

Carbon Isotopes and Photosynthesis:

Carbon isotopes are strongly fractionated during photosynthesis. When plants metabolize carbon dioxide there is preferential utilization of ^{12}C and exculsion of ^{13}C(214). Park and Epstein(214) proposed a model for photosynthetic fractionation of carbon. The four major fractionation sites are indicated in figure I.1 by numbers. The first fractionation step in favor of ^{12}C is a kinetic effect which occurred during uptake of carbon dioxide from atmosphere into the leaf cytoplasm.

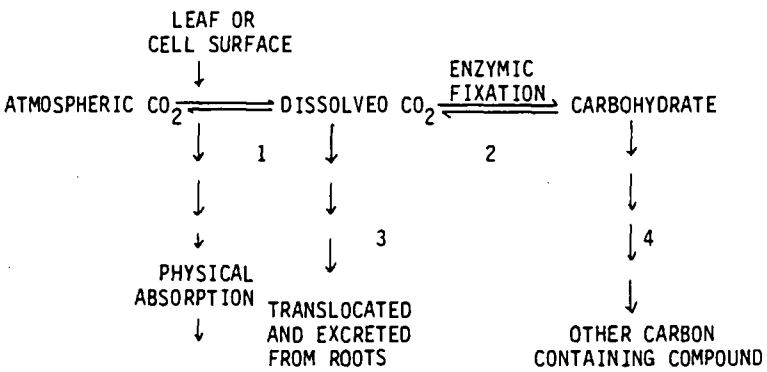

FIGURE I.1 MODEL FOR FRACTIONATION OF CARBON ISOTOPES IN PHOTOSYNTHESIS.

After passing through the membrane, the dissolved carbon dioxide is partitioned into carbohydrate(step 2) and into removal of some of the dissolved carbon dioxide through the vascular system resulting in excretion through roots(step 3). Further fractionation may take place during metabolic processes(step 4) resulting in different del ^{13}C values for different chemical fractions within a plant(215). All four steps affect the final fractionation that is associated with the fixation of carbon dioxide by plants. The relative rates and efficiency of these various steps determine the isotopic composition of the plant. In principle, this allows for plants to have the range of del ^{13}C values from -1 to -38 ppt(216). Execpt for blue-green algae which utilized methane (217)as the carbon source, all plant values in the literature fall within this range.

Three types of photosynthesis occur in the plant world, commonly referred to as the C_3, C_4 and Crassulacean acid metabolism(CAM) pathyways. Plants with the C_3,C_4 and CAM photosynthetic pathyways show characteristically different discriminations against ^{13}C during photosynthesis(218). C_3 photosynthesis, discovered in experiments with algae, spinach and barley(219), convert carbon dioxide from air to a phosphoglycerate compound with three carbon atoms during the first step of photosynthesis(220). A different photosynthetic pathyways was identified in Hawaiian sugarcane, with conversion of carbon dioxide to dicarboxylic acid, a four carbon compound(221). Hence, designations C_3 and C_4 respectively are used to distinguish

between plants utilizing the two different pathyways.

With further reseach, yet another photosynthetic pathyway was discovered, CAM(222). The CAM pathyway, found in succulents such as the cactus, has little bearing on this discussion. An examination of characteristics distinguishing three groups of higher plants are well tabulated (223). C_4 plants with Kranz leaf anatomy(224) and rapid carbon dioxide uptake(225), exhibit del ^{13}C values near a mean of -12.5 ppt. In contrast, plants with C_3 photosynthesis have slow rates of carbon dioxde uptake and average about -26.5 ppt in del ^{13}C values(226). The difference in isotopic composition has become one of standard methods by which C_4 plants can be dintinguished from C_3 plants and, a great variety of plant types has been studied (227,228).

Carbon Isotopes and Environment:

The largest factor affecting carbon isotopic compositions is the existence of C_3,C_4 and CAM photosynthetic options. Craig(212) suggested that environmental effects may account for some of the isotopic fractionation observed in organisms. Under different environmental conditions the del ^{13}C values of C_3 plants may vary from -20ppt to -35ppt, whereas C_4 plants may vary from -9ppt to -16ppt(228). Figure I.2 shows the normal distribution for a sample of 351 south african grass species that includes both C_3 and C_4 type. The two ranges do not overlap, a fortunate circumstance which make possible a number of applications(229). O'Leary(210) reviewed other environmental variables that can

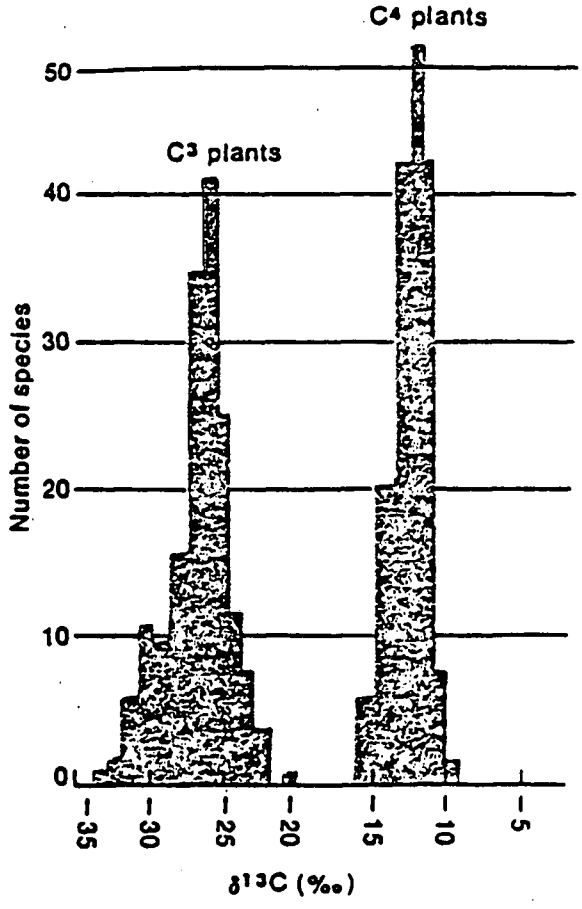

FIGURE I.2 THE DEL ^{13}C VALUES OF 351 SPECIES OF GRASSES ILLUSTRATING THE DISTINCTION BETWEEN C_3 AND C_4 PLANTS.

affect del ^{13}C values; temperature, fertilization, carbon dioxide concentration and light intensity,etc. Some special circumstances can be observed in the dense forest (230). For example, where rotting leaf litter on the ground releases large amounts of carbon dioxide that is isotopically light, while the forest canopy traps the air and prevents it from mixing effectively with free atmosphere. The result is a gradient in the isotope values from the forest floor to the open air high above it, with a corresponding gradient in the plant foilage(231).

However, in a less well understood phenomenon, different plant species with the same photosynthetic pathway may have different del ^{13}C values,though they grow side by side(229). Plants of the same species growing in different environments have slightly different del ^{13}C values as well(229). These variations are more marked in C_3 plants which exhibit wider range of isotopic values than C_4 plants(226,232). C_3 plants grow in a much wider range of habitats, and this may prove that the fractionation factor varies with photorespiration, which varies with sunlight(233,234).

$^{13}C/^{12}C$ Ratios in Metabolites:

Isotopic discrimination is a common feature of biosynthesis in nature. Closer scrutiny indicates that small isotopic differences between plant organs do exist(235-237). A number of investigators have studied the isotopic compositions of various subfractions of the plant. These studies span anatomical variations(214,215),

metabolic fractions(238,239), individual compounds(239,240) and individual carbon atoms(241-243).

Different classes of substrates, amino acids and lipids vary from one another in del ^{13}C values as a result of isotopic fractionation during biosynthesis(238). Such fractionation is indicated by the markedly larger $^{13}C/^{12}C$ ratio of atmospheric carbon dioxide than the total plant carbon(212). To a lesser degree, carbon isotope fractionation occurs in the conversion of carbohydrate to lipid(215) and during the synthesis of cell wall material(215) and amino acids(240). Altogether, isotopic fractionation during the biosynthesis result in different $^{13}C/^{12}C$ ratios in the various classes of metabolites(239). For example, table I.3 compares isotopic values for various fractions of potato tuber tissue(238).

Park and Epstein(215) found a sizeable difference in del ^{13}C values between plant lipids and whole plant carbon for a number of plants. Small differences were also noted between sugars, cellulose and lignin fractions. However, the situation is even more complicated from this,isotopic compositions are far from uniform even for all of the carbons of a particular amino acids(240). On the other hand, Parker(236) found that the different fatty acids of a given organism have identical isotopic compositions as would be expected if they arose through the same synthetic pathway.

Comparison of the major plant constituents in cotton and sorghum has been made by Whelan et al.(239). All

TABLE I.3 CARBON ISOTOPE ABUNDANCE FOR CHEMICAL FRACTIONS OF POTATO TUBER.

	PPT
POTATO TUBER TISSUE	-25.8
SUGARS	-23.8
STARCH	-25.5
PROTEIN	-26.6
ORGANIC ACIDS	-26.9
AMINO ACIDS	-27.1
LIPID	-34.6

fractions(lipids,carotenoids,proteins,amino acids,organic acids, and sugars) of sorghum differ substantially from all fractions of cotton. In both plants lipids and carotenoids are enriched by about 5% in ^{12}C with respect to the total carbon of the whole leaf. Monson and Hayes(241,242) recently reported that intramolecular isotopic fractionation of carbon has been correlated directly with fatty acid biosynthetic pathway. Definition of metabolic pathways and mechanisms can be provided by determination of intramolecular carbon isotopic distributions(243).

Measurement of $^{13}C/^{12}C$ Ratio:

To specify the small differences in isotopic composition between carbon bearing materials, the $^{13}C/^{12}C$ ratio of a given sample is compared in a mass spectrometer with the ratio of an agreed standard. The difference between the sample and the standard is known as relative ^{13}C content, designated by "del" ,and is measured in parts per thousand or per mil(ppt)(244). The function defining the values reported is; (245)

$$\text{Del (ppt)} = \frac{(^{13}C/^{12}C)_{\text{sample}} - (^{13}C/^{12}C)_{\text{standard}}}{(^{13}C/^{12}C)_{\text{standard}}} \times 1000$$

Where $^{13}C/^{12}C$ is the ratio of the abundances of the ions of

the mass 45 ($^{13}C^{16}O_2$) and mass 44 ($^{12}C^{16}O_2$) (212).

From this expression it can be seen that if there has been no change in the $^{13}C/^{12}C$ ratio during photosythetic carbon fixation, the del ^{13}C value will equal zero. If however, there has been discrimination against $^{13}CO_2$ the del ^{13}C value will be a negative number. Thus if a sample of carbon dioxide proves to have a $^{13}C/^{12}C$ ratio which is less than that of the standard by 10 per mil,or 1%, it is said to have a del ^{13}C value of -10ppt(238).

The commonly agreed standard reference for del ^{13}C measurement, known as the Chicago PDB marine carbonate standard, is derived from a piece of Cretaceous marine fossil, Belemitella Americana, from the Peede formation in South Carolina(212,244). But current measurements are secondary standards prepared by the national bureau of standards(4). The isotopic ratio for PDB is $^{13}C/^{12}C$ =0.01124(214), but is assigned to zero; organic matter is invariably depleted in ^{13}C compared to PDB, so del ^{13}C values of organic materials are negative (229).

Applications in Apple Juice Adulteration:

There has been considerable observations(246-248) in detecting the fraudulent addition of C_4 plant derived high fructose corn syrup (HFCS) to C_3 derived materials. Bricout and Koziet (249) considered isotopic analysis as a new way of characterizing synthetic substances. Its application to food adulteration has been

reviewed by Doner(250). In apple juices, Doner et al. (5) measured the variability of the del ^{13}C value in 40 pure apple juices and provided the base line data to detect the undeclared addition of cane or corn sugars to apple juice. No significant variations in del ^{13}C values with regard to variety or geographic origin of the apple was found. Donor and Phillips (4) suggested that the samples with del ^{13}C values less negative than -20.2 ppt, 4 standard deviations from the mean of pure juices, can be classified as adulterated with high probability. Pure apple juices possess del ^{13}C values over a range -22.5 to -27.9 ppt. A'brams(251) reported similar ranges, from -22.5 to -27.2 ppt, for 93 samples. Coppola (252) described slightly more negative value, -22.1 ppt to account for the precision of the method and the natural variability of apple juice. Brause and Raterman (2)determined the del ^{13}C values from some commercial apple juices and the value between -23.4 and -24.7 ppt, fall within the range reported for authentic apple juice (4). They chose -22.0 ppt, slightly more negative than the previous value (4), as a limit value for pure apple juice and described that del ^{13}C profile would be abnormal to the addition of HFCS and cane sugar.

If the added sugar exceeds 10 % level, the result are quite definitive(253) but this method cannot detect the minor adulteration of products that may contain below 30 % added sweeteners. Parker (245) demonstrated the detection of low level(5%) of HFCS in suspected orange juice by measuring the del ^{13}C values from whole juice, pulp, and soluble fraction. Since

HFCS will reside mostly in the soluble fraction, differences between del ^{13}C value in soluble fraction and the pulp fraction can be sensitive indication of adulteration. Presumably this technique could be applied to natural apple juice with suspended solids. Another draw back is that, beet sugar and beet sugar invert can not be distinguished from the apple juice in the del ^{13}C value. Since sugar beet operates the same C_3 photosynthetic system as apple juice.

Bricout and Merlivat (254,255) described $^{18}O/^{16}O$ and $^{2}H/^{1}H$ ratios for differentiating the juice drinks prepared from juice and those prepared from diluted concentrates. ^{2}H and ^{18}O concentration showed that metabolic water in apple juice was richer in the heavy isotopes than rain water used to reconstitute juice. While the isotope composition of reconstituted juice resembled that of the water used for dilution(256,257). Measurement of two isotopes together rather than ^{2}H only yields more information (258). This method enables differentiation of apple drinks prepared from juices and those prepared from diluted concentrates.

TITLE: APPLE JUICE COMPOSITION: SUGAR, ORGANIC ACID, PHENOLIC ACID, AND UV-SPECTRAL PROFILES.

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RUNNING HEAD: Apple Juice Composition

Technical Paper No._____ from the Oregon Agricultural Experiment Station.

APPLE JUICE COMPOSITION: SUGAR ORGANIC ACID, PHENOLIC ACID, AND UV-SPECTRAL PROFILES.

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ABSTRACT

Eight apple samples which included Golden Delicious, Jonathan, Granny Smith and McIntosh varieties and the following geographic origins-Argentina, Mexico, New Zealand, Michigan and Washington were examined. Juices were prepared in the pilot plant and the following determinations made: yield, pH, brix, total acidity and total sugars. High Performance Liquid Chromatography (HPLC) was used for glucose, fructose, sucrose, sorbitol, quinic, malic, citric, shikimic, fumaric acid, hydroxymethylfurfural(HMF) and chlorogenic acid determinations. L-malic acid was measured by enzymic procedure.

While sugar and sorbitol within range of previously reported studies, evidence for reduced sucrose and sorbitol content as result of postharvest storage. Glucose/fructose ratios were almost identical with those previously reported. The HPLC procedure afforded good resolution between malic and quinic acid; quinic acid was second to malic in concentration. The acid profile shows considerable variation; citric acid levels appeared to be influenced

by postharvest storage. Considerable variability in HMF and chlorogenic acid content was also observed. No discernible trend in chemical compositions of apple juices was found between foreign and United States grown apples. Spectrophotometry was applied to characterize each variety using polyclar AT, normal, derivative and fluorescence spectra. Enhancement of spectral shape in second derivative spectra was obtained.

INTRODUCTION

Mattick and Moyer(1) extensively studied the chemical composition of 31 different apple samples representing the principle varieties and major growing regions of the United States over 3 successive years. Ash, brix, pH, proline, specific gravity, total acidity, glucose, fructose, sucrose, sorbitol, and glucose/fructose ratio before and after inversion and minerals were included in their analyses. There are some reports in the literature on composition of apples from different countries, e.g. Argentina (2), Canada (3) and New Zealand(4), but only a limited number of properties were reported.

According to Smock and Neubert(3), apple varieties differ from state to state, the chemical composition among varieties varying over wide ranges and influenced by various factors. Most apples grown in the United States are sold fresh,whole and unprocessed (6); therfore, a considerable amount of apple juice consumed in the United States is manufactured from imported concentrates. Brause and Raterman(7) used a series of instrumental analyses for verification of apple juice authenticity. They proposed sugar profiles, K, chlorogenic acid and del ^{13}C values as a matrix of compositional standards for pure apple juice.

There have been several investigations to verify the authenticity of apple juice products on the basis of sugar analyses(8-10), acid analyses(9,11,12) amino acids (9,13,14),

microbiological assay(15) and sodium content (16,17). Some European countries,such as West Germany(18) and Holland(19) proposed standard values and ranges (RSK-values) as specific reference numbers for apple juice. Hofsommer and Bielig (20) noted that these values must not be slavishly adhered to but are valid as an aid in evaluation of juice. Unfortunately each criterion and method has its limitations to solve the highly sophisticated adulteration problem.

There is a need for combined powerful testing methodology and standardization of analytical techniques for the detection of adulteration. Coppola (21) recently demonstrated the usefulness of HPLC in the detection of apple and cranberry juice adulteration. The purpose of this study was to develop and improve the analytical procedures for determining the authenticity of apple juice and to extend the compositional data bank to include juices of foreign origin.

METHODS

MATERIALS:

Apples were obtained from growers, processors or by local purchase. The geographic origin, variety and history of samples used in this work are summarized in table II.1. Commercial pear, grape and pineapple concentrates were obtained from local processors.

PREPARATION OF APPLE JUICE:

Wash apples throughly and sort before processing. Crush 20 Lb of apples using hammer mill (The W.J. Fitzpatrick, Co., model D, Chicago,Ill.) Place the macerated fruit in canvas sieve cloths, evenly spread in 2 to 3" layers. Fold the corner of cloth over the pulp; alternately stack envelopes of fruit and wooden racks. Place in a hydraulic press and press according to the following program: 200 psi to 1500 psi for 30 min. Clarify the crude extracts by pectic enzyme treatment (Pectinol 60G, Rohm & Haas, Co. Philadelphia, PA) 7.5 mL/L, 50 ^{o}C x 2 h. Filter using filter pump(FMI Model RP, Oyster Bay, NY) containing a filter pad (AMF, cat.No. B0303-50D, Meriden,Conn.) Precoat the filter pad with about 1 cm thickness of filter aid, Hyflo super-cel(Fisher Scitific Co., Pittsburgh, PA). Collect 6.0 L of clarified juice in plastic bottle and store -20 ^{o}F without further heat treatment(Figure II.1)

APPARATUS:

(a) Liquid Chromatography-Varian 5000 series liquid chromatograph (Varian Aerograph, Walnut creek,CA) equipped with column heater. For the sugars; Varian Aerograph refractive index (RI) detector, 7.8 x 300 mm; particle size 9 µm, Aminex HPX-87 carbohydrate column (Bio-Rad Laboratories, Richmond,CA); 3.6 x 40 mm cation (BrownLee Lab. Santa Clara, CA) microguard column; Column temperature 87 ^{o}C. Filter mobile phase through 0.45 µm millipore filter, degas by use of magnetic stirrer, injector loop volume 10 µL.

For the organic acids; Varian variable wavelength detector mode UV-50 at 226 nm, 4.0 x 300 mm MCH-10 Micro Pak column (Varian Aerograph), 3.6 x 40 mm RP-18(BrownLee Lab.) microguard column. Column temperature 25 ^{o}C. Mobile phase 2.0 % KH_2PO_4 and 2.0 % NaCl in deionized water, adjust to pH 2.4 with phosphoric acid. Filter through 0.45 µm millipore filter; degas by use of magnetic stirrer. Flow rate, 0.5 mL/min; injector loop volume, 25 µL.

For phenolics; Varian variable wavelength detector mode UV-50 at 324 nm, 4.0 x 300 mm MCH-10 Micro Pak column (Varian Aerograph), 3.6 x 40 mm RP-18 (BrownLee Lab.) micro guard column, column temperature 25 ^{o}C. Filter mobile phase methanol and 0.1 M KH_2PO_4 through 0.45 µm millipore filter. Run linear gradient (2 %/min) from 10 % methanol to 40 % methanol in 15 min at 2 mL/min, maintain isocratically for 5 min , return the solution to original composition in 10 min; injector loop volume, 25 µL.

(b). Integrator:

Operate Hewlett Packard model 3380A recording integrator at 0.5 cm/min, X 1 attenuation and 0.1 mv full scale.

(c). UV-Visible Spectrophotometer:

Perkin Elmer model 550 UV-Visible double beam Spectrophotometer. Varian DMS 80 UV-Visible Spectrophotometer and Varian model 9170 recorder, Intel 8085 A microcomputer. 10 mm quartz cell, chart speed 4.5 cm/min for normal and 2.5 cm/min for derivative mode.

(d). Spectrofluorometer:

Aminco Bowman Spectrophotofluorometer (SPF) model SPF-125, high pressure xenon arc source lamp, IP 21 photomultiplier detector. Set slit widths 1 mm, 1mm, percent full range scale 3.

REAGENTS:

(a). Solvents-methanol, LC and spectral grade (J.T.Baker, chem., Phillipsburg, NJ). Filter through 0.45 µm durapore filter (HV type, Millipore Lab. Bedford,MA) before use.

(b). HPLC standard sugar solution-1 % of each sugar. Add 1 g of glucose, fructose, sucrose (sigma chemical,Co.), sorbitol (Fisher Scientific,Co.)and Mannitol(Mallinckrodt chemical,Co.) to 100 mL volumetric flask and dilute to volume with deionized water.

(c). HPLC standard organic acid solution-0.1 % of each acid. Add 0.1 g of quinic(ICN Pharmaceutical,Inc.) malic, fumaric(sigma chemical,Co.), citric(Allied chemical,Co), shikimic

(Calbiochemical,Co.) and internal standard, isoleucine (Nutritional Biochemical,Co.) to 100 mL volumetric flask and dilute to volume with deionized water.

(d). Phenolic standard solution for HPLC, TLC and UV-Spectrophotometry-0.1 % of each phenolic acid. Add 0.1 g of chlorogenic, ferulic, phloritin, phloridzin, kaempherol, quercetin, epicatechin and hydroxymethylfurfural (Sigma chemical,Co.), caffeic, rutin and catechin (ICN Pharmaceuticals,Inc.) to 100 mL volumetric flask and dilute to volume with spectral grade methanol.

Sugars, acids and phenolics wre stored under vacuum over P_2O_5 at least 24 h before weighing.

(e). TLC solvent system:

n-butanol-acetic acid-water (BAW, 12/3/5) and 5 % acetic acid.

(f). TLC visualization spray reagents:

Diazotized p-nitroaniline(DPNA)-mix 10 mL 0.5 % p-nitroaniline in 2 N HCl, 1 mL 5 % $NaNO_2$, 30 mL 20 % sodium acetate. Ammonia solution-mix 50 mL 0.88 N NH_4OH, equal volume water.

Vanillin-mix 10 % ethanolic solution of vanillin, equal volume conc. HCl

(g). Spectral shifting agents:

Sodium methoxide(2.5 %)-pipet 10 mL of 25 % sodium methoxide(Aldrich Chem.Co.) into 100 mL volumetric flask and dilute to volume with methanol. Sodium acetate-powder, anhydrous, analytical reagent grade. Boric acid-powder, anhydrous, analytical reagent grade. Aluminium chloride solution(5%)-dissolve 5 g of fresh,dry $ALCl_3$ in methanol to make 100 mL solution.

HPLC DETERMINATION OF SUGARS:

Pass 5.0 mL juice through Sep-Pak C18 cartridge (Waters Associates, Milford, MA). Discard first 2 mL of eluate and collect next 3 mL; pass through the 1 mL anionic-exchange resin(AG 2-X8, chloride form, 200-400 mesh, Bio-Rad Lab.) contained in a swinnex filter(Millipore Co.,Bedford,MA) unit with 0.45 μm millipore filter and collect in a disposable tube. Mix one part of sample with two parts of internal standard solution and two parts of deionized water. Detector response factor (k) for individual sugars were determined by internal standard method. Calculate quantities of individual sugars by following formula:

$$\text{Sugar (mg/mL)} = \frac{As}{Ais} \times \frac{Cis}{K} \times \frac{DF}{R}$$

where Cis=concentration of internal standard; K= detector response factor; As and Ais= total peak areas of sample and internal standard; DF= dilution factor; and R= percent recovery.

HPLC DETERMINATION OF ORGANIC ACIDS:

Filter 5 mL apple juice through the Sep-Pak C18 cartridge. Discard first 2 mL eluate, collect next 3 mL. Dilute one part of sample with two parts of internal standard solution and two parts of deionized water. The detector response factor(K) were determined by internal standard method. Quantitation of individual acids were calculated using the formula as for sugars.

HPLC DETERMINATION OF PHENOLIC ACIDS:

Filter 5 mL apple juice through 0.45 µm millipore filter. The detector response factor (K) was calculated as area per ug by 6 repeated injections of each standards. The response factor (area/µg) for each standard was used to convert peak area into µg sample and calculation back to µg per mL as follows;

$$\text{Phenolics } (\mu g/mL) = \frac{\text{peak area}}{\text{response factor}} \times \text{injection volume} \times \text{dilution factor}$$

PREPARATION FOR GELATIN TREATMENT:

Add 1g of gelatin (A.R.Zacher,Co.,Fresno,CA) to 100 mL volumetric flask, dilute to volume with water. Heat gently up to 90 ^{o}C, stirring continuously(22). Cool and pipet 1mL of this solution 1nto 50 mL centrifuge tube containing 25 mL juice, stirring and standing until the flocculent formed. Centrifuge (2000 rpm x 10 min) and filter through 0.45 µm millipore filter.

ENZYMATIC DETERMINATION OF L-MALIC ACID:

Determine the L-malic acid content using the L-malic acid test kits, following directions (23) from Boehringer Mannheim Biochemicals, Indianapolis,IN. Samples were diluted 1:100 and the absorption monitored at 340 nm.

ISOLATION OF PHENOLICS FOR TLC:

Pass 200 mL apple juice through 30 g hydrated polyclar AT

(GAF, Co., New York,NY). Wash thoroughly with 2 L water. Elute the column with 1 L methanol and 500 mL 0.01 % methanolic HCl. Concentrate the methanolic extract to dryness on rotary evaporator(waterbath temperature, 40 ^{o}C, vacuum 760 mm Hg). Dissolve the phenolics in minimal volume of methanol.

TLC OF PHENOLICS:

Apply 3 µL extract to 20 x 20 cm x0.1mm precoated cellulose TLC plate (E.Merck, Germany) and apply also 1 uL of phenolic standards on second plate. Develop first dimension in BAW (7h), air dry, and develop second dimension in 5.0 % acetic acid (2h). Detect under UV 366 nm and after spraying with visualization reagents.

MEASUREMENT OF ABSORPTION SPECTRA:

Dilute 1 mL of juice sample to 10 mL with spectral grade methanol, place in a dark place until a flocculant precipitate forms. Centrifuge at 2000 rpm for 10 min. Use the centrifugate for UV absorption and fluorescence measurements. To obtain the absorption spectra by polyclar AT treatment, treat 45 mL of juice batch-wise by shaking with successive quantities of hydrated polyclar AT until no further color is removed. Pour adsorbate into a 25x100mm column and wash with 450mL water. Discard aqueous washings. Elute the phenolics with 100 mL methanol followed by 50 mL 0.01% methanolic HCl. Combine methanol eluates. Further dilute methanolic samples with equal volume of methanol in cell. Scan from 400 nm to 200 nm with scan rate 120 nm/min and spectral band

width 2 nm. Dilut the standard phenolic compounds to absorbance below 0.6 at 280 nm or 320 nm and measure under same condition.

For blending experiments: adjust the brix of pear, grape and pineapple concentrates to 13.3 brix, mix with apple juice with ratios varying from 1:9 to 5:5.

MEASUREMENT OF DERIVATIVE SPECTRA:

Run first and second derivative spectra using Varian model DMS 80 spectrophotometer. Scan rate 100 nm/min spectral band width 1 nm/min. Before taking derivative spectra, obtain normal zero order spectra first under same conditions. Measure the peak signal amplitude by peak to peak measurement (24) in mm.

MEASUREMENT OF FLUORESCENCE:

Obtain the fluorescence excitation and emission maxima by increasing the wavelength increment 5 nm manually from 200 nm. Adjust the sensitivity of SPF-125 using 1 ug/mL solution of quinine sulfate in 0.1 N sulfuric acid at excitation 350 nm and emission 450 nm before measurement.

MEASUREMENT OF SPECTRAL SHIFT:

Dissolve the TLC spots in 3 mL methanol solution and record the spectrum using 2.5 mm quartz cells. Measure the spectrum again after add 3 drops of 2.5 % NaOMe solution. Clean the cells and repeat the above procedures with 6 drops of 5 % $ALCl_3$ in methanol, NaOAc powder and $NaOAc/H_3BO_3$ powder on the same

solution(25).

RESULTS AND DISCUSSION

DETERMINATION OF SUGARS:

Figure II.2 illustrates a typical HPLC chromatogram of standard sugars. Figure II.3 is a typical HPLC separation of the free sugars and sorbitol in apple juice. Each sugar was clearly resolved in 14 min. Table II.2 reports the detector response factor (K), retention time(tR), percent recoveries and reproducibility for each sugar. Table II.3 summarizes the free sugar and sorbitol content. The major simple sugars are sucrose, glucose, fructose and sorbitol. The mean value of total sugars was 13.2 g/100 mL and ranged from 6.29 to 16.3g/100 mL. In a previous study on the composition of apple juice (1), a similar range (6.08-16.87 g/100mL) and somewhat lower mean value (10.57g/100mL) has been presented. The mean values for glucose and fructose was 3.12 and 8.68 g/ 100mL or 23.4 and 66.0 % of total sugars, respectively. Fructose was shown to be the most abundant sugar in all samples. The fructose content falls within the range of both the literature compilation by Wrolstad and Shallenberger (26) and the extensive study of Mattick and Moyer (1); however, the mean value was higher than they reported (Table II.3). Similar trends were found for glucose content. Reducing sugars seem not to be as influenced by storage as much as sucrose (27). Hulme (28) noted that the change in glucose and fructose appear to be less affected by storage temperature.

The mean value of the glucose/fructose ratio was 0.36 with a % CV of 22.2. Mattick and Moyer (1) and Wrolstad and Shallenberger(26) presented almost identical values, 0.36 and 0.37 respectively(Table II.3). The range varied betwen 0.20 and 0.42. Most fruits, such as blackberry, grape, strawberry and cherry have an invert pattern or a glucose concentration which exceeds fructose in peach and plum, Apples are exceptional in that fructose is present in amounts up to 3 times greater than glucose (29). The range of glucose/fructose ratio for normal apple juice was reported by Sheu and Wiley(30) to be from 0.24 to 0.64. Industrially prepared juice in Holland was from 0.3 to 0.5(19). In the German RSK-values, the ratio of 0.30 is considered acceptable, and values higher than 0.5 are indicative of treatment with sugars rich in glucose (18). This ratio is not altered by reverse osmosis concentration (30), however, sucrose hydrolysis would affect this ratio. Mattick and Moyer (1) reported the glucose/fructose ratio after hydrolysis. Ratios ranged from 0.16 to 0.60 before inversion, but varied between 0.30 and 0.70 after inversion. Therefore, even considering the hydrolysis of sucrose, the glucose/ fructose ratio should not exceed 0.70 in authentic apple juice.

The mean value of sucrose was 1.39 g/ 100mL and varied from 0.21 to 3.15 with a high %CV of 64.6. The range of sucrose falls within those of previous studies whereas the mean value is lower than that reported value (Table II.3). The 81 season Golden Delicious (WA) sample contained very low quantities of sucrose when compared to other samples. An explanation is that this sample was

subjected to long term storage before processing (Table II.1). A possible source of loss in sucrose content could be the utilization of sucrose in the apples for respiration during storage. The level of sucrose is very susceptible to storage conditions. Gorin (27) reported that during five months storage of Golden Delicious apples, the sucrose content decreased from 24.8 % to 11.1 % while glucose and fructose remained practically constant. Gorin (31) reported a sucrose decrease from 1.77g/100mL to 1.64g/100mL during storage of apple juice for 24 days at 15 ^{o}C. The glucose exceeded sucrose content in this study which is in agreement with Hulme(28) but at variance with Mattick and Moyer(1) and Wrolstad and Shallenberger(26). Variation of sucrose content with maturity (29,32) has been clearly observed, and with heat treatment(87 ^{o}C for 1,4,8,12 and 20min) sucrose content partially hydrolyzed (8). The high %CV of sucrose relative to other sugars may be due to storage, maturity and processing effects. The ratios of sucrose to reducing sugars vary from 0.02 to 0.31 with a high %CV of 69.2. This ratio was affected by maturity (33), and variability in sucrose content directly affects the ratio.

Apples contain substantial amounts of sorbitol (34). The mean value was 0.37 g/100mL, and varied from 0.18 to 0.50g/100mL. Sorbitol content can be a useful indicator for the dilution of sorbitol containing fruit such as apple with sugar or corn syrup (35), and the adulteration of apple with pear which contains high amounts of sorbitol(8,9). Recent findings indicate that the content of sorbitol in apple fruits is largely dependent upon storage

conditions (36,37). Sorbitol content is reported decrease over the six months storage of apples at 1 ^{o}C (32) and the accumulation of sorbitol at low temperature as the result of injury by core flush or low-temperature break down of tissue is documented in the literatures(32,36,38-40). Mattick and Moyer (1) reported sorbitol contents ranging from o.16 to 1.20 g/100mL with a mean of 0.524 g/100mL; this represents a higher mean and a wider range than the results in Table II.1 and the German RSK-value, 2-7g/L(18). Variety , storage and small sample size effects may account for such deviation.

DETERMINATION OF ORGANIC ACIDS:

A typical HPLC separation of an aqueous standard solution containing quinic, malic, shikimic, citric and fumaric acid is shown in Figure II.4. Figure II.5 illustrates a typical HPLC chromatogram of apple juice. Each peak was identified by comparision of its retention time with that for standards and by spiking the sample. The total analysis time was less than 18 min, the acids being eluted according to decreasing polarity. The peak after the solvent is believed to be due to naturally occurring sugars; this was supported by reacting the post column eluents with Fehling's solutions. Standard sugars were also injected to measure the retention time; most sugars were eluted around 5.2 min. By continuous monitoring of the column effluent at 226 nm, the interference from sugars was nil. The percent recovery (R) and response factors(K) are summarized in the Table II.2. The %CV of

retention and response factor for the 6 runs of each acid was generally less than 5.5 %.

A strong cation ion exchange resin column(Aminex HPX-87) was utilized in preliminary analyses, but the malic acid peak was not resolved completely from the quinic acid peak. This system also required a temperature of 80 ^{o}C(11). When the C18 column and the Sep-Pak C18 cartridge were used for sample preparation we obtained good reproducibility and reduced sample preparation time.

The quantities of organic acids and pH values are tabulated in Table II.4. The pH of the juices varied from 3.43 to 4.25 and the total acids ranged between 612 and 1295 mg /100mL. It has been found that acidity varies considerably from variety to variety; in Mattick and Moyer's study of 93 samples (1) the acidity varied from 0.15 to 0.91 % ; a literature compilation(41) reports malic acid range from 548 to 1085 mg/100g. The main non-volatile acid is malic acid. Malic acid constitutes over 60.5-89.7 % of total acids in all samples with 14.6 % CV. A similar range was reported for Canadian apple juices (3),and this range also falls within the values 5.1-9.1 g/L, reported for normal apple juice in Germany (42). Somewhat wider ranges for % malic (35.7 to 91.3 %) of total acids has been reported by Wrolstad et al. in a literature compilation (41).

Quinic acid content ranged from 60 to 394mg/100mL . Quinic was the next in importance to malic, containing from 9.13 to 38.6 % of total acids. Higher levels of quinic acid was found in these samples than reported previously in Canada, 2.9-6.1 % (3). Citric acid is third in concentration, ranging from 0.95 to 40.6 mg/100mL

with a mean of 10.4 mg/100mL. This value is lower than the mean value of 20 mg/100mL reported by Evans et al.(11). Ryan (3) found small amounts of citric acid (10-15mg/100mL) in 10 Canadian apple juices and less than 5mg/100mL in another 10 samples. Evans et al. (11) and Dukel (19) proposed that citric acid should not exceed 20 mg/100mL in pure apple juices. Evans et al. (11) stated that some European countries allow a maximum of 40mg citric acid/100mL and Bielig and Hofsommer (13) reported citric acid up to 35 mg/100g as a standard value. One sample in this study (Golden Delicious,81,WA) contained high 40.6mg citric acid/100mL. This may be due to the effects of postharvest storage. (This sample showed low sucrose and sorbitol values.) Accumulation of citric acid in pulp tissue over the three months storage has been observed by Hulme and Wooltorton(43).

Traces of shikimic and fumaric acid was also detected. These acids showed a higher %CV than malic acid. Shikimic acid varied between 0.09 and 2.04mg/100mL with a mean of 1.0mg/100mL or 0.13 % of total acids. This acid may arise directly from its closely similar alicyclic analogue, quinic (43,44). Ryan (3) also detected traces of shikimic acid in Canadian apple juice. Highest fumaric acid content was 0.03 % of total acids. Three samples were below the detectable amount of 0.005mg/100mL. Fumaric acid, due to its double bond, is about 100 times as sensitive to UV detection as malic (12). Hence this acid can be detected and quantitated even though present in low amounts. Presence of citramalic acid in McIntosh apple juice, and succinic acid in Golden Delicious(Mexico)

sample were tentatively identified, but other organic acids such as glycolic, glyoxylic, phosphoric, oxalic acid which have been reported in apples(45), were not detected.

The acid profile showed much more variation both within and between the different samples than did the sugar profile. In the preparation of apple juice and juice products, however, the manufacturer usually resorts to blending of varieties of different acid content in order to obtain a uniform product of the desired acidity.

Synthetic food grade malic acid can be distinguished from natural malic acid as synthetic malic acid is a racemic (DL) mixture while natural malic acid in fruit has the L-configuration(46). When L-malic acid is considerably lower than the total malic acid, it is likely that synthetic malic acid is present (12). Since in the synthesis of malic acid, some fumaric acid formed(12), the presence of fumaric acid in large amounts may also indicate adulteration with synthetic malic acid. The standard L-malic acid used in this study when analysed, contained 0.2%fumaric acid. Table II.5 presents the differences between HPLC and enzymic determination of malic acid(23). The paired t-test showed no evidence of a statistically significant ($p<0.01$) difference between the two methods. Junge and Spadinger (12) and Evans et al. (11) agreed that the difference should not be greater than 0.3 g/L for authentic apple juices. But four of the eight samples in this study of authentic juices did exceed 0.3g/L. This illustrates the quantitative difficulties inherent with HPLC and enzymic

determination of malic acid and the need for also considering fumaric content in determining whether adulteration with synthetic malic has occurred. They(11,12) also considered a concentration greater than 3.0mg/L of fumaric acid in apple juice to be an indication of the presence of synthetic malic acid. None of the samples in Table II.5 approached that value. Fumaric acid can also be formed from natural malic acid when fruit juice is subjected to heat as in the processing of juice concentrates. Evans et al. (11) observed the formation of fumaric acid as much as 10mg/L during juice concentration. An improved method is needed to determine D-and L-malic acid. Separation of the stereoisomers by HPLC on chiral stationary phase(HPLC-CSP) has recently been demonstrated (47,48). Such a technique for D-and L-malic if developed would greatly improve the reliability for detection of synthetic malic acid.

DETERMINATION OF PHENOLIC ACIDS:

Figure II.6 illustrates the separation of commonly occurring polyphenolic compounds and hydroxymethylfurfural (HMF) using methanol/KH_2PO_4 with gradient elution. HMF, Chlorogenic acid, caffeic, p-coumaric and ferulic acid were completly resolved in 30 min. Better separation was obtained with use of a gradient system than the isocratic elution with acidic methanol used by Brause and Raterman (7). Compounds more polar than phenolic acids including sugars or organic acid esters of phenylpropanoids are eluted with the solvent. Retention time and response factor (K) are

presented in Table II.6. A typical chromatogram of phenolics from apple juice is shown in Figure II.7. Each peak was identified by comparing their retention time with standards. Peaks 1 and 2 were identified as HMF and Chlorogenic acid respectively. Peaks 3, 4, and 5 have the same retention times with caffeic, p-coumaric and ferulic acid respectively: Examination of a commercial apple juice revealed a profile which was quantitatively very different from the eight authentic samples (Figure II.8). Polymeric procyanidins also make up the large portion of apple juice phenolics (49). The juice samples in our study had not been treated with fining agents, or pasteurized as is common commercial practice. We suspected that gelatin fining in particular may have influenced the phenolic profile. Table II.7 (McIntosh,MI)shows how the area of peaks 3,4 and 5 decreased from 30 to 50 % while chlorogenic acid decreased about 4.7 % after gelatin fining. Tetrameric and polymeric procyanidins are particularly affected by gelatin fining (50,51). These observations supported that peaks 3,4, and 5 are coeluting with polymeric procyanidins. Gelatin fining's effectiveness is dependent upon a number of factors including temperature, pH and the concentration of ferric and other ions (52). In this limited study, gelatin fining appears to have had little effect on the low molecular weight compounds such as chlorogenic acid. Wilson (53) demonstrated that the procyanidin isomers of each molecular mass had a wide spread of retention times, and recently Lea (54) observed that large amounts of phenolic acids are eluted in a non-ionic form at similar retention times to procyanidins. Coelution of

procyanidins and different operations of fining techniques can obviously effect the profile and quantitation of phenolics.

Table II.8 gives the quantitative results for chlorogenic acid and HMF content. HMF content ranged from 1.84 to 15.4 mg/L and the mean was 8.14 mg/L. In commercial apple juices from the Danish market, HMF was found to vary from 0 to 11 mg/L for 11 samples(55). HMF is a dehydration product of hexoses. It is present in large quantities in caramel coloring and high concentrations in apple juice can be indicative of the presence of caramel (56). Commercial caramel preparations contained HMF level between 14000 and 18000 mg/L (56). HMF formation in fruit juices(57-59) is influenced by storage, heating and compositional factors. Wucherpfenning and Burkardt (60) mentioned that the concentration and type of sugar , quantity and structure of amino acids as well as the pH all have an effect on HMF formation.

There is a considerable variation in the chlorogenic acid content. It ranged from 1.5 to 228.0 mg/L with a mean of 47.0 mg/L . Brause and Raterman (7) reported chlorogenic acid content of 66 to 232 µg/g for juices they made, and 0 to 208 µg/g for commercial juices. They indicated that chlorogenic acid should appear at levels greater than 50 µg/g for normal apple juice. They considered juices below 50µg/g to be "overprocesed" or adulterated with either beet, cane, invert sugar or HFCS(7). Six of the eight authentic samples in this study(Table II.8) are below 50mg/L.

The ratio of HMF to chlorogenic acid, varied from 0.03 to 8.53 with significant variability, 179.2 % CV. Except for two

samples made from freeze-dried apples, the ratio did not exceed 1. Considerable variation in this ratio limits its utility only to special cases such as dilution and colorant addition. If the juice is diluted, the caramel color would be added to maintain the color specification, it will increase the ratio of HMF to chlorogenic considerably. Fortunately chlorogenic acid is too expensive to be cost-effective as an additive to cover such fraud (7).

TLC DETECTION OF PHENOLIC COMPOUNDS:

A two-dimensional chromatogram of the phenolic compounds in McIntosh apple•juice is shown in Figure II.9. Spots exhibited blue fluorescence under UV light. The color varied from light to dark blue depending on the phenolics present. Table II.9 lists the Rf values and reactions to various diagnostic sprays. Several groups of spots behave similarly and each reacted with diazotized p-nitroaniline(DPNA). This reagent undergoes a coupling reaction with phenolic compound giving azo dyes (61). Some of these compounds were further characterized by their spectral properties in the UV regions.

Spot 1 changes from bright blue to a slightly blue-green color when fumed with ammonia vapor (62) and showed a maxima at 328 nm and minima at 267 nm. When $AlCl_3$ reagent was added there was no shift in absorption maxima (Figure II.10). The bathochromic shift by sodium methoxide (NaOMe) (Figure II.11) represented the presence of phenolic hydroxyl groups (25) and a hypsochromic shift from NaOAc (Figure II.12) indicates a free carboxyl groups in this compound

(63). According to UV spectra and Rf value, this spot is tentatively identified as a caffeic acid derivative. Spots 7 and 8 resembled chlorogenic acid in mobility; they both displayed a slightly purple-blue fluorescence, changed to a yellow-green after fuming with ammonia vapor (64) and gave a slightly brown color with DPNA. They have a maxima at 323 -327nm and minima at 260-268 nm, showed a slight bathochromic shift on the addition of $AlCl_3$ and gave a positive reaction to the NaOMe. Sondheimer (65) reported the presence of isochlorogenic, band 510, neochlorogenic and chlorogenic acid in McIntosh apple. Spots 13 and 14 may possibly correspond to the cis-isomers of spots 7 and 8 respectively; This is based on their high Rf value(66) and similar color reaction under UV.

Spots 2 and 6 showed the same blue color under UV and were slightly blue-green with ammonia. Spot 2 showed maxima at 325 and 286 nm and minima at 269 nm. There was no shift with $AlCl_3$ and a positive response to the NaOMe reagent. Spot 6 showed maxima at 321 nm and 278 nm, a minima at 263 nnm and responded similarly to the shifting agents as spot 2. The UV spectra of these compounds gave tentative identifications as the trans, cis analogs of p-coumarylquinic acid derivative, P-coumarylquinic acid has two maxima at 326-327 nm and 293 nm in 95 % ethanol(67). Williams(68) reported the presence of P-coumarylquinic in apple. Spot 16 fluoresced to purple-blue in UV and slightly blue-green in ammonia, it has maxima at 320 nm and 293 nm, minima at 268 nm and shows a positive response to NaOMe; its characteristics could not be matched with any known phenolic compound. Spot 4 has dark color

under UV with and without ammonia, maxima at 307 nm ,shoulder at 296 nm and minima at 246 nm. Bathochromic shift on the addition of NaOMe indicated the availability of hydroxyl group, but a negative response to NaOAc. Its color under UV and absorption spectra led to the tentative identification of the compound as a p-coumaric acid ester. The color of spots 5 and 12 were colorless under UV and appeared to orange-red color when chromatogram was sprayed with the vanillin-HCl reagent,indicating these spots might be either catechin or leucoanthocyanidins (69). Spot 9 has a maxima at 325 nm and a minima at 262 nm, and slightly shifted by the NaOAc reagent. Its similar behavior with spot 1 and Rf value tentatively identified it as cis-isomer of spot 1. Spot 15 appeared to be polymeric in nature because of this low Rf value in 5% HOAc (67). Anhydrous $NaOAc/H_3BO_3$ is used to analyze spectral shift of flavonoids (67) but no spots responded to this reagent. Kaempherol and quercetin glycosides are reported in apple (70) but they are not so readily detected because of their faint or absence of color(71).

CHARACTERIZATION OF APPLE JUICE SPECTRA:

Figure II.13 illustrates the typical absorption curve of apple juice. The specific shape of the curves was quite similar between samples except for the differences in the absorption intensities. The spectrum typically consists of two absorption maxima in the ranges 319-322 nm (band I) and 279-281 nm (band II). Characteristic feature of these spectra was the low intensity of band I relative to band II.

Figure II.14 shows the absorption curves isolated by polyclar AT. Golden Delicious (WA), Jonathan and Granny Smith (New Zealand) samples revealed an additional absorption maxima around 223-224 nm. Caffeic, ferulic, p-coumaric,dihydrochalcones and some flavonols exhibit a subsidary maxima in that region (Table II.10). Table II.10 lists the absorption maxima and minima of phenolic compounds which were previously reported to be widely distributed in apple (71). The UV spectra of caffeic and chlorogenic are similar. Catechin and (-)epicatechin exhibited absorption maxima near to 279 nm and minima at 250 nm. Some flavonols, such as kaempherol, quercetin showed maxima in the range of 356-364 nm and minima around 281 nm. From these observations, the cinnamic acid derivatives, caffeic, p-coumaric, ferulic and chlorogenic acid would influence the intensity of band I and catechins, dihydrochalcones, flavonols and subsidary peak from some cinnamic acids would contribute to the band II.

Table II.11 gives the absorption characteristics of apple juice samples. The absorption values of two maxima varies with different varieties. The A320nm varied from 0.154 to 0.469 and A 280 nm varied from 0.234 to 0.621. The mean value of A320 nm/A280 nm ratio was 0.60 with a 16.7 %CV. Similar values were observed from two different growing seasons of Golden Delicious (WA). McIntosh sample showed relatively strong intensity in both A320 nm and A280 nm with a ratio of 0.76, but for Jonathan which has a strong absorption at 280 nm the ratio was 0.48. It appears that variety may influence the UV data.

In the sum of two absorption maxima 320 nm and 280 nm, the mean was 0.663 with 32.4 %CV. The highest value was obtained from McIntosh sample and the lowest from Golden Delicious (82, WA). Average value of A320 nm, and A280 nm was 0.248 and 0.415 respectively with A320 nm varing more than A280 nm.

Figure II.15 shows a typical UV spectra from pear,grape and pineapple juice concentrates. Pear and grape juices exhibit very similar absorption curve to the apple juice. Pineapple juice can easily be distinguished from apple juice as it has a strong absorption near 280 nm and the A320nm/ A280 nm ratio was 0.37, which is very low compared to the apple juice. To test the usefulness of absorption ratio and total absorption, different blends of apple juice with pear, grape and pineapple juice concentrates were analyzed. As the content of grape or pear juice increased, the A320nm/A280nm ratio decreased gradually from 0.65 to 0.63, and from 0.65 to 0.46 with pineapple juice up to 50 % level. But the total absorption at 320nm and 280 nm fluctuated as the content of these fruit solids in apple juice increased(Table II.12). At the 10 % level blending, the sum of these absorbances decreased from 0.609 to 0.482 with grape, to 0.301 with pear and to 0.522 with pineapple juice, indicating the presence of those fruits solids in apple juice, but these values are still within the range found for pure apple juices in this study:0.388-1.090 . To detect the presence of other fruit juices such as pear, pineapple and grape in the apple jucie, the ratio and total absorption of A320nm and A280 nm will be of limited value.

Table II.13 lists the excitation and emission maxima. Excitation maxima ranged from 284 nm to 296 nm and emission maxima ranged from 325 nm to 365 nm. There is no discernible differences in the exciatation and emission maxima from apple,pear,grape, or pineapple juices. Figure II.16 illustrates the typical first order($dA/d\lambda$) derivative spectra of apple juice. The maxima in the zero order absorption band occurrs at zero ($\Delta A=0$) in the first order derivative spectra. All apple juice samples have $\Delta A=0$ around 313-316 nm, 274-276 nm and some samples have also near 220-234 nm. No considerable difference in the first order derivative spectra was found, but it can be distinguished from pineapple juice (Figure II.17). The order of peak amplitude for apple juice was 224nm>282nm>330nm except for McIntosh and Golden Delicious (WA), which have the order of 224nm>330nm>282nm.

The second order derivative($d^2A/d^2\lambda$) spectra showed enhanced resolution of absorption maxima (Figure II.18). The zero order absorption maxima appears as a mininum and the points of inflection appear as maxima, thus original zero order spectra is considerably sharpened in the second order derivative spectra. The absorption maxima around 320nm of McIntosh, resolved to 5 minima at 324nm, 320 nm, 312 nm, 304nm and 296nm in the second order spectra (Figure II.18) and for most samples, the extremes (maxima,minima) occurred at different wavelength (Table II.14). All samples showed noticeably strong amplitude around 274-284nm (peak AB) and 220-240nm (peak CD) in the Figure II.19. Thus the peak AB and CD was selected to measure the peak amplitude by peak to peak measurement. Table

II.15 presents the results of peak amplitude ratios. The ratio varied from 0.22 to 0.61. Two Golden Delicious samples from Washington showed similar values, 0.22 and 0.25 respectively. The two Argentine samples showed high values relative to other samples, Granny Smith was 0.51 and Deliciosa was 0.61. Recently, peak amplitude ratio was introduced for comparative identification of structurally similar compounds (72) and quantitation (73) of drugs. The ratio of pear juice was 0.14, pineapple juice was 0.04 and white grape juice was 0.08. These results indicate that derivative spectra may be useful for fingerprinting authentic juices.

CONCLUSIONS

From the results obtained in this work, it can be concluded that postharvest storage and processing conditions played an important role in the variability of apple juice components whereas geographic origins had little influence. This is especially true in sugar profiles where sucrose and sorbitol content was influenced by storage effects. Less effect was observed for glucose /fructose ratios.

Compositional data from Argentine, New Zealand and Mexican apples do not significantly different from those grown in United States. With the use of High Performance Liquid Chromatography (HPLC) and Sep-Pak C18 cartridge, it was possible to get high recovery and reproducibility in sugars, acids and phenolic acids. Free sugars and sorbitol contents fall within the ranges

previously reported, and glucose/fructose ratios were almost identical with literature values. The main non-volatile acid is malic acid with lesser amounts of quinic acid. No interference in the determination of malic acid was observed. Small amounts of citric and trace of fumaric and shikimic acid have been detected. Coelution of polymeric procyanidins with phenolic compounds was observed by comparisions with commercial juice and gelatin fining experiments. Extremely high variability of HMF and Chlorogenic acid contents limits their application.

Spectrophotometery offers potential help in screening juices for authenticity. The spectrum typically consists of two absorption maxima at around 320 nm and 280 nm. The A320nm/A280nm ratio was relatively constant. The ability to detect and measure the minor spectral features is considerably enhanced in the derivative mode.

On the basis of tabulated values in the literature and this study, the following characteristics for authentic apple juice are suggested: For sugar profile; Data base in literature (1,7,26) are sufficient for sugar profile. Useful critera are in particular glucose/fructose ratio and sorbitol level. Sorbitol and sucrose are highly variable due to processing and storage. For acid profile; Recommendation's from Evans et al. (11) and Junge and Spadinger (12) for differences between HPLC and enzymic determinations may be too low. Four out of eight samples in authentic apple juice exceeded 0.3g/L. This value must be used in combination with fumaric acid contents. Improved methodology is needed to detect both D-and L-malic acid on the same time. High variability in citric contents

requires more studies to be done need on this acid contents in authentic apple juice. For phenolic profile; Chlorogenic acid levels proposed by Brause and Raterman (7) should be revised. High variability in this compound due to different fining, juice preparation techniques, storage and analytical methodology can influence the phenolic profile. For UV-profile; Derivative spectra can be a potential screening method.

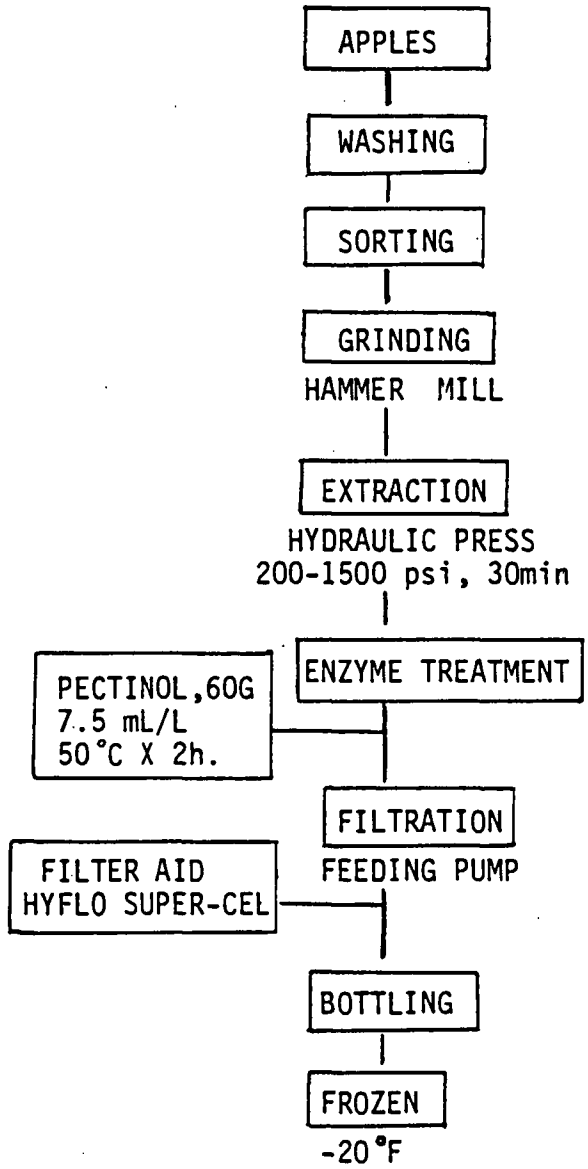

FIGURE II.1 FLOW SHEET FOR THE PROCESSING OF APPLE JUICE.

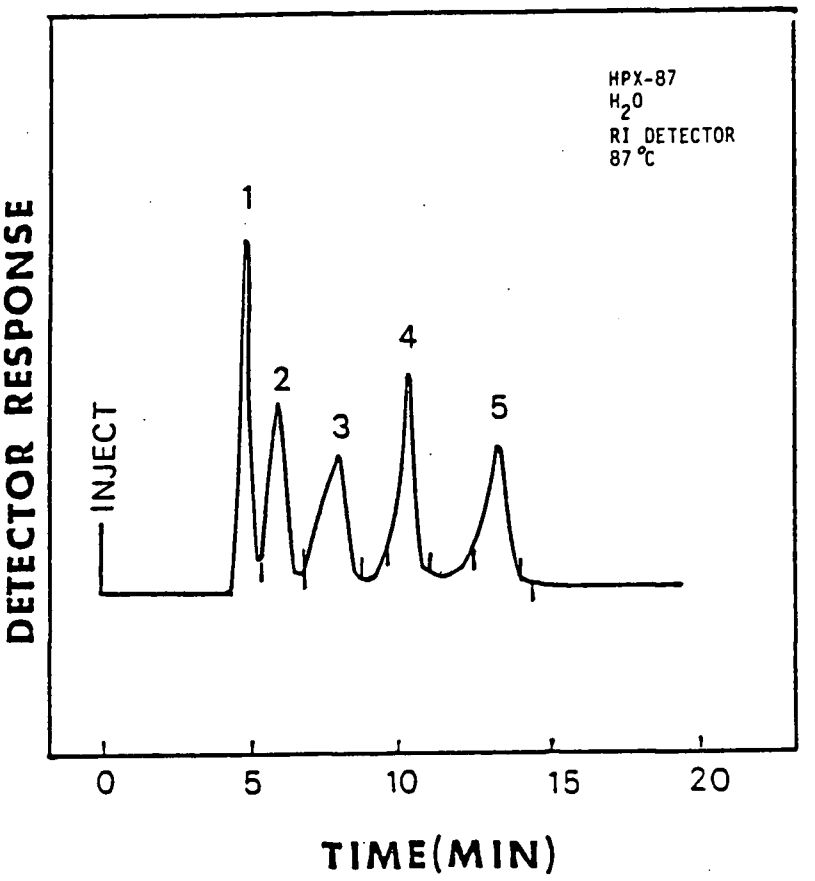

FIGURE II.2 TYPICAL HPLC SEPARATION OF STANDARD SUGARS.
1. sucrose 2. glucose 3. fructose 4. mannitol
5. sorbitol

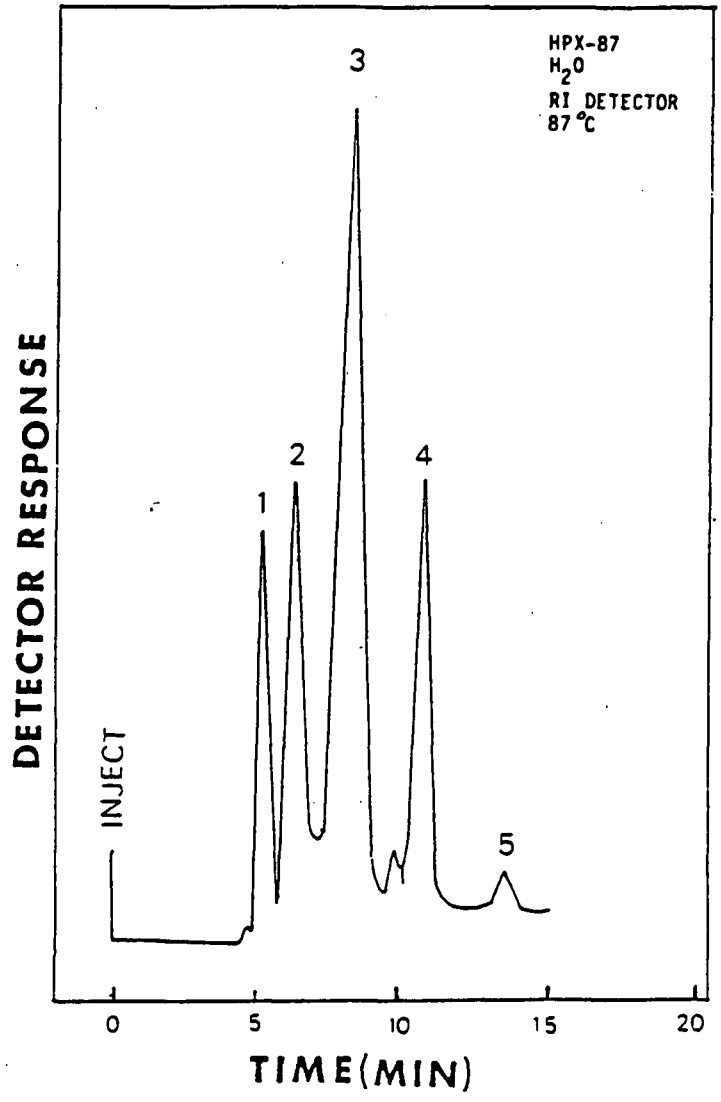

FIGURE II.3 TYPICAL HPLC SEPARATION OF SUGARS FROM APPLE JUICE. 1. sucrose 2. glucose 3. fructose 4. mannitol(int. std.) 5. sorbitol.

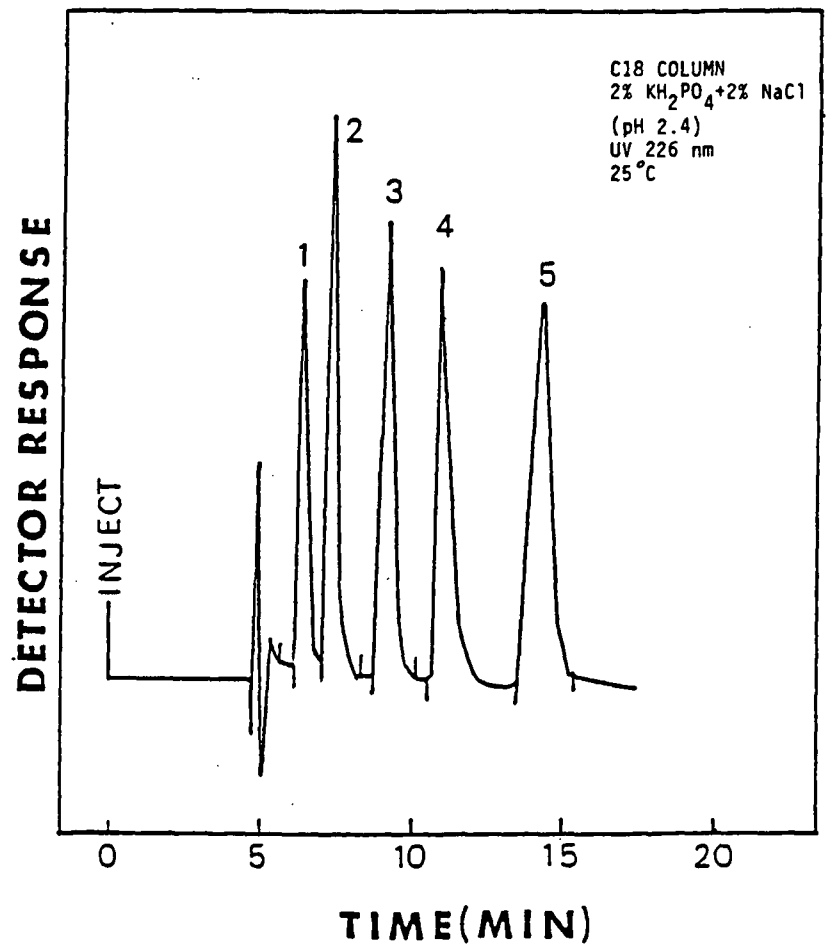

FIGURE II.4 TYPICAL HPLC SEPARATION OF STANDARD ORGANIC ACIDS.
1. quinic 2. malic 3.shikimic 4. citric 5. fumaric

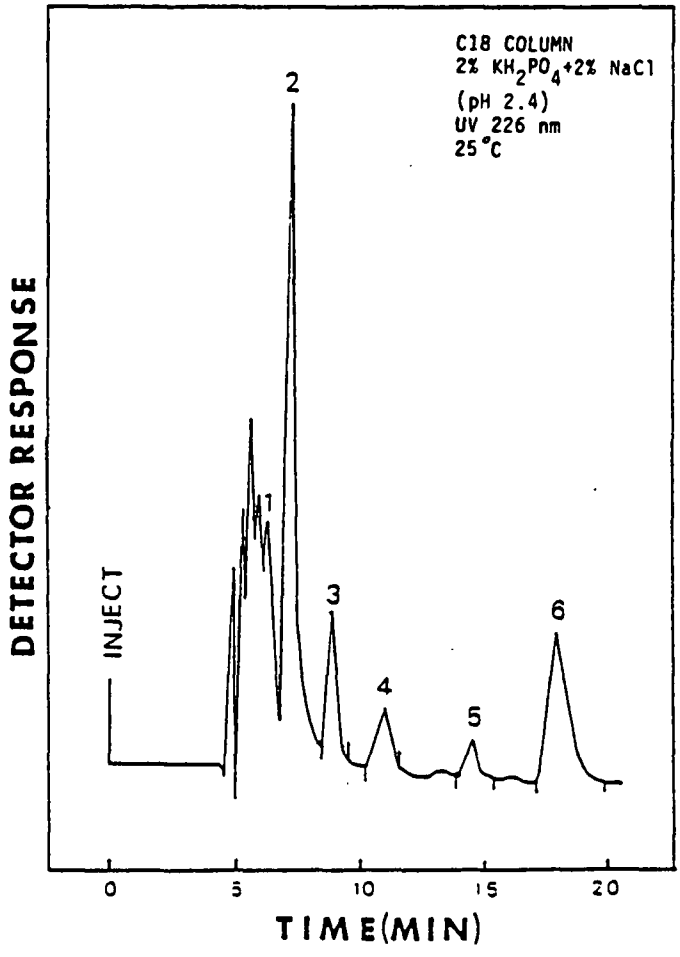

FIGURE II.5 TYPICAL HPLC SEPARATION OF ORGANIC ACIDS FROM APPLE JUICE.
1. quinic 2. malic 3. shikimic 4. citric
5. fumaric 6. isoleucine(int.std.).

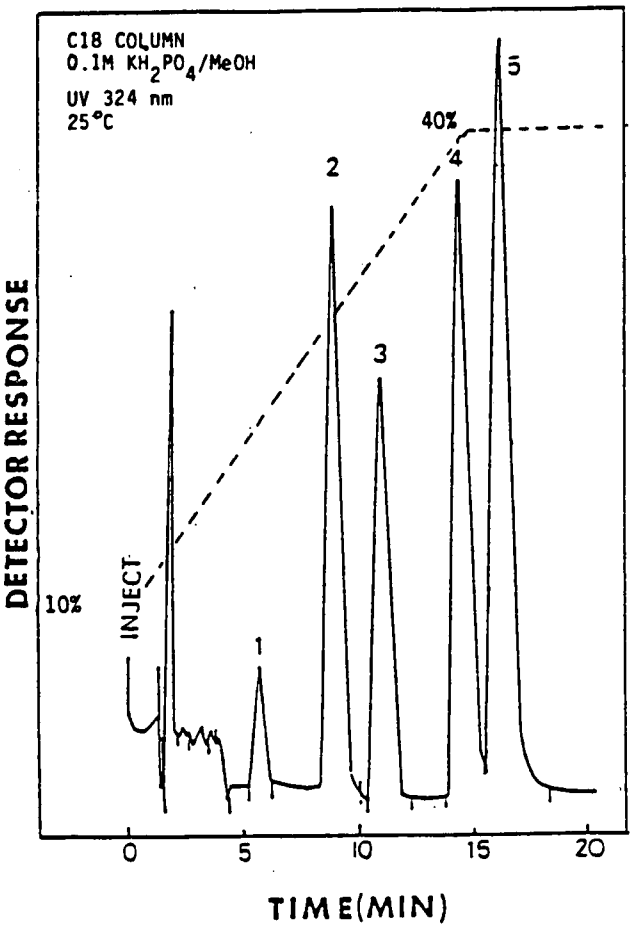

FIGURE II.6 TYPICAL HPLC SEPARATION OF STANDARD PHENOLIC ACIDS.
1. HMF 2. chlorogenic 3. caffeic 4. p-coumaric
5. ferulic acid.

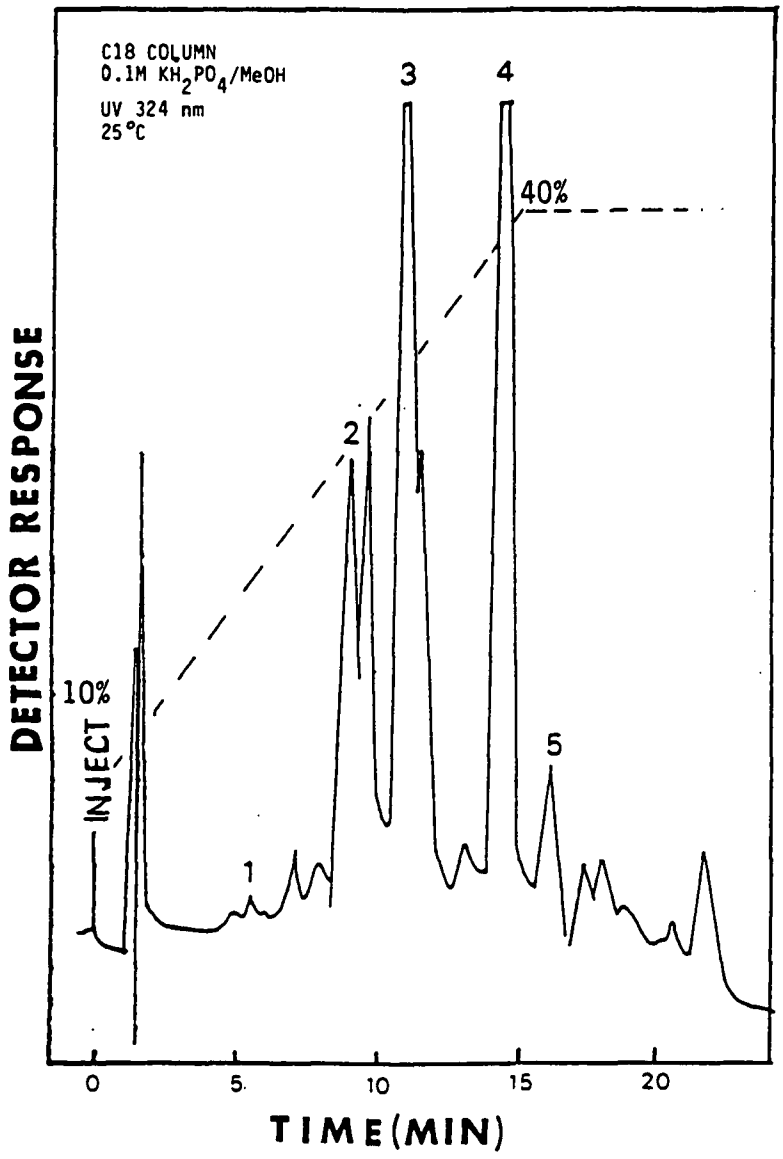

FIGURE II.7 TYPICAL HPLC SEPARATION OF PHENOLIC ACIDS IN APPLE JUICE.
1.HMF 2. chlorogenic 3. caffeic
4. p-coumaric 5. ferulic acid.

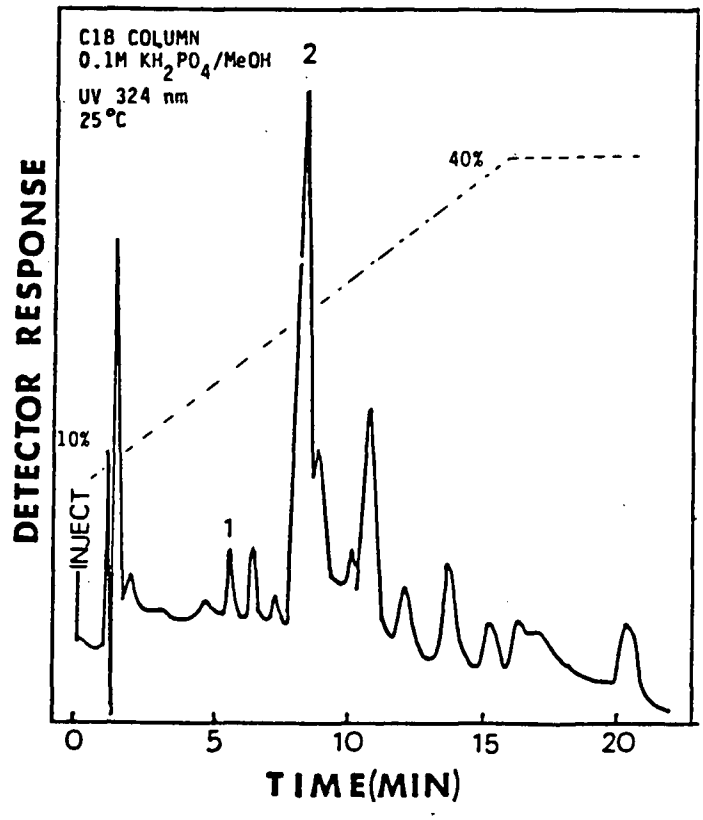

FIGURE II.8 TYPICAL HPLC SEPARATION OF PHENOLIC ACIDS FROM COMMERCIAL APPLE JUICE.
1. HMF 2. chlorogenic

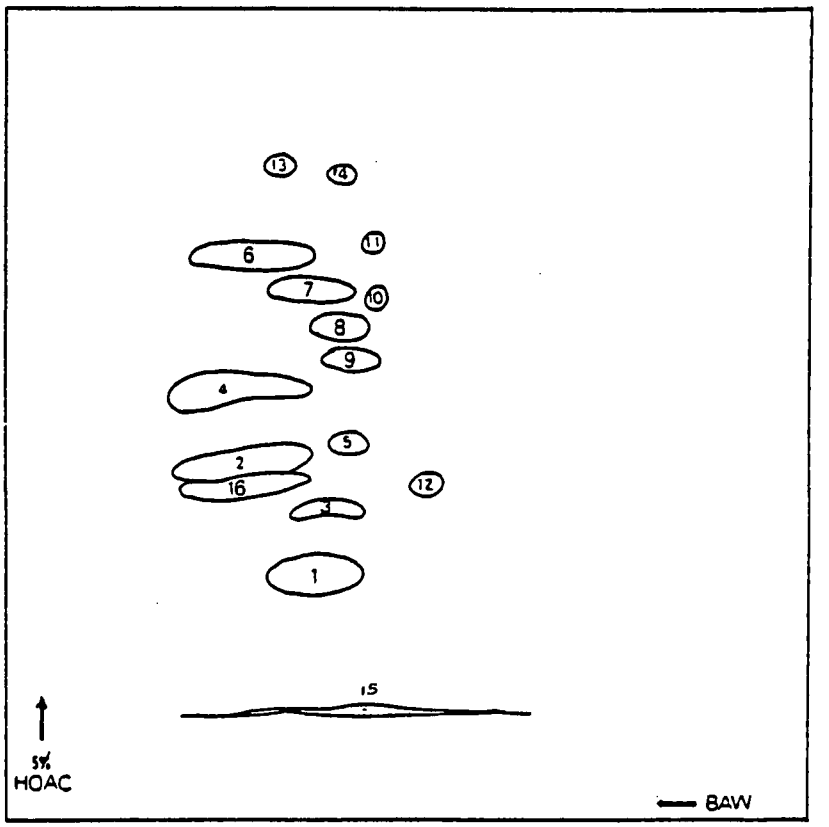

FIGURE II. 9 TWO-DIMENSIONAL TLC CHROMATOGRAM OF THE POLYPHENOLS IN MCINTOSH APPLE JUICE.

1.caffeic acid 2. trans-p-coumarylquinic 4. p-coumaric ester
5. catechin or leucoanthocyanidin 6. cis-p-coumarylquinic
7. chlorogenic acid isomer 8. chlorogenic acid isomer
9. cis-caffeic acid 12. catechin or leucoanthocyanidin
13. cis-chlorogenic acid isomer 14. cis-chlorogenic acid isomer.

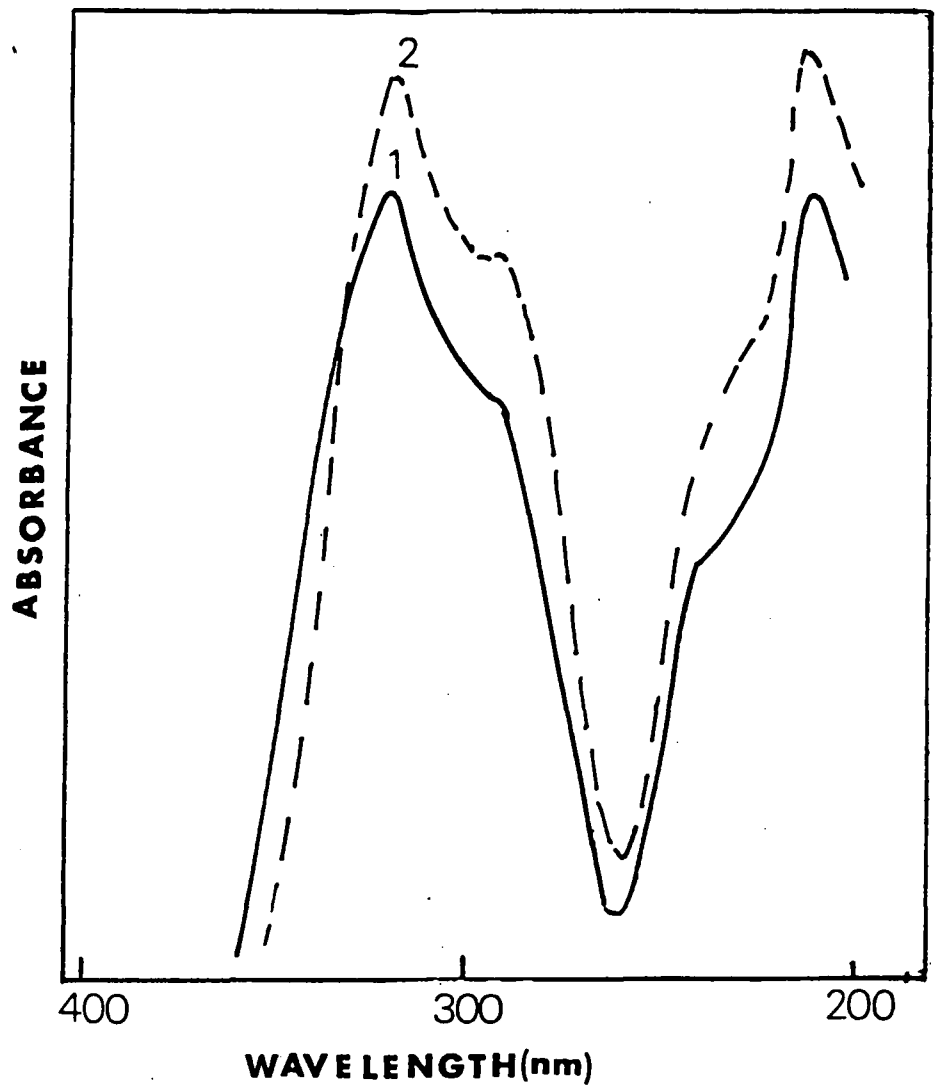

FIGURE II.10 UV ABSORPTION SPECTRUM OF SPOT 1.
1. MeOH 2. MeOH + $AlCl_3$

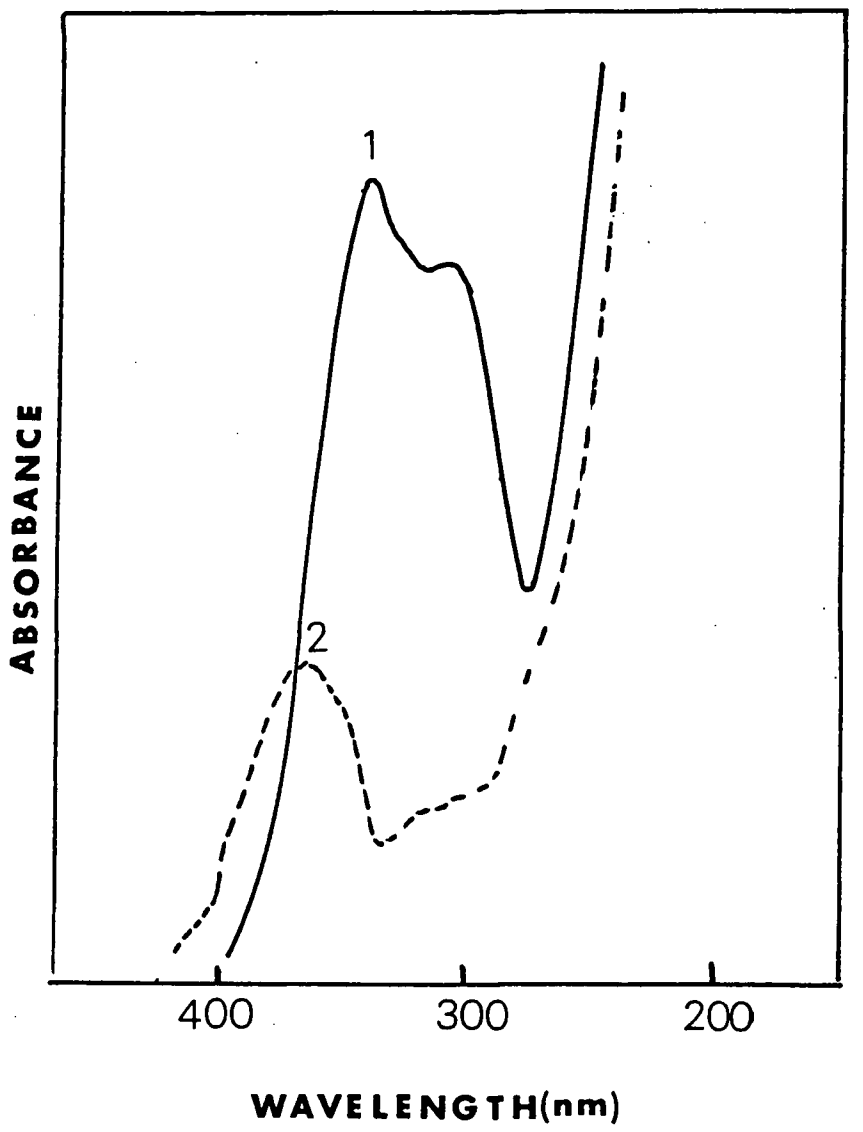

FIGURE II.11 UV ABSORPTION SPECTRUM OF SPOT 1.
1. MeOH 2. MeOH + NaOMe

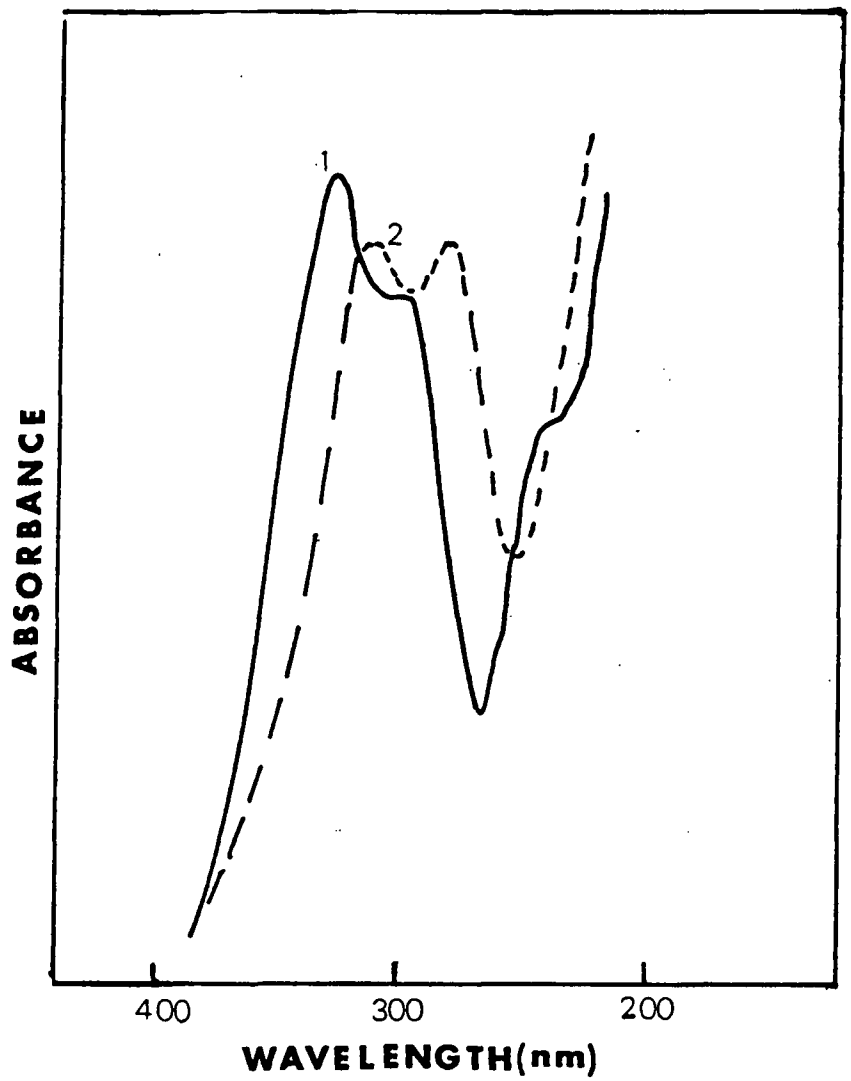

FIGURE II. 12 UV ABSORPTION SPECTRUM OF SPOT 1.
1. MeOH 2. MeOH + NaOAc

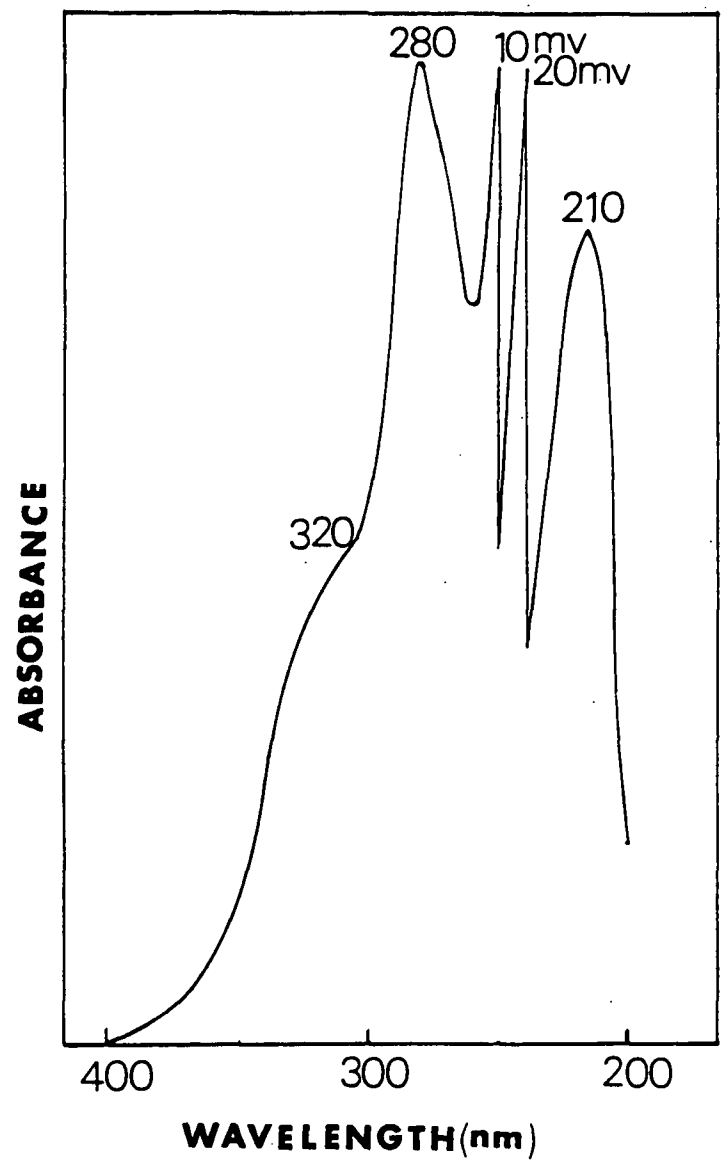

FIGURE II. 13 TYPICAL UV ABSORPTION CURVE OF APPLE JUICE

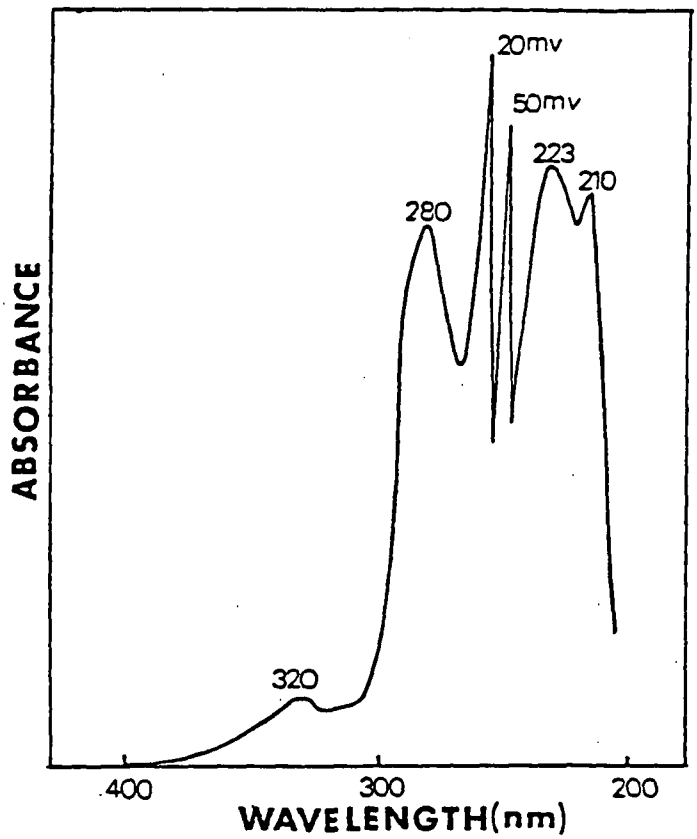

FIGURE II.14 TYPICAL UV ABSORPTION CURVE OF APPLE JUICE PURIFIED WITH POLYCLAR AT.(GOLDEN DELICIOUS, 81 WA).

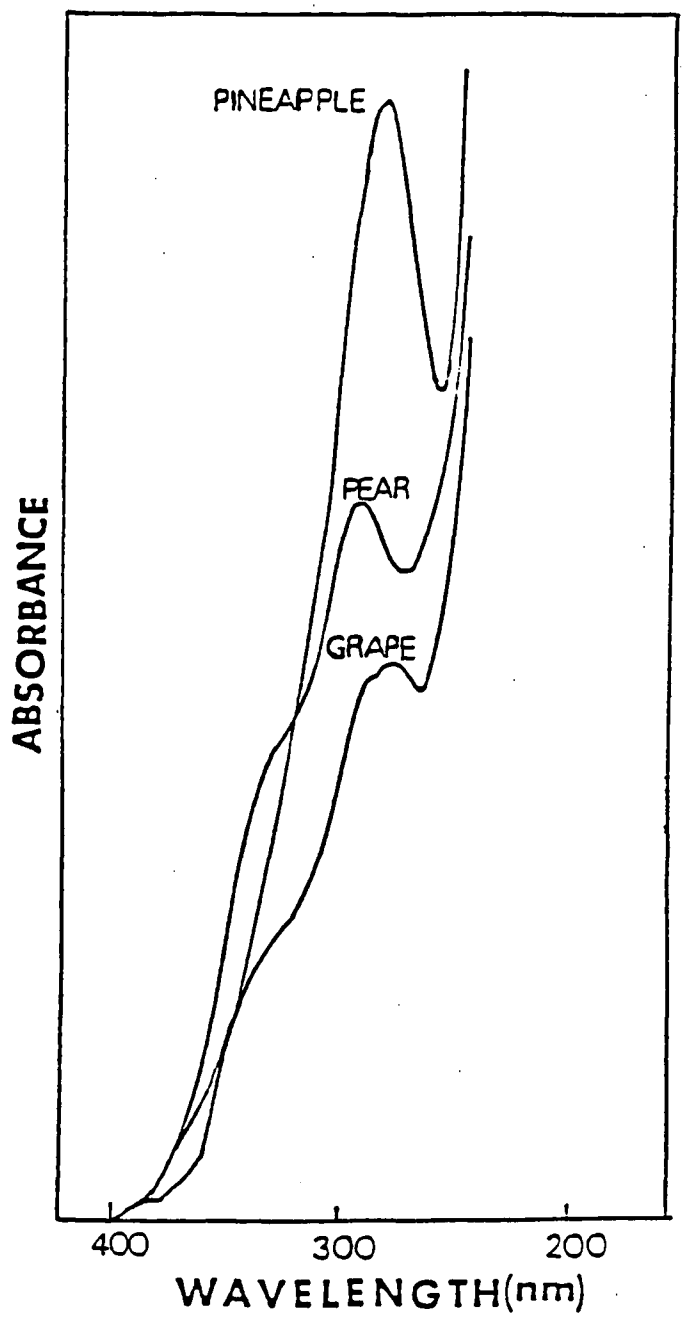

FIGURE II.15 TYPICAL UV ABSORPTION CURVES FROM PEAR PINEAPPLE AND WHITE GRAPE JUICE CONCENTRATES.

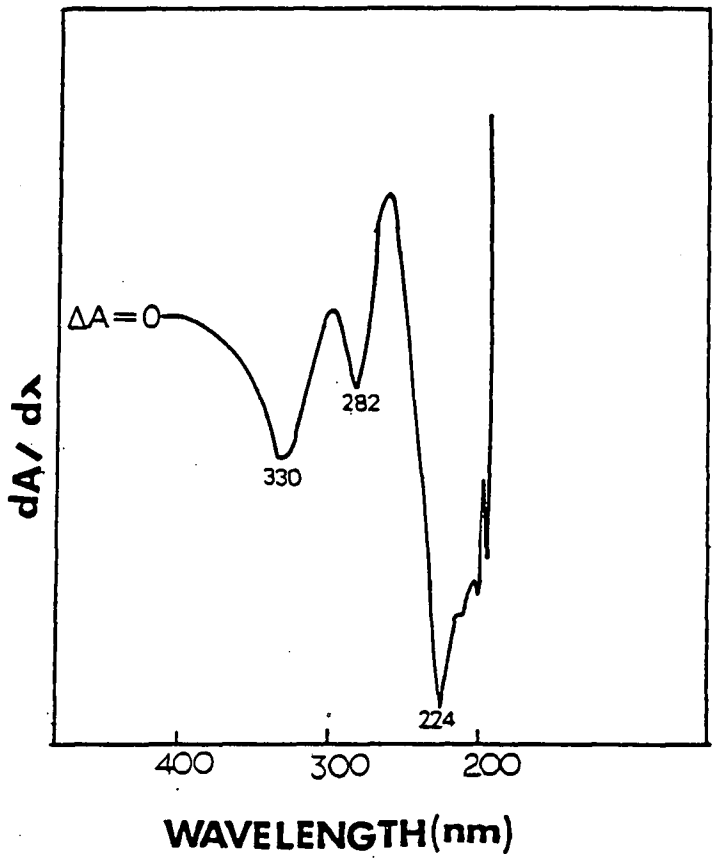

FIGURE II. 16 TYPICAL FIRST-ORDER DERIVATIVE SPECTRA OF APPLE JUICE (MCINTOSH, MI).

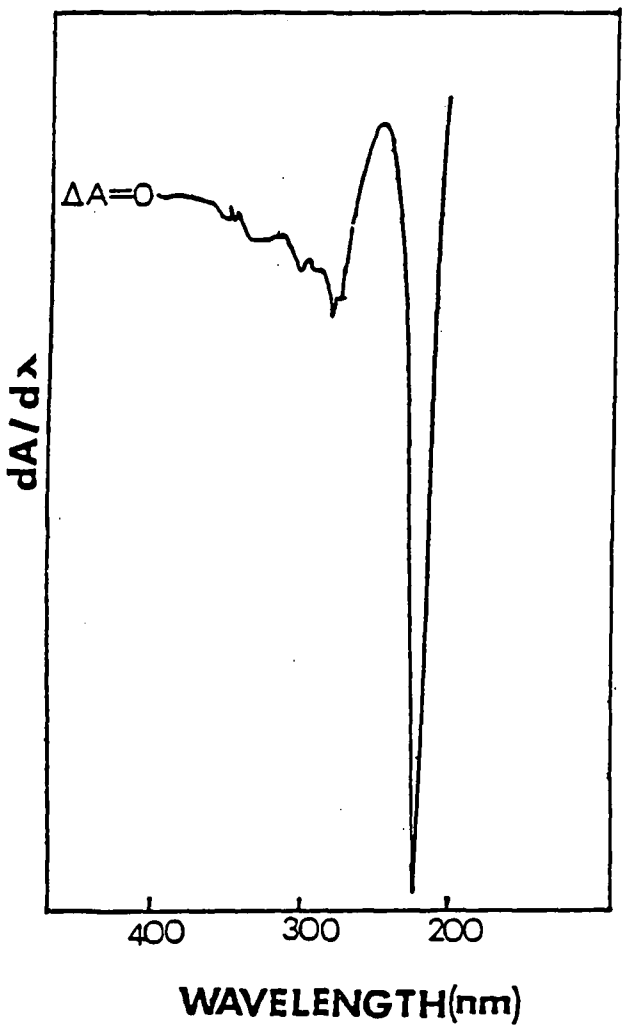

FIGURE II.17 TYPICAL FIRST-ORDER DERIVATIVE SPECTRA OF PINEAPPLE JUICE CONCENTRATE.

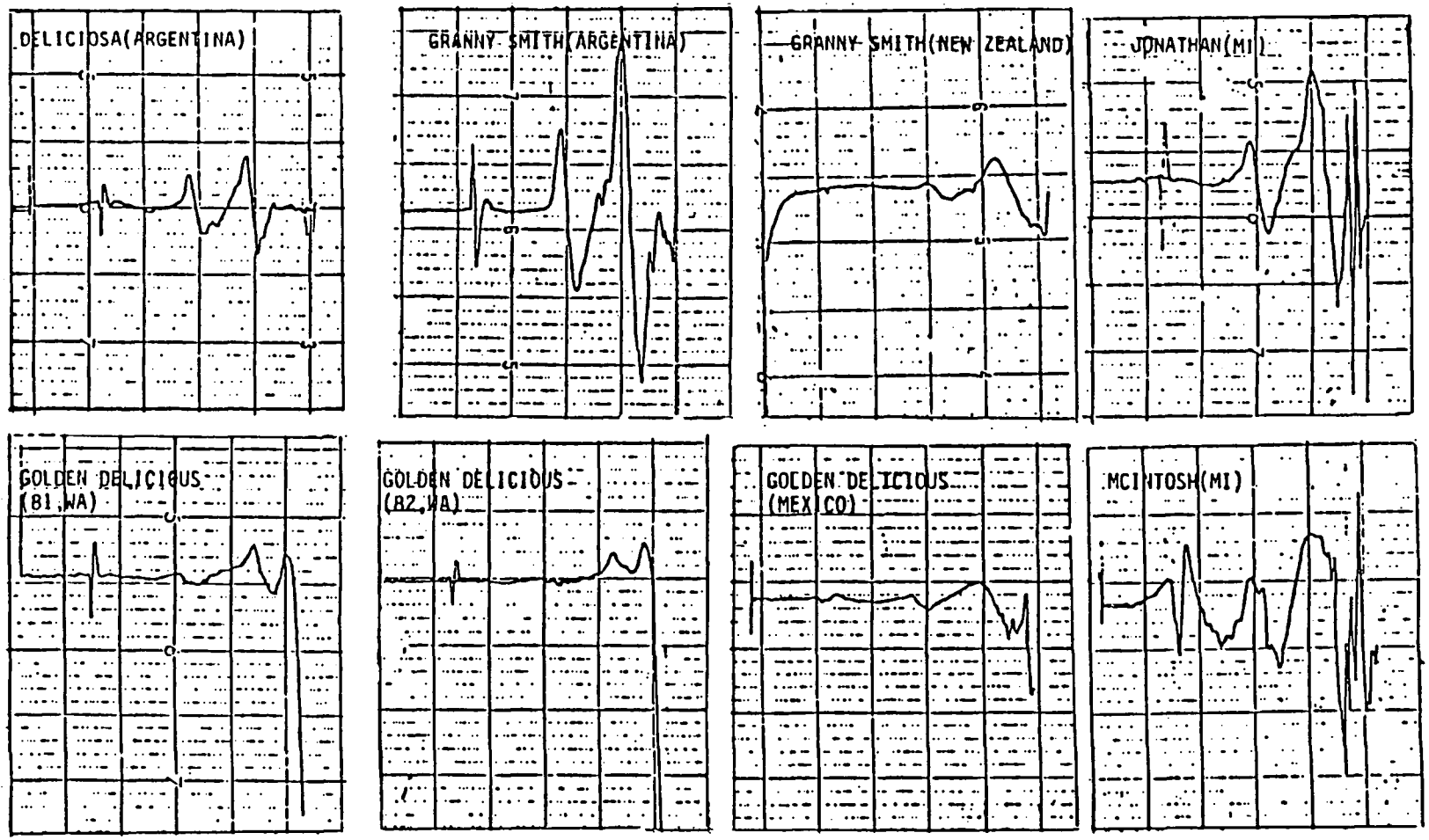

FIGURE II.18 TYPICAL SECOND-ORDER DERIVATIVE SPECTRA OF APPLE JUICES.

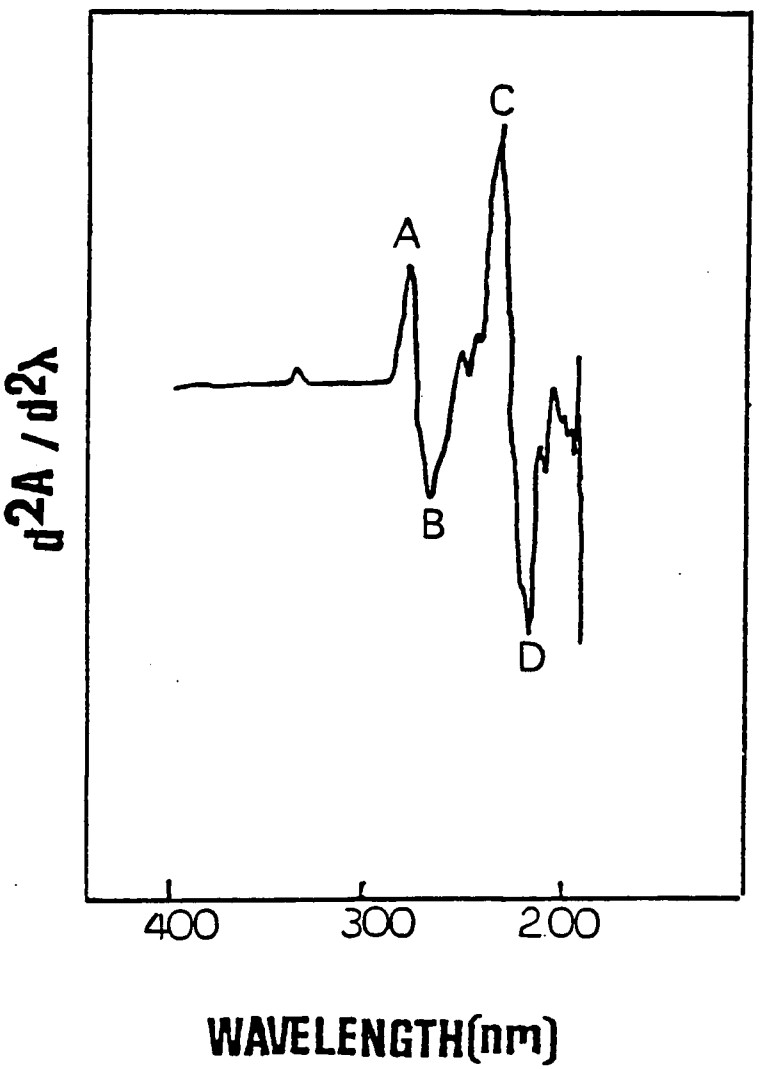

FIGURE II.19 TYPICAL SECOND-ORDER DERIVATIVE SPECTRA OF APPLE JUICE(GRANNY SMITH,ARGENTINA).

TABLE II.1 VARIETY, GEOGRAPHIC ORIGIN AND HISTORY OF APPLES.

VARIETY	GROWING SEASON	GEOGRAPHIC ORIGIN	DATE OBTAINED	DATE PROCESSED
GOLDEN DELICIOUS	1981	WA, USA	4/20/82	6/20/82
GOLDEN DELICIOUS	1982	WA, USA	10/21/82	10/26/82
GOLDEN DELICIOUS	1983	MEXICO	1/5/84	1/6/84
JONATHAN	1982	MI, USA	10/29/82	1/4/83
MCINTOSH	1982	MI, USA	10/29/82	1/4/83
[a] GRANNY SMITH	-	ARGENTINA	8/18/82	8/19/83
[a] DELICIOSA	-	ARGENTINA	8/18/82	8/19/83
GRANNY SMITH	-	NEW ZEALAND	5/24/83	5/24/83

[a] FREEZE DRIED APPLES.

TABLE II.2 DETECTOR RESPONSE FACTOR(K), % RECOVERY AND REPRODUCIBILITY.

STANDARDS	K	%CV	t_R (min)	%CV	%RECOVERY	COLUMN
SUCROSE	1.12	6.4	4.91	0.1	91.9	AMINEX HPX-87
GLUCOSE	1.14	9.8	5.98	1.3	90.2	"
FRUCTOSE	1.06	4.7	8.11	0.3	86.1	"
SORBITOL	0.76	8.5	10.6	0.2	97.9	"
QUINIC	2.43	3.3	6.35	2.3	96.3	C18
MALIC	3.08	3.8	7.02	0.3	98.6	"
SHIKIMIC	226	3.9	8.60	0.7	77.5	"
CITRIC	4.32	5.3	10.7	0.1	88.6	"
FUMARIC	545	3.5	14.9	0.3	91.4	"

TABLE 11. 3 FREE SUGARS AND SORBITOL CONTENT OF APPLE JUICES[a].

APPLE JUICES	FRUCTOSE g/100mL	%TS	GLUCOSE g/100mL	%TS	G/F	SUCROSE g/100mL	%TS	TOTAL SUGARS g/100mL	SORBITOL g/100mL	%TS+S	TOTAL REDUCING SUCROSE
GOLDEN DELICIOUS (81,WA)	9.16	69.2	3.87	29.2	0.42	0.21	1.60	13.24	0.30	2.2	0.02
GOLDEN DELICIOUS (82,WA)	8.38	64.8	2.93	22.6	0.35	1.63	12.6	12.94	0.38	2.9	0.14
GOLDEN DELICIOUS (MEXICO)	7.72	58.7	2.29	17.4	0.30	3.15	23.9	13.16	0.28	2.1	0.31
GRANNY SMITH (NEW ZEALAND)	9.80	63.8	4.36	28.4	0.44	1.21	7.90	15.37	0.41	2.6	0.09
GRANNY SMITH (ARGENTINA)	9.74	65.8	4.00	27.0	0.41	1.07	7.20	14.81	0.39	2.6	0.08
DELICIOSA (ARGENTINA)	10.8	66.3	4.25	26.2	0.39	1.23	7.60	16.25	0.49	2.9	0.08
MCINTOSH(MI)	9.57	71.3	1.87	13.9	0.20	1.98	14.8	13.42	0.39	3.8	0.17
JONATHAN(MI)	4.27	67.9	1.41	22.4	0.33	0.61	9.70	6.290	0.39	4.3	0.11
MEAN	8.68	66.0	3.12	23.4	0.36	1.39	10.7	13.18	0.37	2.7	0.13
	[b](6.00)	(55.0)	(2.35)	(21.0)	(0.37)	(2.51)	(24.1)	(11.0)	(0.51)	(4.7)	
	[c](5.81)	(54.9)	(2.09)	(19.7)	(0.36)	(2.68)	(25.4)	(10.6)	(0.52)	(4.7)	
SD	2.00	3.81	1.16	5.44	0.08	0.90	6.63	3.03	0.11	0.47	0.09
	[b](0.59)	(4.87)	(0.84)	(6.77)	(0.14)	(0.94)	(7.72)	(1.63)	(0.33)	(2.71)	
	[c](1.22)	(6.62)	(0.68)	(5.21)	(0.11)	(1.01)	(8.47)	(1.80)	(0.21)	(1.30)	
%CV	23.1	5.80	37.0	23.3	22.2	64.6	62.2	23.0	29.6	17.2	69.2
	[b](9.80)	(8.80)	(35.8)	(32.2)	(38.2)	(37.2)	(31.9)	(14.8)	(64.2)	(57.2)	
	[c](21.0)	(12.1)	(32.4)	(26.5)	(29.2)	(37.7)	(33.4)	(17.0)	(40.8)	(28.0)	
RANGE	4.27-10.8	58.7-71.3	1.41-4.36	13.9-29.2	0.20-0.42	0.21-3.15	1.6-23.9	6.29-16.25	0.18-0.50	2.1-3.6	0.02-0.31
	[b](5.01-7.04	45.0-64.9	1.17-3.64	10.6-33.1	0.17-0.65	1.10-3.78	11.1-36	9.04-13.98	0.21-1.01	1.5-9.4)	
	[c](3.20-10.5	39.0-70.0	0.89-3.99	10.0-31.0	0.16-0.60	0.88-5.62	8.0-46.0	6.08-16.87	0.16-1.20	1.6-8.8)	

[a] VALUES ARE BASED ON 13.3°BRIX. [b] FROM WROLSTAD & SHALLENBERGER (26). [c] FROM MATTICK & MOYER (1).

TABLE II.4 NON-VOLATILE ORGANIC ACIDS IN APPLE JUICE[a]

APPLE JUICES	QUINIC mg/100ml	%	MALIC mg/100ml	%	SHIKIMIC mg/100ml	%	CITRIC mg/100ml	%	FUMARIC mg/100ml	%	TOTAL mg/100ml	pH
GOLDEN DELICIOUS (81, WA)	200	29.6	433	64.2	1.56	0.23	40.6	6.02	0.04	0.01	675	3.73
GOLDEN DELICIOUS (82, WA)	189	24.7	567	74.0	1.04	0.10	8.40	1.09	0.04	0.01	765	3.96
GOLDEN DELICIOUS (MEXICO)	61.8	10.1	548	89.6	1.05	0.17	0.95	0.16	0.18	0.03	612	3.96
JONATHAN(MI)	149	16.8	729	82.1	1.35	0.15	8.70	0.98	-	-	888	3.50
MCINTOSH(MI)	394	30.4	897	69.2	0.56	0.04	4.40	0.34	-	-	1295	3.43
GRANNY SMITH (ARGENTINA)	229	25.7	648	72.8	0.09	0.01	13.3	1.49	0.01	0.01	891	3.75
DELICIOSA (ARGENTINA)	313	38.6	490	60.5	2.04	0.25	5.35	0.66	-	-	810	4.25
GRANNY SMITH (NEW ZEALAND)	59.7	9.13	575	89.7	0.37	0.06	6.20	0.97	0.05	0.01	641	3.58
MEAN	199	23.2	611	75.3	1.00	0.13	10.4	1.46	0.06	0.01	822	3.77
SD	115	10.3	147	10.9	0.65	0.09	12.9	1.89	0.07	0.01	219	0.27
% CV	57.7	44.4	24.0	14.6	64.3	69.9	125	129	109	11.0	26.6	7.29
RANGE	59.7	9.13	433	60.5	0.09	0.04	0.95	0.16	0.01	0.01	612	3.43
	394	38.6	897	89.7	2.04	0.25	40.6	6.02	0.18	0.03	1295	4.25

[a] VALUES ARE BASED ON 13.3 °BRIX.

TABLE II. 5 COMPARISION BETWEEN HPLC AND ENZYMIC DETERMINATION OF MALIC ACID[a].

APPLE JUICES	HPLC(g/l)	ENZYMIC(g/l)	DIFFERENCES(g/l)	FUMARIC(g/l)
GOLDEN DELICIOUS (81,WA)	4.33	3.75	0.58	0.4
GOLDEN DELICIOUS (82,WA)	5.67	5.27	0.40	0.3
GRANNY SMITH (ARGENTINA)	4.90	4.78	0.12	-
GRANNY SMITH (NEW ZEALAND)	5.75	5.61	0.14	0.5
DELICIOSA (ARGENTINA)	4.90	4.78	0.12	-
MCINTOSH(MI)	8.97	7.61	1.36	-
JONATHAN(MI)	7.29	6.09	1.20	-
GOLDEN DELICIOUS (MEXICO)	5.48	5.27	0.21	1.8

[a]VALUES ARE BASED ON 13.3°BRIX.

TABLE 11.6 RESPONSE FACTOR AND REPRODUCIBILITY OF STANDARD PHENOLICS AT 324nm.

COMPOUNDS	RESPONSE FACTOR (PEAK AREA/µg)	%CV	RETENTION TIME (min)	%CV	COLUMN
HMF	240	7.4	5.5	4.1	C18
CHLOROGENIC	3400	3.4	8.7	2.2	"
CAFFEIC	5110	8.3	10.7	2.0	"
P-COUMARIC	4640	6.6	14.2	1.4	"
FERULIC	5360	9.5	15.8	1.4	"

TABLE II. 7 PERCENT DECREASE OF PEAK AREAS AFTER GELATIN TREATMENT.[a]

PEAKS[b]	% DECREASE OF PEAK AREA
1	0.00
2	4.70
3	30.2
4	33.5
5	49.6

[a] =0.04 (%w/v).

b =McIntosh.

TABLE II.8 CONCENTRATION OF HMF AND CHLOROGENIC ACID IN APPLE JUICES[a]

APPLE JUICES	HMF(mg/l)	CHLOROGENIC(mg/l)	HMF/CHLOROGENIC
GOLDEN DELICIOUS (81,WA)	2.98	3.35	0.89
GOLDEN DELICIOUS (82,WA)	2.02	3.75	0.54
GOLDEN DELICIOUS (MEXICO)	14.4	67.2	0.21
GRANNY SMITH (NEW ZEALAND)	9.80	35.9	0.27
GRANNY SMITH (ARGENTINA)	12.8	1.50	8.53
DELICIOSA (ARGENTINA)	15.4	6.40	2.41
MCINTOSH(MI)	5.85	228	0.03
JONATHAN(MI)	1.84	30.0	0.06
MEAN	8.14	47.0	1.62
SD	5.68	76.6	2.89
% CV	69.8	162.9	179.2
RANGE	1.84-15.4	1.5-228	0.03-8.53

[a] VALUES ARE BASED ON 13.3°BRIX.

TABLE II.9 Rf VALUES, COLOR REACTION AND ABSORPTION CHARACTERISTICS OF POLYPHENOLIC COMPOUNOS FROM MCINTOSH APPLE JUICE.

SPOT	Rf BAW	Rf 5% HOAc	UV	UV/NH_3	OPNA	VANILLIN	λ MAX (nm)	λ MIN (nm)	$AlCl_3$	NaOAc	NaOAc/H_3BO_3	NaOMe
1	0.70	0.22	BB	SBG	BRN	-	328,320[a]	267	-	-15	-	+16
2	0.84	0.38	PB	SBG	BRN	-	325,286	269	-	-	-	+30
3	0.78	0.31	BB	FG	8RN	-	325	256	-	-	-	+30
4	0.87	0.49	P	P	8RN	-	307,296[a]	246	-	-	-	+45
5	0.65	0.42	C	C	-	PINK	-	-	-	-	-	-
6	0.80	0.70	SPB	SBG	F8RN	-	321,278	263	-	-	-	+30
7	0.72	0.65	SPB	SYG	FBRN	-	327	268	+3	-	-	+40
8	0.66	0.60	SPB	SYG	FBRN	-	323,318[a]	260	+4	-	-	+40
9	0.62	0.54	SPB	SYG	FBRN	-	325	262	+2	-3	-	-
10	0.65	0.62	FB	FB	FBRN	-	-	-	-	-	-	-
11	0.61	0.70	FB	FB	F8RN	-	-	-	-	-	-	-
12	0.51	0.35	C	C	-	PINK	-	-	-	-	-	-
13	0.70	0.82	FB	SYG	FBRN	-	-	-	-	-	-	-
14	0.65	0.80	FB	SYG	FBRN	-	-	-	-	-	-	-
15	0.56	0.00	C	C	-	-	-	-	-	-	-	-
16	0.84	0.33	P8	SBG	8RN	-	320,293	268	-	-	-	+45

a = SHOULOER, B=BLUE, C=COLORLESS, G=GREEN, Y=YELLOW, P=PURPLE, B=BRIGHT, F=FAINT, S=SLIGHT.

TABLE II.10 ABSORPTION MAXIMA ANO Rf VALUES OF STANDARO POLYPHENOLS.

COMPOUNOS	SPECTRUM IN MeOH MAX.	MIN.	Rf BAW	5% HOAc	COLOR UV	VIS.
CAFFEIC	316, 287[a]	256	0.75	0.21,0.56	SB	-
CHLOROGENIC	324, 298[a]	264	0.64	0.59,0.70	SB	-
P-COUMARIC	308, 298[a]	244	0.85	0.34,0.67	OP	-
FERULIC	312, 292[a]	254	0.80	0.28,0.59	PB	-
KAEMPEROL	364, 320[a]	282	0.76	0.0	C	Y
QUERCETIN	356, 310[a]	281	0.54	0.30	C	FY
CATECHIN	279	250	0.63	0.37	C	PINK
(-)EPICATECHIN	279	250	0.52	0.29	C	PINK
PHROLIDZIN	283	244	0.72	0.32	FT	-
PHLORETIN	283	244	0.84	0.01	FT	-
RUTIN	352	280	0.55	0.31	FT	-

[a] IS SHOULDER.

S=SLIGHT, B=BLUE D=DARK, P=PURPLE , C=COLORLESS, F=FAINT, T=TAN

Y=YELLOW

TABLE II.11 ABSORPTION CHARACTERISTICS OF APPLE JUICES.[a]

APPLE JUICES	A320nm	A280nm	SUM A320+A280nm	RATIO A320/A280
GOLDEN DELICIOUS(81,WA)	0.178	0.280	0.458	0.64
GOLDEN DELICIOUS(82,WA)	0.154	0.234	0.388	0.66
GOLDEN DELICIOUS(MEXICO)	0.370	0.556	0.908	0.63
MCINTOSH(MI)	0.469	0.621	1.090	0.76
JONATHAN(MI)	0.225	0.486	0.711	0.48
GRANNY SMITH(ARGENTINA)	0.193	0.329	0.522	0.59
DELICIOSA(ARGENTINA)	0.232	0.380	0.612	0.61
GRANNY SMITH(NEW ZEALAND)	0.178	0.386	0.564	0.46
GOLDEN DELICIOUS (81,WA,RECONSTITUTE)	0.238	0.352	0.590	0.69
JONATHAN (MI,RECONSTITUTE)	0.265	0.522	0.787	0.50
MEAN	0.248	0.415	0.663	0.60
SD	0.096	0.126	0.215	0.10
%CV	38.7	30.4	32.4	16.7
RANGE	0.154	0.234	0.388	0.46
	0.469	0.621	1.090	0.76

[a] VALUES ARE BASED ON 13.3°BRIX.

TABLE II.12 ABSORPTION CHARACTERISTICS OF APPLE JUICE AFTER BLENDING WITH SEEDLESS WHITE GRAPE, PEAR AND PINEAPPLE JUICE CONCENTRATES WITH DIFFERENT LEVELS.[a]

BLENDING(%)		A320nm	A280nm	SUM A320+A280nm	RATIO A320/A280nm
APPLE JUICE	100	0.240	0.369	0.609	0.65
GRAPE JUICE	10	0.189	0.293	0.482	0.65
	20	0.177	0.278	0.455	0.64
	30	0.178	0.288	0.466	0.62
	40	0.172	0.288	0.460	0.60
	50	0.173	0.298	0.471	0.58
	100	0.159	0.317	0.476	0.50
PEAR JUICE	10	0.119	0.182	0.301	0.65
	20	0.203	0.313	0.516	0.65
	30	0.153	0.238	0.391	0.64
	40	0.218	0.341	0.559	0.64
	50	0.231	0.366	0.597	0.63
	100	0.254	0.409	0.663	0.62
PINEAPPLE JUICE	10	0.197	0.325	0.522	0.61
	20	0.197	0.350	0.547	0.56
	30	0.142	0.275	0.417	0.52
	40	0.212	0.438	0.650	0.48
	50	0.211	0.460	0.671	0.46
	100	0.240	0.643	0.883	0.37

[a] VALUES ARE BASED ON 13.3°BRIX.

TABLE II.13 FLUORESCENCE EXCITATION AND EMISSION MAXIMA.

JUICES	EXCITATION MAXIMA(nm)	EMISSION MAXIMA(nm)
GOLDEN DELICIOUS(81,WA)	284	360
GOLDEN DELICIOUS(82,WA)	285	360
GOLDEN DELICIOUS(MEXICO)	285	355
MCINTOSH(MI)	284	365
JONATHAN(MI)	284	335
GRANNY SMITH(ARGENTINA)	290	325
DELICIOSA(ARGENTINA)	296	361
GRANNY SMITH(NEW ZEALAND)	290	338
PEAR	280	341
SEEDLESS WHITE GRAPE	285	350
PINEAPPLE	300	350

TABLE II.14 ABSORPTION CHARACTERISTICS OF APPLE JUICE.

APPLE JUICES	MeOH λ max.(nm)	PVPP λ max.(nm)	DERIVATIVES 1st. (ΔA=0)	2nd. max.	2nd. min.
GOLDEN DELICIOUS (81,WA)	320 278	321 276 223	314 276	285 274 230	280 272 215
GOLDEN DELICIOUS (82,WA)	319 277	323 278 223	314 276	284 276 229	280 270 214
GOLDEN DELICIOUS (MEXICO)	319 280	320 279	314 274	282 235 212	278 216 208
JONATHAN(MI)	320 279	322 277 224	314 275 234	282 230 212	278 224 208
MCINTOSH(MI)	319 280	322 284	313 278	320 316 292 282 274 248 233 228 220 216	318 312 286 276 268 244 232 222 212 208
GRANNY SMITH (ARGENTINA)	319 278	320 278	316 274 220	284 258 235 220 212	276 252 226 216 209
DELICIDSA (ARGENTINA)	323 277	320 279	314 274 234	284 268 240 224 218 210	274 264 234 220 216 208
GRANNY SMITH (NEW ZEALAND)	319 278	323 278 223	318 274 220	280 264 229	268 260 220

TABLE II.15 PEAK AMPLITUDE RATIO IN SECOND-ORDER DERIVATIVE SPECTRA.

APPLE JUICES	RATIO(PEAK AB/ PEAK CD)
GOLDEN DELICIOUS(81,WA)	0.22
GOLDEN DELICIOUS(82,WA)	0.25
GOLDEN DELICIOUS(MEXICO)	0.30
JONATHAN (MI)	0.40
MCINTOSH (MI)	0.38
GRANNY SMITH(ARGENTINA)	0.51
DELICIOSA (ARGENTINA)	0.61
GRANNY SMITH(NEW ZEALAND)	0.27
PEAR	0.14
SEEDLESS WHITE GRAPE	0.08
PINEAPPLE	0.04

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TITLE: ISOTOPIC COMPOSITION OF CARBON IN APPLES AND THEIR SUBFRACTIONS: JUICE, SEEDS, SUGARS, ACIDS, AND PHENOLICS.

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RUNNING HEAD: Apple Juice Composition

Technical Paper No._____ from the Oregon Agricultural Experiment Station.

ISOTOPIC COMPOSITION OF CARBON IN APPLES AND THEIR SUBFRACTIONS: JUICE, SEEDS, SUGARS, ACIDS, AND PHENOLICS.

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ABSTRACT

The $^{13}C/^{12}C$ ratios of 8 authentic apple juices and their major constituents: pulp, seed, sugars, organic acids and phenolics were determined by Mass spectrometry. Sugars and organic acids were separated by ion-exchange chromatography; Polyvinylpyrrolidone (PVPP) was used to isolate phenolics from apple juices. Pulp and seeds were collected from macerated fruit and press-cake respectively. The mean value for all juice samples was -24.2 ppt and the coefficient of variation was 2.36%. Foreign samples from Argentina, Mexico and New Zealand did not differ from domestic samples. The isotopic composition of subfractions ranged from -22.0 to -31.0 ppt. The non-volatile organic acid fraction yielded more negative values than the sugar fraction. The phenolic fraction showed the most enriched in ^{12}C. Linear regression between the juices, pulp, and sugars showed a high correlation, but the acid correlation was low.

INTRODUCTION

Isotopic carbon measurements are currently used to monitor the purity of apple juice. Doner et al.(1) measured the variability of the $^{13}C/^{12}C$ ratio in 40 pure apple juices to determine base line values for a method to detect the undeclared addition of corn or cane syrups in apple juice. A collaborative study (2) was conducted to determine the precision and accuracy of stable isotope ratio analysis (SIRA). Doner and Phillips(2) reported that samples with del ^{13}C values less negative than -20.2 ppt, 4 standard deviation from the mean of pure juices, can with a high degree of confidence, be classified as adulterated. The Processed Apples Institute(PAI) reported(3) del ^{13}C values from 31 different apple juice samples over 3 years, the results agreeing closely with Doner et al.(1). Brause and Raterman (4) analysed the del ^{13}C values from commercial apple juices and proposed values more positive than -22.0 ppt should be considered as adulterated. Coppola(5) recently described the detection of gross adulteration of apple juice and considered the $^{13}C/^{12}C$ ratio, more positive than -22.1 ppt as adulterated with cane or corn sugar.

Many reports(6-8) indicate that environmental effects may account for some of the isotope fractionations observed in organisms. The isotopic compositions of various subtractions of the plant also have been investigated by number of workers(9,10). Jacobson et al.(11) found that amino acids contain less ^{13}C than organic acids, which in turn contains less ^{13}C than sugars, in

potato tubers.

One objective of this study was to expand the data base in the literature to include foreign samples. We are also interested in seeing how chemical composition influences SIRA and if analysis of subfractions would offer potential for increased sensitivity in detecting adulteration.

METHODS

REGEANTS :

(a) Ion-exchange resins : Wash 2 g cation exchange resin(Bio-Rad, AG 50W-X4, 200-400 mesh, hydrogen form) with 25 mL deionized water. Wash 2 g anionic exchange(Bio-Rad AG 1-X8, 200-400 mesh, acetate form) with 2 times of 5 bed volume of 1N NaOH. Rinse with deionized water until eluant is less than pH 9.

(b). Polyvinylpyrrolidone polymer(PVPP) : Wash PVPP according to procedure described by Loomis(12).

ISOLATION OF SAMPLES :

Samples of pulp was taken after milling in the pilot-plant manufacture of the juice(13) and seeds were collected from press-cake. For sugars; Pass 20 mL apple juice through a 15 mm id X 7 cm column containing AG 50W-X4, cation exchange resin, and a 15 mm id X 7 cm column containing AG 1-X8, anion exchange resin. Wash with deionized water and collect 200 mL eluant in volumetric flask.

For acids: recover acids from anionic column by washing with 50 mL 15% H_3PO_4, followed with 50ml deionized water and collect in volumetric flask.

For phenolics: apply 300mL of juice to 7.0 cm Buchner funnel containing 50 g hydrated PVPP on whatman No.1 paper. Rinse PVPP and sample residue 10 300 mL portions of deionized water. Recover PVPP adsorbate and place in column. Elute phenolic compounds with 10

300 mL portions of absolute methanol followed by 3 300 mL portion of 0.1% methanolic HCl.

Concentrate sugar, acid and phenolic extracts to dryness on rotary evaporator(water bath temperature, 35 ^{o}C, vacuum 30" Hg). Dissolve each fraction in 50 mL of deionized water. Freeze Sugar, acid, phenolic and pulp fractions at -30 ^{o}F during overnight; freeze dry(temperature -50 ^{o}C, vacuum 30" Hg, Hull Co., Hatboro,PA). For Seeds; dry(vacuum oven, 50 ^{o}C, 10" Hg), crush and powder with mortar and pestle.

DETERMINATION OF DEL ^{13}C VALUE :

Samples are sent to Coastal Science Laboratories, 5321 Industrial Oaks Blvd. Suite 103, Austin, Texas 78735 for combustion and analysis.

RESULTS AND DISCUSSION

The del ^{13}C values for 8 apple juice samples, their subfractions and previously reported values are summarized together in Table III. 1. For the 5 different geographical origins and 2 different growing seasons of apples, the isotope composition in each fraction of apple is quite uniform. There are no significant differences in del ^{13}C values with regard to apple variety or different geographic origins such as Mexico, New Zealand, Argentina and United States.

The overall mean value of the juice is -24.2 ppt with a standard deviation of 0.57. The range from -23.4 to -25.1 ppt falls within those of the previous works (1,2,3) with a lower % CV, probably because of the small sample size in this study. In the range of del ^{13}C value for sub-fractions, sugars and organic acids are from -22.2 to -24.8 ppt with 4.1% CV and from -25.4 to -29.6 ppt with 5.2 % CV respectively. The range and % CV of juices still have less variation than those of the sugars and organic acids.

The pulp fraction has an essentially identical value with the juice. The sugars showed slightly less negative values than the juice and a higher % CV than those of the juice and pulp. The organic acids are enriched by about 3.1 ppt in ^{12}C with respect to the total carbon of juice, revealing a more negative value than the sugars. This agrees with previous observation in potato (11). While only two phenolic samples were analyzed, that fraction showed the most enrichment. A variety of studies (10,11,14,15) have indicated that different metabolites of a particular class have quite different isotopic composition, and subsequent metabolism of photosynthetic products is also accompanied by isotope fractionation.

Regarding the influence of composition on the del ^{13}C values of apple juice, Figure III. 1-3 illustrates the linear regression between del ^{13}C values of juice and each fraction. Pulp has the highest correlation (r=0.93) and sugars also showed a high correlation (r=0.82). As carbohydrate contributes to about 97%

of the apple juice solids(1,16) and also constitutes most of the soluble solid materials in the flesh of the apple(17), the high correlation between pulp, sugars and juice is not suprising. High correlation pulp and juice suggests a useful procedure for more sensitive detection of HFCS in "natural" apple juice which contains pulp. Parker(18) isolated the soluble, juice and pulp fraction to detect the low concentration (5%) of HFCS addition. Since HFCS will reside mostly in the soluble fraction, differences between the pulp and soluble fraction can enhance the sensitivity. But the acid fraction did not indicate a significant relationship (r=-0.14).

In Figure III. 4, the correlation between del ^{13}C values of juices and total sugar content is shown. It suggests possible correlation with total sugar content (r=0.65). To further test this possible relationship, we ran the linear regression between del ^{13}C values (3) and total sugar content of the 93 samples reported in the Processed Apples Institute(PAI) sponsored study (3,19). The correlation was 0.31. Figure II.5 shows the relationship between the del ^{13}C of juice and total acid content. No apparent relationship (r=0.16) was found. A similar result (r=-0.13) was obtained when we tested the correlation between total acidity and del ^{13}C of the 93 samples of A'brams(3) and Mattick and Moyer's (19) works. Wide variability (19,20) and small proportion of total acids (0.2-1.0 % as malic) when compared to sugars(19,21,22) may explain the low relationship. The proportion of the total carbon in the phenolic fraction for apple juice is also small and unlikely to significantly affect the del

^{13}C value for the juice, even though that fraction is most enriched in ^{12}C. Total sugars and total acidity do not show high correlations between del ^{13}C values. Previous studies (1-4) did not reveal any relationship between del ^{13}C and variety or growing region. There is no reliable predictor of del ^{13}C. Thus an adulterator practicing low level adulteration (10-20 %) with corn syrup or cane sugars runs the risk of having samples outside the range of data base (less negative than -20.2 ppt) unless he has del ^{13}C analyses for each lot.

Figure III. 6 shows the correlation between the sugar and acid fractions. One might expect a sample with a less negative sugar del ^{13}C value to have a correspondingly less negative del ^{13}C acid value. However, a low correlation resulted. By eliminating two outlying samples, r increased to 0.77. This suggests that a more negative sugar value is accompanied by a more negative del ^{13}C value for acids, but this needs to be documented with larger sample numbers.

It has been observed (23) that less variation for del ^{13}C values occurs with orange juice than with apple or honey. This allows for more sensitive detection of HFCS in the first commodity. A more restrictive geographic origin and less variation in composition(24,25) of orange juices compare to apple juices may contribute to this uniformity. Those variations are more marked in C_3 plant which exhibits a wider range of isotope values than C_4 plant(26,27). Wide variability in del ^{13}C value of apple juice has been considered in detecting the minor adulteration of

products. It may be suggested that the wide geographic distribution, environmental and intrinsic variations in del ^{13}C values are major factors. Our preliminary experiment indicates that small proportions of total acids and phenolics do not seem to be significant when compared to the natural variability in the isotopic composition of carbon in apple juices.

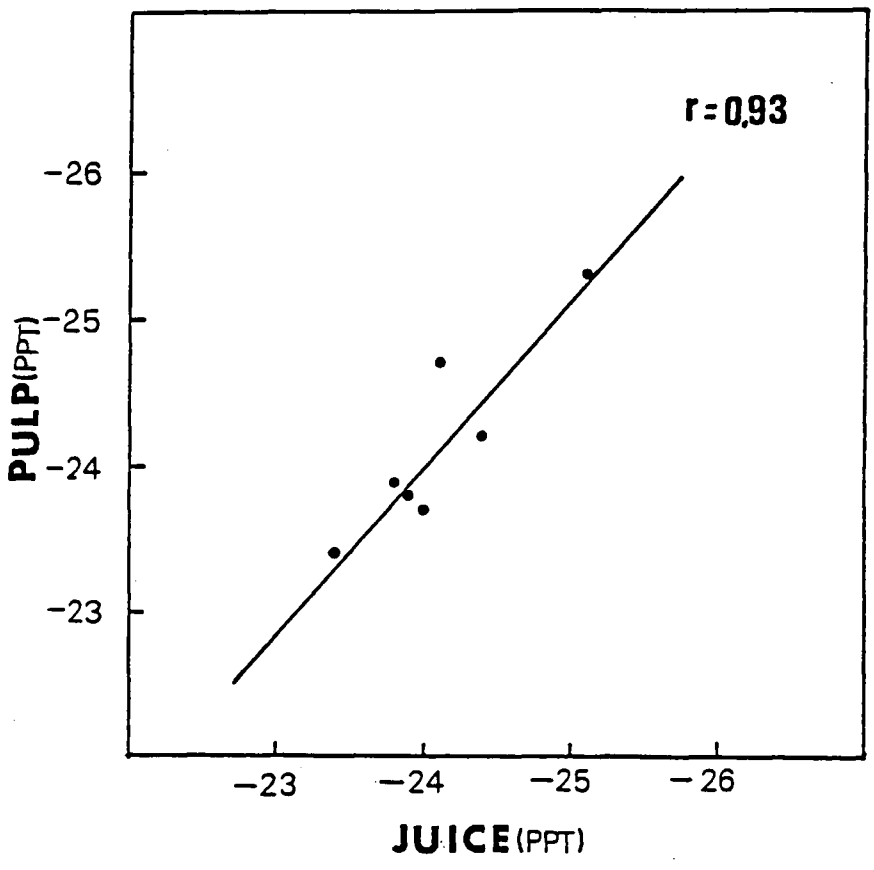

FIGURE III.1 CORRELATION BETWEEN DEL ^{13}C OF JUICE AND THE PULP.

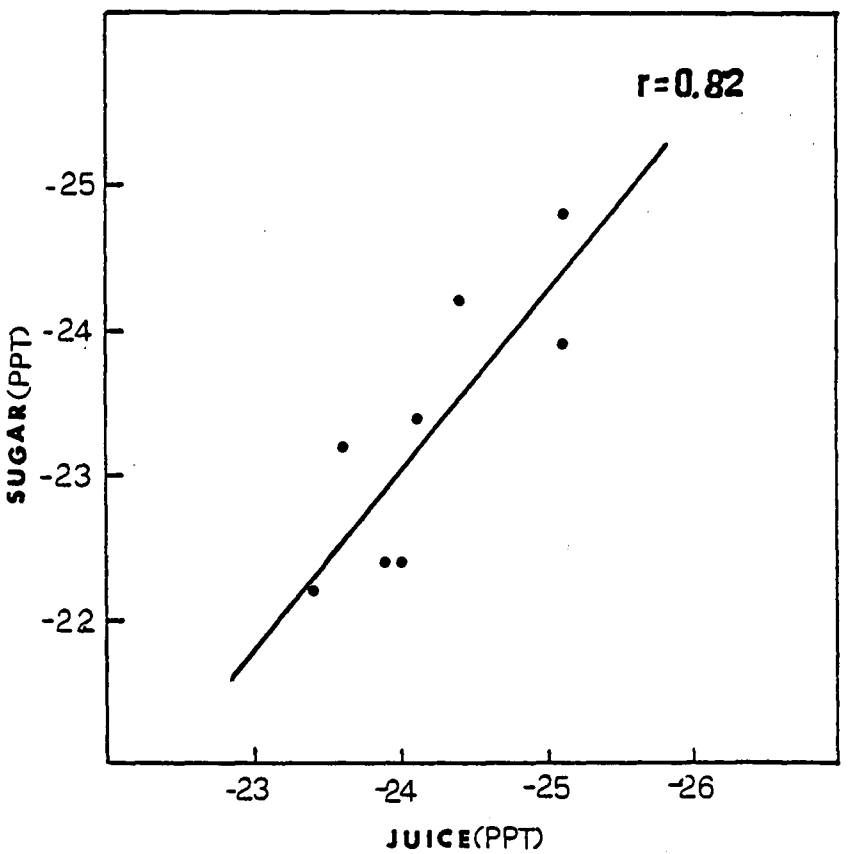

FIGURE III.2 CORRELATION BETWEEN DEL ^{13}C OF JUICE AND THE SUGAR.

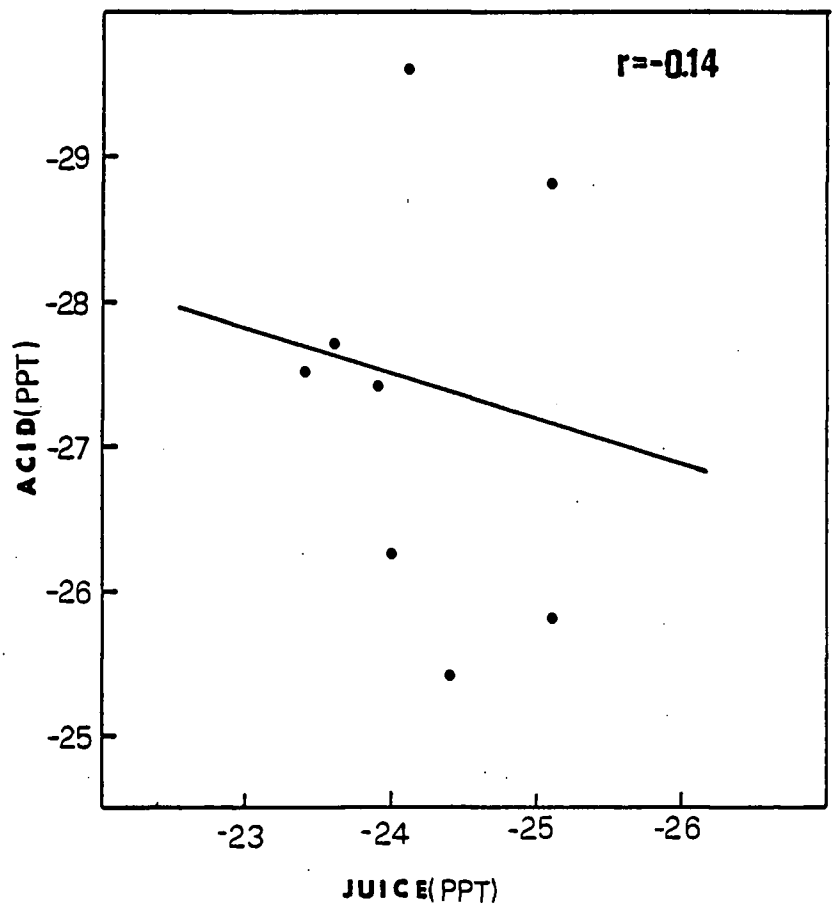

FIGURE III.3 CORRELATION BETWEEN DEL ^{13}C OF JUICE AND THE ACID.

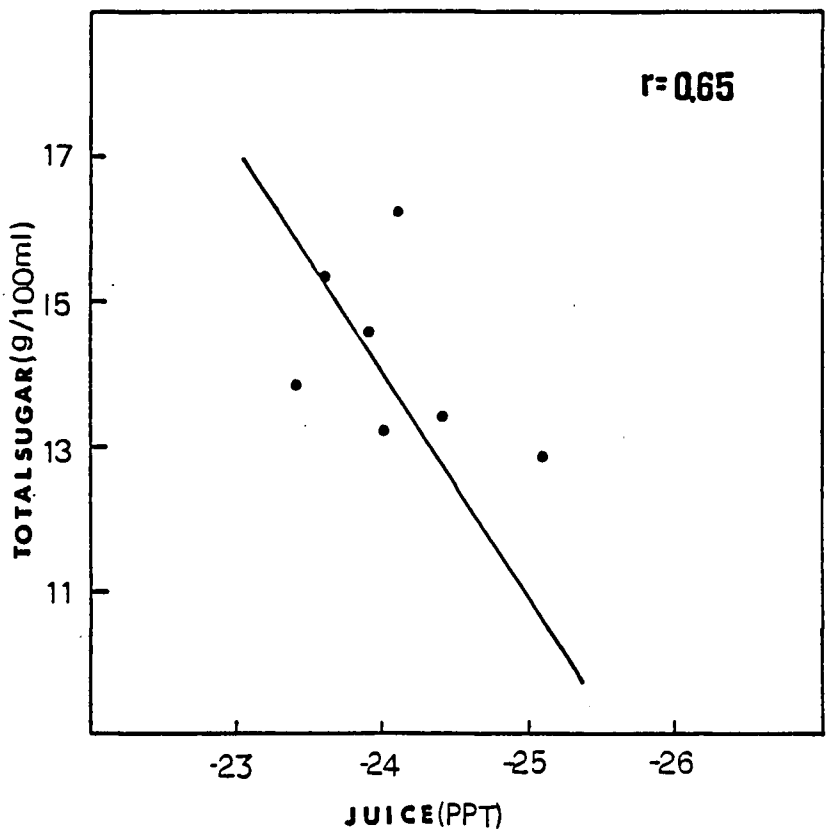

FIGURE III.4 CORRELATION BETWEEN DEL ^{13}C OF JUICE AND THE TOTAL SUGARS.

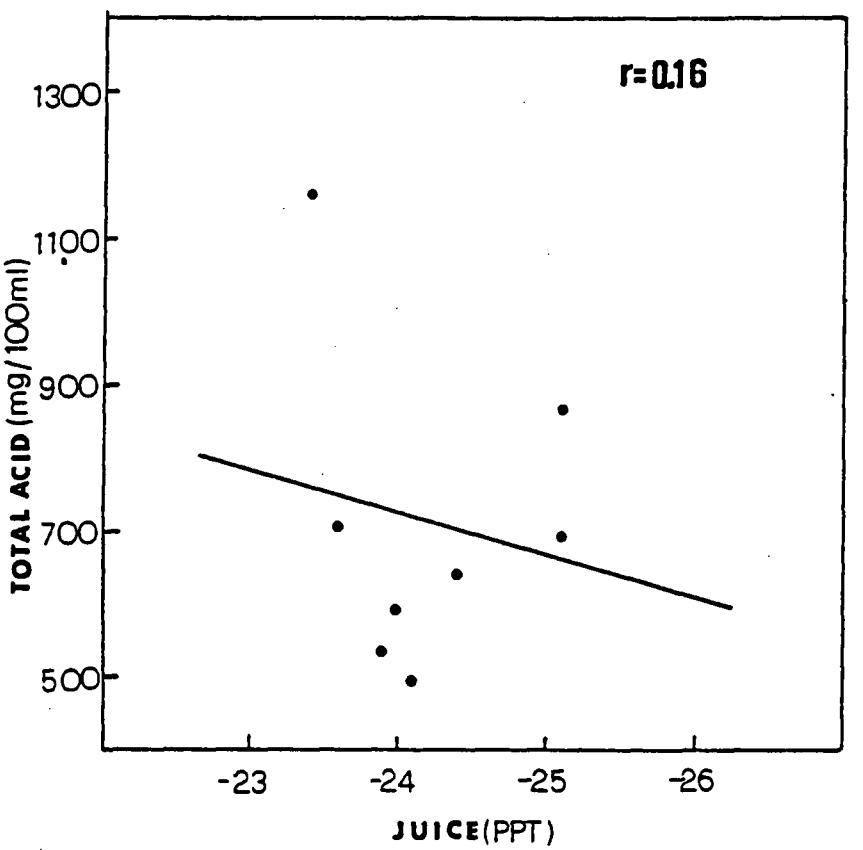

FIGURE III.5 CORRELATION BETWEEN DEL ^{13}C OF JUICE AND TOTAL ACIDS.

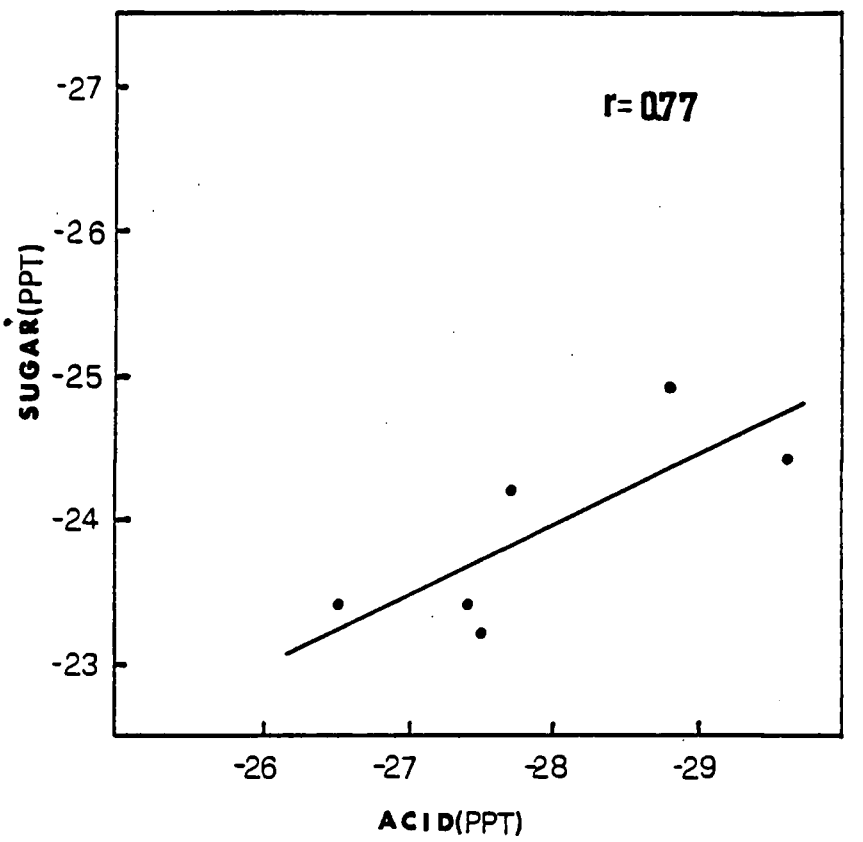

FIGURE III.6 CORRELATION BETWEEN DEL ^{13}C OF ACID AND THE SUGAR.

TABLE III.1 OEL ^{13}C VALUES FOR APPLE JUICES

JUICES	NO.OF SAMPLES	OEL ^{13}C PDB± 0.2 PER MIL. PULP	JUICE	SUGARS	ACIDS	PHENOLICS	SEEDS
GOLDEN DELICIOUS (81,WA)	1	-23.7	-24.0	-22.1	-26.5		-24.8
GOLDEN DELICIOUS (82,WA)	1	-25.3	-25.1	-22.0 -24.8	-25.8		
MCINTOSH(MI)	1	-23.4	-23.4	-22.2	-27.5		
JONATHAN(MI)	1	-25.3	-25.1	-23.9	-28.8		
GRANNY SMITH (ARGENTINA)	1	-23.7 -23.8	-23.8 -24.0	-22.4 -22.4	-27.5 -27.2		
DELICIOSA (ARGENTINA)	1	-24.7 -24.7	-24.0 -24.1	-23.4 -23.4	-29.6		
GRANNY SMITH (NEW ZEALAND)	1		-23.6	-23.2	-27.7	-31.0	
GOLDEN DELICIOUS (MEXICO)	1	-24.2	-24.4	-24.2 -24.3	-25.4 -25.5	-26.5	
MEAN		-24.3	-24.2 [a](-25.3)[b](-25.6)	-23.2	-27.2	-28.8	
SD		0.76	0.57 [a](1.28),[b](1.05)	0.97	1.39		
%CV		3.17	3.14 [a](5.04),[b](4.10)	4.18	5.11		
RANGE		-23.4 -25.3	-23.4 -25.1 [a](-22.5 -27.9) [b](-22.5 -27.2)	-22.0 -24.8	-25.4 -29.6	-26.5 -31.0	

[a]FROM OONER & PHILLIPS(2), [b]FROM A'BRAMS(5).

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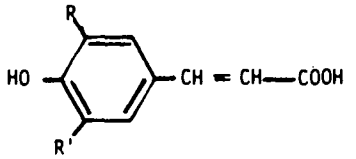
APPENDIX

APPENDIX

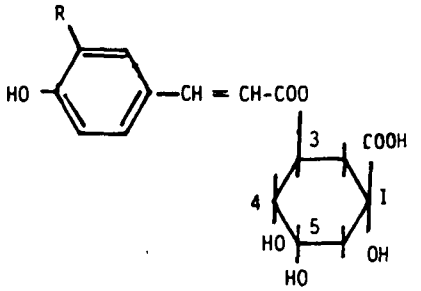
SOME OPEN CHAIN PHENYLPROPANE DERIVATIVES AND STRUCTURES.



R=R'=H; P-COUMARIC ACID

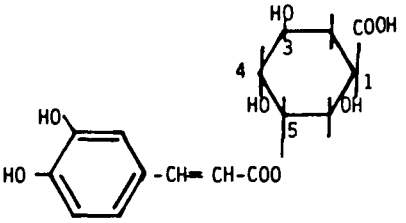
R=OH, R'=H; CAFFEIC ACID

R=OCH_3, R'=H; FERULIC ACID

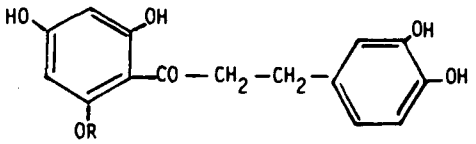


R=OH CHLOROGENIC ACID

R=H P-COUMAROYLQUINIC ACID



NEOCHLOROGENIC ACID



R=H; PHLORETIN
R=GLUCOSE; PHLORIOZIN