A Chain Analysis of the Production of ‘Healthy’ Apple Juice. The Case of Polyphenolic Antioxidants.

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A Chain Analysis of the Production of 'Healthy' Apple Juice. The Case of Polyphenolic Antioxidants.

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CHAPTER 1

Introduction. Polyphenolic Antioxidants in Apple and Apple Juice

1.1. HEALTH AS A QUALITY PARAMETER IN THE FOOD CHAIN

Nowadays there is an increasing interest among consumers on how food products can contribute to their health. Chronic diseases such as cardiovascular diseases, cancer, diabetes, obesity, osteoporosis and dental disease are a growing burden to which, in 2001, worldwide, approximately 59% of the reported deaths and 46% of the diseases could be attributed. Due to shifting dietary patterns together with decreased physical activity, these numbers are expected to increase. Diet is assumed to play a key role as a risk factor for chronic diseases (*1*). High intake of fruits and vegetables has been widely acknowledged in epidemiological research to have a protective effect against various cancers (*2*, *3*, *4*), and to provide protection against cardiovascular disease (*5*, *6*, *7*). However, in a recent large prospective study (with a follow-up of 5.4 years) no association was found between total or specific vegetable and fruit intake with breast cancer risk (*8*), and in another prospective study (follow up of 12–14 years) no association between fruit and vegetable intake (either total or of any particular group) and overall cancer incidence was observed (*7*). In the last study an association with protection against cardiovascular diseases was confirmed.

In 1976 Kühnau suggested that apart from vitamins and trace elements, secondary plant metabolites, as part of our diet, are necessary for maintaining or improving health or general well being. He referred to phenolic acids and their derivatives, and flavonoids (*9*). Flavonoids have been regarded as interesting compounds for a long time, mainly due to their contribution to plant and fruit color (*10*). Nowadays inverse relations with aging diseases such as coronary heart diseases have been described for flavonoids (*11*, *12*, *13*, *14*), which greatly renewed interest in these components. But apart from flavonoids, other health-protecting substances in fruits and vegetables are present, e.g. potentially anticarcinogenic compounds such as carotenoids; organosulfides; glucosinolates, phytosterols; folic acid; vitamin C and E; dietary fiber, and protease inhibitors (*3*, *6*, *15*, *16*). These compounds all may function on their own, but also interact with each other and with other components present in the food matrix. Their metabolites are of interest as well.

Not all fruits and only a few vegetables are consumed raw; often they are processed further in industry or at home. In food production and processing many chemical and physical reactions take place (desired as well as undesired), which affect the

structure and composition of a product, and as a result the effect of a product on health may be affected as well. Parameters that are generally used to describe food quality are taste; flavor; color; texture; mouthfeel; microbiological and toxicological safety, nutritive value and keepability. In product design the effects of ingredients and processes on these parameters are investigated. The health protective capacity of a product should be treated as a quality parameter as well, especially if a product is positioned as a healthy food. To be able to do so, the health protective capacity of a food product should be measurable and quantifiable (*17*), and there should be an underlying mechanism or hypothesis relating specific components present in the product with health. This underlying mechanism or hypothesis determines which test system needs to be used for measuring the bioactivity of the compounds of interest, where bioactivity of a compound indicates that it has an impact on metabolism (e. g. the stimulation of detoxification enzymes) (*18*).

As apple is a well-known source of flavonoids and a product that can be eaten raw, but which is processed also to a variety of products, it was chosen as a test product in this thesis to explore possible changes that may occur in the food chain (from harvest, storage until processing and further storage of the product).

Cultivation methods, choice of raw material (apple cultivar), industrial processing, storage procedures, distribution and final processing by the consumer may all affect the final concentrations of the compounds present and also the bioactivity of the product (*19*). Knowledge of these aspects will provide the food processor with information focused on health protecting compounds that can be used in product design and optimization. Furthermore, the changes that occur in the production and processing chain are important for food composition studies and dietary intake estimations in epidemiological research, because quantitative information about variability in active components and processing effects will help in interpreting results of epidemiological studies (*20*, *21*).

1.2. APPLE

The apple belongs to the family of the Rosaceae and the genus Malus. *Malus pumila* (formerly *M. communis* or *Pyrus malus*) is considered to be the parent of most of the cultivated apples. Examples are *M. sylvestris* (wild crab apple) and *M. domestica* (cultivated apple). Botanically speaking a fruit is the ripened ovary, together with its seeds, of a flowering plant, but mostly the term fruit is used to refer to the part of the

seed suitable for human consumption. Apple is a false fruit, as it is mature ovaries fused together with many closely associated parts. Five ovaries of the flower are imbedded in tissue that becomes fleshy and edible (*22*).

1.2.1. Composition.

Apple is composed of water (85%); carbohydrates (11%); dietary fibre (2%); fat (0.6%); organic acids (0.5%); and protein (0.3%). Malic acid is the most abundant acid; furthermore phenolic acids (such as caffeic acid and chlorogenic acid) and vitamins are present. Vitamin C is the most important vitamin in apple with a mean concentration of 120 mg/kg of fresh weight (fw). The observed variation in compound concentrations can be large, e.g. for vitamin C values between 30 and 250 mg/kg are reported. As “special bioactive compounds” present in apple are described (flavonols analyzed as aglycon): quercetin 49 mg/kg; kaempferol 3.3 mg/kg; isorhamnetin 12 µg/kg; glutathione 6.1 mg/kg (*23*).

Quercetin, kaempferol and isorhamnetin are examples of flavonoids; the latter belong together with (poly)phenolic acids to the compound class of the polyphenolics. Polyphenols are compounds in which phenolic hydroxyl groups (hydroxyl groups connected to an aromatic ring) are present (*24*). They are secondary plant metabolites and are therefore found in fruits and vegetables. Primary metabolites (e.g. amino acids, sugars, fatty acids) are involved in metabolism, growth, maintenance and survival of plants, whereas secondary plant metabolites are synthesized in plants from a few key intermediates of primary metabolism (*25*). They are produced usually at relatively low concentrations in the plant tissues and accumulate there.

There are great interspecies differences in polyphenolic contents of fruits and vegetables, qualitatively and quantitatively. Between varieties of the same species also large differences may exist. Furthermore flavonoids are not distributed equally within the fruit: differentiation between fruit tissues (pulp, seeds, skin, etc) occurs (*24*, *26*). A large variety in polyphenolic compounds exists. The simple forms are water soluble and found mainly in vacuoles, polymerized forms can be less soluble and lignins are completely insoluble in water.

1.2.1.1. Flavonoids.

In plants more than 6400 flavonoid compounds have been found up to now and in general they are glycosylated, hydroxylated, acylated or methoxylated (*27*, *28*). Their basic structure is derived from the 2-phenyl-benzo-γ-pyrane or flavane nucleus (*9*), see Figure 1. Flavonoids can be subdivided in various classes that differ in the oxidation level of the central pyran ring. Three of the classes are very widespread in fruits and are quantitatively dominant: anthocyanins, flavonols, and flavanols (Figure 1). The other classes are less abundant (except in certain special cases, such as *Citrus*): flavones; flavanones; flavanonols or dihydroflavonols; chalcones; dihydrochalcones. Isoflavonoids are another large and very distinctive subclass of the flavonoids. They have a limited distribution in the plant kingdom: they are almost entirely restricted to the subfamily *Papilionoideae* of the *Leguminosae* of which soy is an example (*29*).

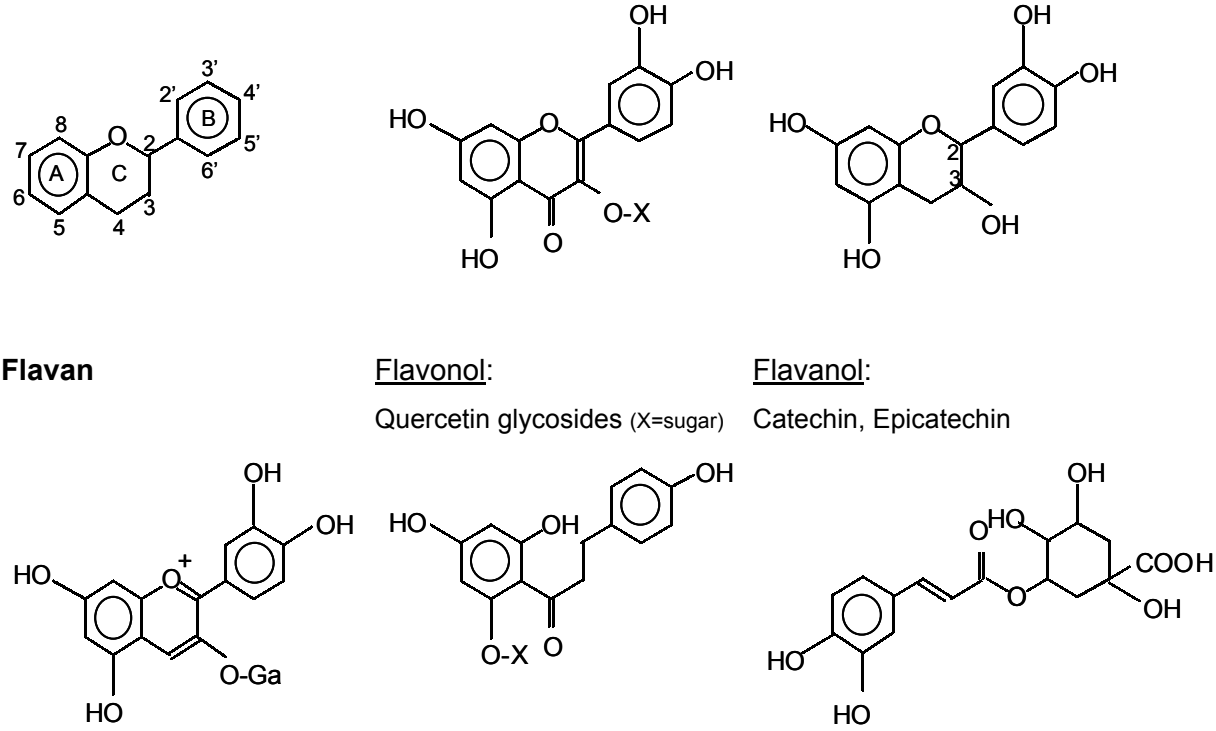

Figure 1. Structures of polyphenolic compounds present in apples (*24*, *30*, *31*, *32*, *33*).

Certain flavonoids contribute to flower color, thereby attracting insects (and playing a role as pollination and seed dispersal factors). They also impart to fruit color as copigments, thus stimulating the appetite for food. Furthermore, in food they impart to flavor by providing astringency (catechins and leucoanthocyanidins) and taste (*34*).
Flavonoids undergo changes (usually oxidative) during storage and processing of the raw plant material (*34*), knowledge on their (bio)-chemical and physical properties is important in food production. Numerous polyphenols and certain flavonoids are substrates for polyphenoloxidase. This enzyme plays a role in the browning of fruits caused by damage, such as cutting. In the presence of oxygen these substrates form brown complexes (*24*, *35*).

Flavonols.

Flavonols are present in 80% of the plants and in fruits, where they can be a precursor for the formation of anthocyanidins (*9*). The color of flavonol (glycosides) is ranging from ivory to yellow (*34*).
Flavonol glycosides are not substrates for polyphenoloxidase, because of the sterical hindrance of the sugars bound to the C3-atom in the flavonol skeleton (*36*).
Flavonol glycosides are better water-soluble than their corresponding aglycones. In apple flavonols are mainly present as quercetin glycosides, with six different glycosides possible: Q-3-galactoside (Q-3-Ga or hyperin); Q-3-arabinoside (Q-3-Ar, with arabinose in two conformations (furanose = avicularin and pyranose = guaijaverin or foeniculin); Q-3-rhamnoside (Q-3-Rh or quercitrin); Q-3-xyloside (Q-3-Xy or reynoutrin); Q-3-glucoside (Q-3-Gl or isoquercitrin); and Q-3-rutinoside (Q-3-Ru or rutin) (*37*). Q-3-Ru is the only Q-disaccharide (Q-3-O-Gl-Rh). Apple peel contains the highest quercetin glycoside concentrations. In the flesh and core hardly any quercetin glycosides are found.

Flavanols (or Catechins).

Rich sources of catechins are tea, chocolate, apples and pears. In contrast with flavonols and anthocyanidins, catechins are not glycosylated, but present as aglycones. (+)-Catechin and (–)-epicatechin are both a 3,5,7,3',4'-pentahydroxyflavan, and differ only from each other in configuration at the C_3 atom: catechin has a S-configuration and epicatechin a R-configuration. Flavanols appear in monomeric form as well as in oligomeric form (procyanidins for example are

dimers of catechin and epicatechin). Catechins can be galloylated, but these forms have not been found in apple (*38*). Catechins are colorless and oxidation sensitive (*9*). They are together with chlorogenic acid, good substrates for the enzyme polyphenoloxidase (*24*, *35*). (–)-Epicatechin is the predominant form of the catechins present in apple (*38*). Catechin and epicatechin are present in both flesh and peel (*39*). Apple without peel contains about 75% of the total catechins concentration found in apple with peel (*38*).

Anthocyanins.

Anthocyanins are glycosylated or acylated anthocyanidins. The sugars (glucose, galactose, rhamnose, arabinose and xylose) are most often present as 3-monosides, 3-biosides and 3-triosides. Anthocyanins can be acylated with hydroxycinnamic acids, hydroxybenzoic acids, acetic acid and some aliphatic dicarboxylic acids, such as malonic, malic, oxalic and succinic acids (*40*).

Anthocyanins are water-soluble (*40*). In flowers anthocyanins provide the whole range of colors from pink and orange to violet and blue. In fruit they contribute to the purple and red colors. They accumulate in the vacuoles of epidermal and subepidermal cells. Usually the anthocyanins are in solution within the vacuole, but sometimes they are located in spherical vesicles, called ‘anthocyanoplasts’ (*40*). Anthocyanins are present as an equilibrium of four molecular species: a red flavilium cation, a colorless pseudobase, a colorless chalcone, and a blue quinoidal base. Their color is strongly pH-dependent (*33*). Anthocyanins are unstable during chemical and physical processing. They are not substrates for polyphenoloxidase (*33*). Cyanidin galactoside is the most common anthocyanin detected in apple (*24*).

Dihydrochalcones.

Dihydrochalcones differ from the other flavonoids, in that their central pyran ring is open. Chalcones are bright yellow colored (*34*). In apple two phloretin glycosides have been found: phloretin glucoside (or phloridzin) and phloretin xyloglucoside. Phloridzin is found in larger quantities than phloretin xyloglucoside (*41*). Phloridzin is mainly located in the seeds, leaves and stems and only present in minor quantities in the fruits. It is not present in pears (*24*).

1.2.1.2. Phenolic Acids.

Phenolic acids can be distinguished in hydroxybenzoic and hydroxycinnamic acids (such as chlorogenic acid). Their content is generally low in fruits (0–10 mg/kg of FW), but can be high in certain fruits of the Rosaceae family, especially in blackberry (*24*).

Chlorogenic acid is a conjugate of caffeic acid with quinic acid and can be esterified on several positions: 5'-caffeoylquinic acid is called chlorogenic acid; 4'-caffeoylquinic acid (cryptochlorogenic acid); and 3'-caffeoylquinic acid (neochlorogenic acid) (*24*). In apple chlorogenic acid is found in higher concentration (78 mg/kg) (*23*) than cryptochlorogenic acid or neochlorogenic acid (*24*). Chlorogenic acid is the natural substrate for the enzyme polyphenoloxidase (*24*, *35*).

1.2.2. Cultivation.

1.2.2.1. Growth and Maturation of Apples.

The concentrations of phenolics are very high in young fruits and then rapidly decrease during fruit development. This is the result of a dilution of phenolic compounds in the vacuole, because the cell stretches, but the number of cells is fixed (*42*). Furthermore, flavonoid biosynthesis is induced by UV-irradiation. A common response of plant cells to stress such as wounding or infection is the induced incorporation of phenylpropanoids into the cell wall (*43*).

1.2.2.2. Differences within Apple Cultivars.

Between apples of one cultivar polyphenolic concentrations may vary largely. Flavonoid formation is influenced by light (*9*); therefore it is dependent on the apple's position in the tree (at the top or at the lowest branches; shaded or not), and on the tree's position in the orchard. Factors such as fertilization, climate (weather, light and temperature), soil type, crop load, and pruning play a role (*44*). These may cause differences between harvest years (seasonal variation).

1.2.2.3. Differences Between Apple Cultivars.

In Table 1 the concentration of quercetin glycosides in the peel of various apple cultivars is given. It shows that quercetin glycoside content of apple peel is very high. Also the difference between apple cultivars is illustrated. Each cultivar has its own 'quercetin glycoside profile': not all glycosides are present in the same amount.

Polyphenolic profiles can be very fruit specific and are sometimes used to determine if product adulteration has occurred (*45*).

Table 1. Concentration of Quercetin Glycosides in the Peel of Various Apple Cultivars (mg/kg of fw) (*46*)

Compound	Golden Delicious	Red Delicious	Mc Intosh	Spartan
Q-3-Ru	110	117	115	55
Q-3-Gl and Ga	780	604	696	783
Q-3-Xy	180	210	254	244
Q-3-Rh	265	104	361	71
Q-3-Ar	502	546	777	663
total	1837	1581	2203	1756
recalculated to Q aglycone	1215	1047	1471	1216

For apple consumers, concentrations analyzed in the complete or edible apple is more interesting. Comparison of literature data may sometimes be difficult, because the definition of edible (with or without peel) may differ, and as a result which part of the fruit is analyzed. In a study in which the edible apple (with peel) was analyzed, Jonagold apple contains the highest quercetin concentration, that is 72 mg/kg of fw (analyzed as aglycon). In Cox's Orange quercetin concentration was 41 mg/kg; in Elstar 32; in Golden Delicious 25; in Granny Smith 24; and in James Grieve 21 (*47*).

1.2.3. Storage.

Concentrations of quercetin glycosides and cyanidin galactoside in Jonagold and Elstar apple skin remained quite stable upon regular and ULO (ultra low oxygen) storage for up to 6 and 8 months respectively, and after shelf life, while catechin, phloridzin and chlorogenic acid concentrations only showed minor changes (*48*). Factors of importance are storage time and temperature, and storage procedure (under 'controlled atmosphere' or in air).

1.2.4. Processing.

Apples are not only eaten fresh, they are processed also to various apple products, such as apple juice; apple juice concentrate; apple juice blends; applesauce; compote; apple wine; apple cider; cider vinegar; dried apple products; sliced apple

products; baked apple products and canned apple products (*49*). Between 40–60% of apple production is used by the industry (*42*).

During processing apples may undergo various treatments: physical treatments (e.g. peeling; cutting; pressing; centrifugation; micro-, ultra- or nanofiltration; concentration; heating; boiling; drying; lyophilization). Furthermore, chemical treatments can be applied (addition of sugar; enzyme treatment; use of additives such as ascorbic acid, SO_2, citric acid). Each of which may affect the polyphenol composition of the product. In apples the average quercetin concentration was 36±19 mg/kg of fw (analyzed as aglycon) (*47*). In commercially available apple juice only 2.5 mg/L quercetin was detected (*50*), while in applesauce the measured quercetin concentration was 18–22 mg/kg. This indicates that processing of apples causes a decrease in quercetin content. To know whether this loss in flavonoid content is due to degradation of these compounds during processing or to other factors, one should look more precisely into the production processes.

After processing, apple products can be packaged and further stored. Important factors affecting the stability of polyphenolic compounds in packaged juice are pH; gas composition (O_2, CO_2, N_2); storage time and temperature; and packaging material.

1.3. APPLE JUICE

1.3.1. Apple Juice Production.

As an example of apple processing, apple juice production was studied in the research described in this thesis. The basic principle for apple juice production is very simple. It consists of the following steps (*49*): apple fruit selection (on basis of cultivar, maturity and quality), washing and inspection, crushing, milling, or slicing to apple pulp (with a chosen particle size). The pulp consists of disrupted and intact cells as well as cell liquid. Raw juice is obtained by application of a force on the pulp, and the juice is released. Juice yield depends on cultivar, maturity, storage conditions and apple juice production method. Figure 2 shows an overview of various apple juice production methods. Many variations on this scheme are possible.

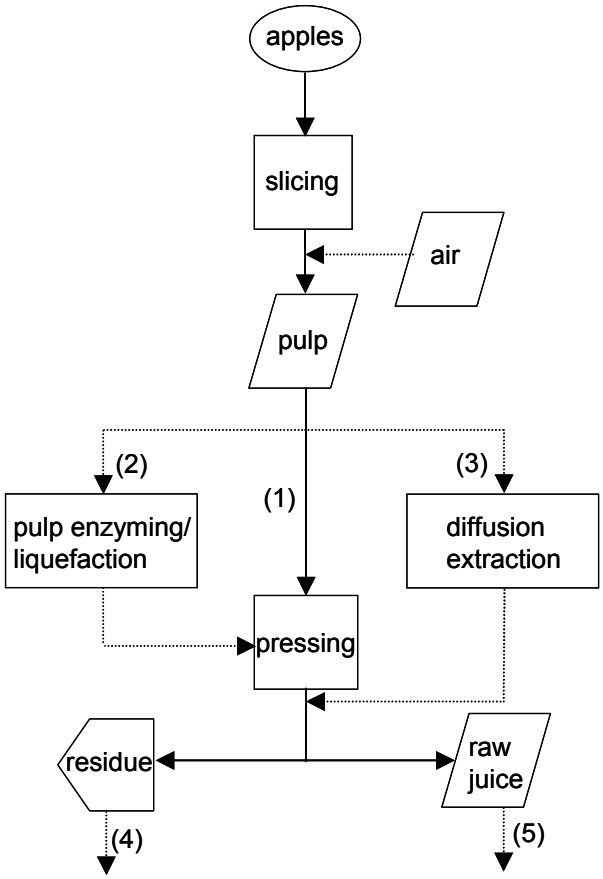

Figure 2. Production of raw apple juice. Dotted lines are optional processing steps.

1.3.1.1. Straight Pressing.

Following route 1 in Figure 2, the pulp is pressed immediately. The pulp is not aerated. This provides a juice yield of 50 tot 70% (*51*). The taste of the juice is corresponding to the characteristic apple taste.

1.3.1.2. Pulp Enzyming.

To obtain a higher juice yield enzymatic treatment of the pulp before pressing is often applied (route 2 in Figure 2). Pectolytic enzymes are used to increase the pressability by degrading pectins in the cell walls. Before addition of pectolytic enzymes, the pulp can be aerated to allow oxidation of polyphenols. This will prevent inhibition of pectolytic enzymes by these compounds (*51*).

Three overlapping processes can be distinguished, depending on the enzyme mixture used, its concentration and the applied time-temperature treatment: maceration, pulp enzyming and liquefaction. In maceration single pectolytic enzymes

are used, resulting in a mixture of intact cells by limited degradation of pectins in the middle lamella of the cell wall. In pulp enzyming a stronger desintegration of the cell wall occurs. In liquefaction a mixture of polysaccharide degrading enzymes is used (pectolytic, cellulolytic en hemicellulolytic). With the last treatment, cell walls may be degraded to such an extent that pressing will not be necessary; in that case centrifugation is a possibility for juice production (*52*).

To improve juice yield a second pressing can be performed on the press cake, after addition of water to it (water extraction), and pectolytic enzymes might be applied again, this provides an extra juice yield of 15 to 20% (*52*). Polyphenolic content (chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, phloridzin, epicatechin and catechin; quercetin glycosides were not determined) after pulp enzyming was lower than after straight pressing, caused by oxidation and complex formation in the press cake. Juice obtained by liquefaction has a higher polyphenol content, probably caused by a release from the cell walls by enzymatic degradation (*52*). In apple juices from three different cultivars prepared by pulp enzyming, Spanos et al. (*53*) found no quercetin glycosides, a low catechin concentration (0–4 mg/L), 1–9 mg/L epicatechin, 5–12 mg/L phloridzin, and 17–59 mg/L chlorogenic acid.

Others reported the presence of traces of quercetin glycosides in apple juice following commercial scale pressing (*54*) and in apple juice prepared in a domestic juice processor (*55*). In other commercially available apple juices (processing method not specified) no catechin and epicatechin were detected at all (*38*).

The above indicates that it is possible to improve total catechin concentration in the juice by choice of processing method. Liquefaction provides an apple juice with higher catechin and epicatechin concentrations than pulp enzyming and straight pressing. A disadvantage of juice obtained by liquefaction is its taste, because a too high polyphenol content may cause a bitter taste (*56*).

1.3.1.3. Diffusion-Extraction.

Batch pressing is the oldest way for obtaining apple juice. To further improve juice yield and to enable a continuous juice production instead of batch production, diffusion-extraction has been proposed (route 3 in Figure 2). In a diffusor-extractor the juice located in the apple cells is transferred to the solvent (water, in countercurrent) by a slow diffusion process. Heating can accelerate the diffusion process. The cell wall is disrupted which facilitates juice release. Factors such as

temperature, apple particle size and concentration differences between released juice and solvent are important (*57*). The temperature that is normally used is between 60 and 65 °C. Juice yields vary from 91 to 95% (*58*). Polyphenol concentration in apple juice obtained by diffusion-extraction is higher than in enzymatically prepared juice. In Table 2 the effect of straight pressing and diffusion-extraction at various temperatures on flavonoid concentration is compared.

Table 2. Effect of Processing Method on Flavonoid Content in Apple Juice (*53*)

Apple cultivar: Red Delicious	Quercetin glycosides (mg/L)	Catechin (mg/L)	Epicatechin (mg/L)	Phloridzin (mg/L)
straight pressing	0	4.0	9.1	16.1
diffusion-extraction				
55 °C	17.5	2.0	4.2	17.4
63 °C	31.2	9.5	20.7	38.7
67 °C	35.5	10.1	24.9	48.9
73 °C	38.2	13.5	44.4	59.1

In apple juice prepared by diffusion-extraction flavonoid concentration is elevated at extraction temperatures higher than 63 °C. Higher temperatures are the cause of polyphenoloxidase inactivation at the beginning of the juice processing, and the oxygen concentration is decreased as well. As a result less oxidation products are formed, and more polyphenols are obtained in the juice. But this may result in a more astringent tasting apple juice with color deviations (*59*).

1.3.2. Further Processing and Storage of Raw Apple Juice.

The raw juice, manufactured by one of the previous methods, can be further processed before it reaches the consumer. Treatments are for example clarification/filtration, concentration, pasteurization, and packaging (Figure **3**).

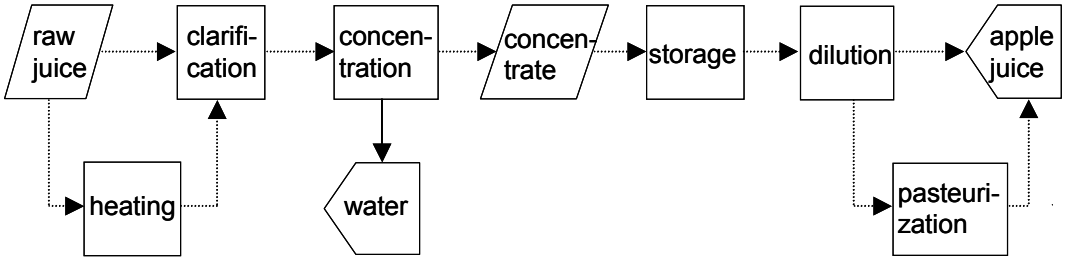

Figure 3. Further processing of raw apple juice.

The raw juice that is obtained after pressing is turbid. Polysaccharides, proteins and polyphenolics are subject to polymerization and condensation, which may or may not precipitate. To prevent precipitation, the juice is clarified by centrifugation, ultrafiltration or by application of clarifying agents (such as gelatin, bentonite, polyvinylpyrrolidon) or by the use of pectolytic enzymes (*42*). The clarified juice can be concentrated to ~70 °Brix to reduce the volume, which makes storage and transport much cheaper. The apple juice or concentrate can be pasteurized. Each treatment mentioned in Figure 3 may result in apple juices with other characteristic sensory properties.

Storage is important at different stages in apple juice processing. Apples are stored before apple juice production, and the produced juice itself can be stored (as a concentrate or as a juice). During storage of apple juice or juice concentrates various changes may occur, for example as a result of browning and degradation reactions. Comparison of apple juices prepared from concentrates that were not stored with concentrates that were stored at 25 °C for 9 months showed that quercetin glycoside concentrations lowered from 8.7 to 4.0 mg/L and phloridzin content was reduced from 3.7 to 2.5 mg/L. In the juice from a concentrate that was not stored 2.9 mg/L of catechin and 6.1 mg/L of epicatechin was present, but in the juice from stored concentrate these compounds were not detected at all (*53*). This indicates that storage of juice concentrates will decrease the flavonoid concentration in apple juice.

1.4. POLYPHENOLIC COMPOUNDS AND HEALTH

1.4.1. Health Aspects.

In general, to many phenolic compounds, anti-inflammatory, antimutagenic and anticarcinogenic properties and capacity to modulate some key cellular enzyme functions are ascribed (*16*, *60*). Furthermore, flavonoids are potent antioxidants, free radical scavengers, and metal chelators, they inhibit lipid peroxidation. Free radical formation is considered to play a key role in the development of cancer and coronary heart disease by attack on biomolecules (lipids, proteins, DNA) or the biomembrane (*43*). Therefore, flavonoids may protect against cancer because of their antioxidant properties, or via induction of detoxification enzymes (*3*, *61*). Recently it appeared that apple extracts in an in-vitro model provided protective effects in hydroxyperoxide-induced neurodegeneration (*62*). Alzheimer's disease is an

example of neurodegenerative diseases. It also appeared that quercetin (tested as aglycon in the same in-vitro model for oxidative stress-induced neurodegeneration) showed a higher protective effect than vitamin C, probably due to the fact that it is also a stronger antioxidant than vitamin C (*63*).

1.4.2. Antioxidant Function.

As mentioned before, one of the characteristics of flavonoids related to their health protecting properties is their function as antioxidants. Various definitions for the term antioxidant exist. An antioxidant is in fact a reductant (or a reducing agent, which means an electron or hydrogen donor) (*64*). But antioxidants can also function by radical scavenging; singlet oxygen quenching; metal ion complexing (metal chelating). In food science the term antioxidant is restricted to chain breaking inhibitors of lipid peroxidation, such as α-tocopherol. However, free radicals generated in vivo can damage many other targets, including proteins, DNA and small molecules. Therefore a broader definition is: “any substance that, when present in low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (*65*). Antioxidants can be divided in natural and synthetic antioxidants; another classification is primary and secondary antioxidants. Primary antioxidants are capable of terminating radical reactions, by functioning as electron donors; secondary antioxidants can delay autoxidation (in lipid peroxidation) by degradation of hydroperoxides to stable end products or by sequestering metal ions (*66*). Furthermore, fat-soluble and water-soluble antioxidants can be distinguished. Ascorbic acid, thiamine and riboflavin are water-soluble antioxidants, while tocopherols (such as vitamin E) and carotenoids (e.g., β-carotene and lycopene) are fat-soluble antioxidants. Antioxidants in the polyphenol and flavonoid groups vary in their hydrophilic–lipophilic properties. Polyphenolic compounds are good antioxidants: phenolic anions can be oxidized to phenoxyradicals by electron donation. These are relatively stable, because they are mesomerically stabilized. These phenoxyradicals may react further to chinones or form complexes (*67*).

During normal aerobic metabolism reactive oxygen species (ROS) are formed *in vivo*, which can cause damage to DNA, proteins and lipids. ROS can be subdivided in radicals (such as superoxide ($O_2^{-\bullet}$), hydroxyl ($^{\bullet}OH$), and peroxyl ($ROO^{\bullet}$)) and non-

radicals (such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), and ozone (O_3)) (*68*). Humans can protect themselves against ROS by endogenous antioxidant systems (such as superoxide dismutases, catalases, and glutathione peroxidases), endogenous antioxidants (thiol-specific antioxidants, metallothioneins, other metal ion-binding and storage proteins, urate, ubiquinol) and by antioxidants originating from their diet (*69*, *70*). If ROS production increases or total antioxidant defenses fall a situation called 'oxidative stress' may result. Oxidative stress is involved in many human diseases (*70*).

Flavonoids such as quercetin are considered to be antioxidants, because they inhibit the peroxidation of polyunsaturated fatty acids by reacting with the peroxyl radical and terminating the chain radical reaction. They can scavenge the superoxide radical as well as the hydroxyl radical. They can also inhibit lipoxygenases and cyclooxygenases. But polyphenolic flavonoids exhibit also prooxidant activities. In that case they act as oxidants by generating reactive oxygen species in the presence of metal ions such as iron or copper (*71*, *72*).

Antioxidant effectiveness depends on molecular structure, concentration, localization, substrate solubility, reaction kinetics and reaction products (73). *In vitro* screening for antioxidant activity can be used, but *in vivo* testing is also important. A compound that is a poor antioxidant in vitro is unlikely to be a good antioxidant *in vivo* (*72*). Furthermore, a compound should be tested at concentrations achievable *in vivo* and biologically relevant ROS should be used (*72*).

1.4.3. Measurement of Antioxidant Activity.

For *in vitro* quantification of antioxidant activity an enormous amount of different methods exist. These tests are based on an oxidizable compound (e.g. PUFA, single or mixture) in a chosen system (such as microsomes, liposomes, LDL, metmyoglobin), in which oxidation is induced, whether or not inhibited by an antioxidant. Reaction products are measured and values obtained with antioxidant are compared with those from reactions without antioxidant.

The various methods differ in the choice of the substrate (the oxidizable compound), the initiator, the measurement of intermediate and/or final reaction products and in the expression of the results (*74*). Antioxidant effectiveness also depends on other components and their possible interaction with antioxidants (*75*). Data obtained by

different antioxidant assays are difficult to compare and interpret, because the tests are based on different principles.

Antioxidant assays can be divided in tests that are based on the ability of an oxidant to scavenge radicals and tests that measure the ability of an antioxidant to protect a lipid substrate from oxidation. Examples of methods in which radical scavenging is determined and which are often used are the TEAC I-III assays. Such an assay uses the ABTS radical and the results are described by the TEAC-value (Trolox Equivalent Antioxidant Capacity), which gives the activity of an antioxidant compared to the activity of 1mM of Trolox (a synthetic water-soluble antioxidant) (*76*). The higher a TEAC-value, the stronger the antioxidant is. There are three versions available: TEAC-I is used for hydrophilic antioxidants; TEAC-II for lipophylic antioxidants; and TEAC-III can be used for both (77).

Other tests that determine radical scavenging are the DPPH (*2,2*-diphenyl-*1*-picrylhydrazyl) assay (*78*); DMPD (*N,N*-dimethyl-*p*-phenylenediamine) assay (*79*); TRAP-system (Total Radical-trapping Anti-oxidant Parameter) (*80*, *81*); FRAP-assay (Ferric Reducing/Antioxidant Power) (*82*); TOSC (Total Oxidant Scavenging Capacity) assay (*83*); and ORAC (Oxygen Radical Absorbance Capacity) assay (*84*). Kim et al (*85*) used VCEAC (Vitamin C Equivalent Antioxidant Capacity), to describe results obtained in assays in which ABTS or DPPH radicals were scavenged. In the PCL (Photochemiluminescence) assay free radicals are generated photochemically and a sensitive detection is possible by using chemiluminescence (*86*).

In the FRAP assay the measured reducing capacity does not necessarily reflect antioxidant activity, DPPH radicals can interact with other radicals (alkyl) and in general free radical trapping methods are one-dimensional methods: they do not take into account the complex multistep mechanism of phenolic antioxidants, their multiple actions in complex biological systems, partitioning effects and the significant effect of substrates on antioxidant effectiveness (*75*). When a substrate is absent (such as in the ABTS assay) the results do not adequately mimic the processes in food and biological systems (*74*).

The ability to protect a lipid substrate from oxidation can be measured in triacylglycerols, methyl esters, free fatty acids, or incorporated into various biological particles such as lipoproteins or liver microsomes. Whether the antioxidants function in aqueous, bulk lipid or in heterophasic systems is very important for the choice of measurement system (*75*).

Advantages and drawbacks of the different methods have been extensively reviewed (*74*, *75*, *77*, *87*, *88*). Although many different methods to assess antioxidant activity exist, it is likely that by using one antioxidant test system, comparisons within a production and processing chain can be made.

1.4.4. Dietary Intake.

The structural diversity of polyphenols makes the estimation of their content in food difficult and therefore the estimation of daily intake as well (*89*). Furthermore, estimates differ in the polyphenolic compounds that are included in the calculations. In 1976 dietary intake of flavonoids has been estimated at 1 g/day, of which anthocyanins contributed 180–215 mg/day, catechins 220 mg/day and biflavans 460 mg/day (*9*). More recently flavonol and flavon intake of 4112 Dutch adults was estimated at 23 mg/day. The major sources of intake were tea (48%), onions (29%), and apples (7%) (*90*). In another Dutch dietary survey (6200 men and women aged 1–97 year), catechin intake (c, ec, gc, egc, ecg, and egcg) was 50 ± 56 mg/day (mean ± SD), while ranging from 0 to 958 mg/day. Major sources were tea, chocolate, apples and pears (*91*). For the Danish population a similar catechin intake (20–50 mg/day) was estimated (*92*).

Finnish flavonoid intake (catechins, flavonols, flavones, and flavanones) based on 1997 food consumption data was estimated to be 55.2 mg/day; anthocyanins were excluded. The major sources of intake were fruits (67%), beverages (25%), vegetables (5%) and berries (3%). Of the fruits oranges provided 54% and apples 4% of the daily intake (*93*). Useful data on anthocyanin intake is not available, mainly due to difficulty of quantification, since their molar absorptivity depends on the balance of the colored and colorless forms, pH value and presence of copigments (*33*).

Recently, polyphenolic intake (phenolic acids, flavonols, catechin monomers, proanthocyanidins, flavanones, anthocyanins) again is estimated at 1 g/day, to be interpreted with caution, because large uncertainties remain due to the lack of comprehensive data on the content of some of the main polyphenol classes in food. It is also very much dependent on dietary habits. In this study phenolic acids accounted for about 30% of the total intake and flavonoids accounted for the remainder (*89*). Up till now there is no recommended daily intake for polyphenolic compounds (*18*).

1.4.5. Absorption and Digestion.

Apart from the concentration and bioactivity of specific components present in the food product, the bioavailability of these compounds is important. In nutritional sciences, bioavailability is a function of the digestibility, the absorbability, and the ability to use a nutrient for metabolic functions (*94*). Next to bioavailability of compounds, tissue distribution, influence of microflora, and influence of genetic variants in metabolizing enzymes are of importance (*95*). According to Stahl et al (*18*) the concept of bioavailability comprises: (i) the amount of an ingested compound that becomes available for absorption in the gastrointestinal tract, i.e. availability for absorption or 'bioaccessibility'; (ii) absorption; (iii) tissue distribution; and (iv) bioactivity or impact on metabolism.

Internal exposure to bioactive compounds is difficult to evaluate. In general, after the consumption of 10–100 mg of a phenolic compound, the maximum concentration observed in human plasma is about 1 μM. But this concentration might be higher caused by the presence of unknown (and unaccounted for) metabolites formed in the body's tissues or by the colonic microflora (*89*).

The pharmacokinetics of individual flavonoids ingested by healthy volunteers as pure compound, plant extract or whole food studied in the last decade was reviewed by Manach et al (*96*). Data indicate large differences among the different types of dietary flavonoids; differences between food sources, and the most abundant flavonoids in the diet do not necessarily produce the highest concentration of flavonoids or their metabolites in vivo. Small intestinal absorption ranges from 0–60% of the dose and elimination half-lives range from 11–28 h for quercetin glycosides and 2–6 h for epicatechin. Absorbed flavonoids undergo extensive first-pass Phase 2 metabolism in the small intestine epithelial cells and in the liver. Metabolites conjugated with methyl, glucuronate and sulfate groups are the predominant forms present in plasma.

Flavonol glycosides were thought to be stable under the conditions of the human stomach and small bowel, but Hollman et al (*97*) showed that intact glycosides of quercetin (Q-3-Ru, Q-3-Gl) may be absorbed from the small intestine, whereas Gee et al (*98*) found that this absorption took place by a mechanism involving the glucose transport pathway. An in-vitro digestion experiment, in which gastrointestinal conditions were simulated, indicated that in some cases glycosides first were converted to aglycones and subsequently degraded (*99*).

It is important to know the optimal plasma levels for bioactive compounds in humans. Plasma levels of vitamin C of larger than 50 μM are considered to be optimal; these levels can be reached in healthy individuals without particular oxidative stress, with a daily intake of 75–150 mg (*6*). For polyphenolic compounds and flavonoids optimal plasma levels are still lacking. They are difficult to establish, because the risk of confounding by other constituents in fruits and vegetables exists.

1.5 CONCLUSIONS

Polyphenolic compounds (such as flavonoids) are present in fruits and vegetables and they may provide protection against aging diseases in humans. The protective mechanism is not clear, but a possible explanation is (amongst others) through their antioxidant activity. Polyphenolic compounds comprise various compound classes, which all have different physicochemical properties. Their distribution in the fruit differs, and some compounds are more stable than others (e. g. a substrate for polyphenol oxidase or not). The effect of storage on the concentration of various compounds present in the apple peel has been described, but not the effect of storage on the antioxidant activity of apple. This is also true for the effect of processing of apples to apple juice and for the effect of storage on apple juice. It is likely that the industrial practice of enzyming and oxidizing polyphenols may affect the antioxidant activity of the juice, as polyphenols are important contributors to antioxidant activity.

Although many different methods to assess antioxidant activity exist, and data obtained by different antioxidant assays are difficult to compare, it is worthwhile to chose one system to investigate the effects on antioxidant activity caused by the apple production and processing chain, i.e. to make comparisons within the production and processing chain is likely to be possible. However it would be better to use more assays.

It is important to know what happens to polyphenolic compounds in the production and processing chain, this knowledge can be used in improved estimations of dietary intakes, which is needed to establish possible associations between fruits and vegetables, the polyphenols they contain, and the occurrence of aging diseases.

1.6 AIM AND OUTLINE OF THE THESIS

The aim of this thesis is to investigate the production chain of apple and apple derived products (harvest, storage, processing and further storage of the product), with focus on polyphenolic compound concentrations and antioxidant activity. Polyphenolic compounds are possibly health-protecting compounds, and therefore changes that occur during the production and processing chain are of interest. Furthermore these changes are important for food processors, food composition studies and epidemiological research.

Polyphenolic compounds were quantified by HPLC, based on a method described by Lister et al (*37*). Optimization of a method to determine the antioxidant activity (by using rat liver microsomes) of individual compounds as well as that of apple juice and extracts of apple products is presented in Chapter 2.

From a production chain perspective the choice of the raw materials that are used is important for the health-protecting potential of the end product. Four apple cultivars (Jonagold, Golden Delicious, Cox's Orange, and Elstar), which can be used as fresh apples or in processed apple products, were compared. To study seasonal differences, apples from three different harvest years were analyzed and the effect of long-term storage, both at refrigerator temperature and under controlled atmosphere conditions was assessed (Chapter 3).

The effect of the apple juice processing method on antioxidant activity was determined. Chapter 4 deals with the effect of processing apples into juice by conventional production methods. Three apple cultivars (Jonagold, Golden Delicious, and Elstar) were compared as raw material used for the processing.

In Chapter 5 the possibility to enhance the antioxidant activity of apple juice by adjustments in the processing method is described.

The effect of heat treatments and storage conditions on the stability of polyphenolic compounds present in enriched apple juice and the antioxidant activity is described and modeled in Chapter 6.

This thesis is concluded with a general discussion of our findings and an estimation of the changes in flavonoid intake as a result of replacing normal apple juice by enriched apple juice in Chapter 7.

1.7. LITERATURE CITED

1. Anon. *Diet, Nutrition and the Prevention of Chronic Diseases*: report of a Joint FAO/WHO Expert Consultation, Geneva, 28 January – 1 February 2002. (WHO technical report series, 916). World Health Organization, Geneva, **2003**.
2. Block, G.; Patterson, B.; Subar, A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18*, 1–29.
3. Steinmetz, K. A.; Potter, J. D. Vegetables, fruit, and cancer prevention: a review. *J. Am. Diet. Assoc*. **1996**, *96*, 1027–1039.
4. Jansen, M. C. J. F.; Bueno De Mesquita, H. B.; Feskens, E. J. M.; Streppel, M. T.; Kok, F. J.; Kromhout, D. Quantity and variety of fruit and vegetable consumption and cancer risk. *Nutr. Cancer* **2004**, *48*, 142–148.
5. Ness, A. R.; Powles, J. W. Fruit and vegetables, and cardiovascular disease: A review. *Int. J. Epidem.* **1997**, *26*, 1–13.
6. Eichholzer, M.; Lüthy, J.; Gutzwiller, F.; Stähelin, H. B. The role of folate, antioxidant vitamins and other constituents in fruits and vegetables in the prevention of cardiovascular disease: the epidemiological evidence. *Int. J. Vitam. Nutr. Res.* **2001**, *71*, 5–17.
7. Hung, H. H.; Joshipura, K. J.; Jiang, R.; Hu, F. B., Hunter, D.; Smith-Warner, S. A.; Colditz, G. A., Rosner, B.; Spiegelman, D., Willett, W. C. Fruit and vegetable intake and risk of major chronic disease. *J. Natl Cancer Inst*. **2004**, *96*, 1577–84.
8. van Gils, C. H.; Peeters, P. H. M.; Bueno de Mesquita, H. B.; Boshuizen, H. C.; Lahmann, P. H.; Clavel-Chapelon, F.; Thiébaut, A.; Kesse, E.; Sieri, S.; Palli, D.; Tumino, R.; Panico, S.; Vineis, P.; Gonzalez, C. A.; Ardanaz, E.; Sánchez, M.-J.; Amiano, P.; Navarro, C.; Quirós, J. R.; Key, T. J.; Allen, N.; Khaw, K.-T.; Bingham, S. A.; Psaltopoulou, T.; Koliva, M.; Trichopoulou, A.; Nagel, G.; Linseisen, J.; Boeing, H.; Berglund, G.; Wirfält, E.; Hallmans, G.; Lenner, P.; Overvad, K.; Tjønneland, A.; Olsen, A.; Lund, E.; Engeset, D.; Alsaker, E.; Norat, T.; Kaaks, R.; Slimani, N.; Riboli, E. Consumption of vegetables and fruits and risk of breast cancer. *J. Amer. Med. Assoc*. **2005**, *293*, 183–193.
9. Kühnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *Wld Rev. Nutr. Diet*. **1976**, *24*, 117–191.
10. Swain, T. Nature and properties of flavonoids. In: *Chemistry and biochemistry of plant pigments*, second edition, vol.1. Goodwin, T. W. (ed.). Academic Press: London, **1976**, p.425–463.
11. Middleton, E. jr; Kandaswami, C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: *The Flavonoids, Advances in Research since 1986*. Harborne, J. B., Ed.; Chapman and Hall: London; **1994**; pp. 619–652.
12. Hertog, M. G. L.; Freskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **1993c**, *342*, 1007–1011.
13. Hollman, P. C. H. Evidence for health benefits of plant phenols: local or systemic effects? *J. Sci. Food Agric.* **2001**, *81*, 842–852.
14. Arts, I. C. W.; Hollman, P. C. H. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr*. **2005**, *81*, 317S–325S.
15. Wattenberg, L. W. Inhibition of carcinogenesis by minor anutrient constituents of the diet. *P. Nutr. Soc.* **1990**, *49*, 173–183.
16. Tanaka, T. Cancer chemoprevention by natural products (review). *Oncol. Rep.* **1994**, *1*, 1139–1155.

17. van Boekel, M. A. J. S.; Jongen, W. M. F. Product quality and food processing: how to quantify the healthiness of a product. *Cancer Lett.* **1997**, *114*, 65–69.

18. Stahl, W.; van den Berg, H.; Arthur, J.; Bast, A.; Dainty, J.; Faulks, R. M.; Gärtner, C.; Haenen, G.; Hollman, P.; Holst, B.; Kelly, F. J.; Polidori, M. C.; Rice-Evans, C.; Southon, S.; van Vliet, T.; Viña-Ribes, J.; Williamson, G.; Astley. S. B. Bioavailability and metabolism. *Mol. Aspects Med.* **2002**, *23*, 39–100.

19. Dekker, M.; Verkerk. R.; Jongen, W. M. F. Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. *Trends in Food Sci. Tech.* **2000**, *11*, 174–181

20. Dekker, M.; Verkerk. R. Dealing with variability in food production chains: a tool to enhance the sensitivity of epidemiological studies on phytochemicals. *Eur. J. Nutr.* **2003**, *42*, 67–72.

21. Dekker, M.; Verkerk. R. Re: Fruit and vegetable intake and risk of major chronic diseases. *J. Natl. Cancer Inst.* **2005**, *97*, 607–608.

22. Lal Kaushal, B. B.; Sharma, P. C. Apple. In: *Handbook of Fruit Science and Technology. Production, Composition, Storage and Processing*. Salunkhe, D. K.; Kadam, S. S.(eds). Marcel Dekker: New York, **1995**, pp. 91–122.

23. Scherz, H.; Senser, F. *Food Composition and Nutrition Tables,* 6th revised and completed edition. Medpharm Scientific Publishers: Stuttgart, **2000**.

24. Macheix, J. J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*. CRC Press: Boca Raton, FL, **1990**; 378p.

25. Eastwood, M. A. Commentary. A molecular biological basis for the nutritional and pharmalogical benefits of dietary plants. *Q. J. Med*. **2001**, *94*, 45–48.

26. Tomas Barberan, F. A.; Clifford, M. N. Dietary hydroxybenzoic acid derivatives - nature, occurrence and dietary burden. *J. Sci. Fd Agric.* **2000b**, *80*, 1024–1032.

27. Bors, W.; Saran, M. Radical scavening by flavonoid antioxidants. *Free Rad. Res. Comms*. **1987**, *2*, 289–294.

28. Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochem*. **2000**, *55*, 481–504.

29. Dewick, P. M. Isoflavonoids. In: *The flavonoids, advances in research since 1986*. Harborne, J. B. (ed.). Chapman and Hall, London, **1994**. p.118–238.

30. Santos Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Fd Agric.* **2000**, *80*, 1094–1117.

31. Tomas Barberan, F. A.; Clifford, M. N. Flavanones, chalcones and dihydrochalcones - nature, occurrence and dietary burden. *J. Sci. Fd Agric.* **2000a**, *80*, 1073–1080.

32. Hollman, P. C. H.; Arts, I. C. W. Flavonols, flavones and flavanols - nature, occurrence and dietary burden. *J. Sci. Fd Agric.* **2000**, *80,* 1081–1093.

33. Clifford, M. N. Anthocyanins - nature, occurrence and dietary burden. *J. Sci. Food Agric.* **2000a**, *80*, 1063–1072.

34. Harborne, J. B. Function of flavonoids. In: *Comparative biochemistry of the flavonoids*. Academic Press: London, **1967**. pp.280–303.

35. Goupy, P.; Amiot, M. J.; Richard-Forget, F.; Duprat, F; Aubert, S.; Nicolas, J. Enzymatic browning of model solutions and apple phenolic extracts by apple polyphenoloxidase. *J. Food Sci.* **1995**, *60*, 497–501, 505.

36. van Buren, J.; de Vos, L.; Pilnik, W. Polyphenols in Golden Delicious apple juice in relation to method of preparation. *J. Agric. Food Chem.* **1976**, *24*, 448–451.

37. Lister, C. E.; Lancaster, J. E.; Sutton, K. H. Developmental changes in the concentration and composition of flavonoids in skin of a red and green apple cultivar. *J. Sci. Fd Agric*. **1994**, *64*, 155–161.

38. Arts, I. C. W.; van de Putte, B.; Hollman, P. C. H. Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. *J. Agric. Food Chem*. **2000**, *48*, 1746–1751.

39. Mayr, U.; Treutter, D.; Santos-Buelga, C.; Bauer, H.; Feucht, W. Developmental changes in the phenol concentrations of 'Golden Delicious' apple fruits and leaves. *Phytochem*. **1995**, *38*, 1151–1155.

40. Strack, D.; Wray, V. The anthocyanins. In *The Flavonoids; Advances in Research since 1986*. Harborne, J. B., Ed.; Chapman and Hall: London, **1994**; pp. 1–22.

41. Lu, Y. R.; Foo, L. Y. Identification and quantification of major polyphenols in apple pomace. *Food Chem.* **1997**, *59*, 187–194.

42. Nicolas, J. J.; Richard Forget, F. C.; Goupy, P. M.; Amiot, M. J.; Aubert, S. Y. Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci.* **1994**, *34*, 109–157.

43. Robards, K; Antolovich, M. Analytical chemistry of fruit bioflavonoids. A review. *Analyst* **1997**, *122*, 11R–34R.

44. Awad, M. A. *The apple skin: colourful healthiness. Developmental and environmental regulation of flavonoids and chlorogenic acid in apples*. Thesis. Wageningen University, **2001b**.

45. Fernández de Simón, B.; Pérez-Ilzarbe, J.; Hernández, T.; Gómez-Cordovés, C.; Estrella, I. Importance of phenolic compounds for the characterization of fruit juices. *J. Agric. Food Chem.* **1992**, *40*, 1531-1535.

46. Dick, A. J. Apple fruit polyphenols and fruit softening. *Bull. Liaison Groupe Polyphénols* **1986**, *13*, 358.

47. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J. Agric. Food Chem*. **1992b**, *40*, 2379–2383.

48. Awad, M. A.; de Jager, A. Flavonoid and chlorogenic acid concentrations in skin of 'Jonagold' and 'Elstar' apples during and after regular and ultra low oxygen storage. *Postharvest Biol. Technol.* **2000**, *20*, 15–24.

49. Bump, V. L. Apple pressing and juice extraction. In *Processed apple products*; Downing, D. L., Ed.; AVI (Van Nostrand Rheinhold): New York, **1989**, 53–83.

50. Hertog, M. G. L.; Hollman, P. C. H.; van de Putte, B. Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *J. Agric. Food Chem.* **1993a**, *41*, 1242–1246.

51. Kilara, A. Enzymes and their uses in the processed apple industry: a review. *Process Biochem.* **1982**, *17*, 35–41.

52. Schols, H. A.; In 't Veld, P. H.; van Deelen, W.; Voragen, A. G. J. The effect of the manufacturing method on the characteristics of apple juice. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 142–148.

53. Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of processing and storage on the phenolic composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572–1579.

54. Price, K. R.; Prosser, T.; Richetin, A. M. F.; Rhodes, M. J. C. A comparison of the flavonol content and composition in dessert, cooking and cider-making apples; distribution within the fruit and effect of juicing. *Food Chem*. **1999**, *66*, 489–494.

55. Pérez-Ilzarbe, J.; Hernández, T.; Estrella, I. Phenolic compounds in apples: varietal differences. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 551–554.

56. Drewnowski, A.; Gomez Carneros, C. Bitter taste, phytonutrients, and the consumer: a review. *Am. Journal of Clin. Nutr*. **2000**, *6*, 1424-1435.

57. von Lüthi, H. R.; Glunk, U. Gewinnung von Apfelsaft durch kontinuierliche Extraktion. *Flüssiges Obst* **1974**, *41*, 498–505.

58. Schobinger, U.; Dousse, R.; Dürr, P.; Tanner, H. Vergleichende Untersuchungen von Preßsaft, Diffusionssaft und Tresterextrakt aus Äpfeln. *Flüssiges Obst* **1978**, *45*, 210–216.

59. von Haug, M.; Gierschner, K. Auswirkung unterschiedlicher industrieller Herstellungsverfahren (Auspressen, Extraktion) auf die phenolischen Verbindungen in Apfelsäften. 1. Mitteilung: Abtrennung und Dünnschichtchromatographie der Pflanzenphenole. *Deut. Lebensm.-Rundsch.* **1979**, *75*, 248–253.

60. Havsteen, B. H. The biochemistry and medical significance of the flavonoids. *Pharmacol. Therapeut.* **2002**, *96*, 67–202.

61. Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985–1012.

62. Heo, H. J.; Kim, D. O.; Choi, S. J.; Shin, D. H.; Lee, C. Y. Apple phenolics protect in vitro oxidative stress-induced neuronal cell death. *J. Food Sci.* **2004**, *69*, s357–s360.

63. Heo, H. J.; Lee, C. Y. Protective effects of Quercetin and Vitamin C against oxidative stress-induced neurodegeneration. *J. Agric. Food Chem.* **2004**, *52*, 7514–7517.

64. Miller, N. J.; Rice-Evans, C. A.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407–412.

65. Halliwell, B.; Aeschbach, R.; Loliger, J.; Aruoma, O. I. The characterization of antioxidants. *Fd Chem. Toxic*. **1995a**, *33*, 601–617.

66. Shahidi, F.; Wanasundara, P. K. J. P. D. Phenolic antioxidants. *Crit. Rev. Food Sci.* **1992**, *32*, 67–103.

67. Engbersen, J. F. J.; de Groot, Æ. *Inleiding in de bio-organische chemie*. Pudoc, Wageningen **1988**.

68. Aruoma, O. I. Nutrition and health aspects of free radicals and antioxidants. *Fd Chem. Toxic.* **1994**, 32, 671–683.

69. Halliwell, B.; Murcia, M. A.; Chirico, S.; Aruoma, O. I. Free radicals and antioxidants in food and in vivo: what they do and how they work. *Crit. Rev. Food Sci.* **1995b**, *35*, 7–20.

70. Halliwell, B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans – Commentary. *Free Radical Res.* **1996**, *25*, 57–74.

71. Sahu, S. C. Dual role of flavonoids in mutagenesis and carcinogenesis. *Environ. Carcino. & Ecotox. Revs* **1994**, *C12*, 1–21.

72. Halliwell, B. Antioxidant characterization - Methodology and mechanism. *Biochem. Pharmacol*. **1995c**, *49*, 1341–1348

73. Thomas, M. J. The role of free radicals and antioxidants: how do we know that they are working? *Crit. Rev. Food Sci.* **1995** *35,* 21–39.

74. Antolovich, M.; Prenzler, P. D.;Patsalides, E.; McDonald, S.; Robards, K. Methods for testing antioxidant activity. *Analyst* **2002**, *127*, 183–198.

75. Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Fd Agric*. **2000**, *80*, 1925–1941.

76. Miller, N. J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem*. **1995**, *43*, 1794–1801.

77. Schlesier, K.; Harwat, M.; Bohm, V.; Bitsch, R. Assessment of antioxidant activity by using different in vitro methods. *Free Radical Res.* **2002**, *36*, 177–187

78. Soler Rivas, C.;Espin, J. C.; Wichers, H. J. An easy and fast test to compare total free radical scavenger capacity of foodstuffs. *Phytochem. Analysis* **2000**, *11*, 330–338.

79. Fogliano, V.; Verde, V.; Randazzo, G.; Ritieni, A. Method for Measuring Antioxidant Activity and Its Application to Monitoring the Antioxidant Capacity of Wines. *J. Agric. Food Chem.* **1999**; *47,* 1035–1040.

80. Wayner, D. D. M., Burton, G. W.; Ingold, K. U.; Barclay, L. R. C.; Locke, S. J. The relative contribution of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochim. Biophys. Acta* **1987**, *924*, 408–419.

81. Ghiselli, A., Serafini, M.; Maiani, G.; Azzini, E.; Ferro Luzzi, A.. A fluorescence-based method for measuring total plasma antioxidant capability. *Free Rad. Biol. Med.* **1995**, *18*, 29–39.

82. Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.

83. Lichtenthaler, R.; Marx, F.; Kind, O. M. Determination of antioxidative capacities using an enhanced total oxidant scavenging capacity (TOSC) assay. *Eur. Food Res. Technol*. **2003**, *216*, 166–173.

84. Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clin. Chem*. **1995**, *41*, 1738–1744.

85. Kim, D. O.; Lee, K. W.; Lee, H. J.; Lee, C. Y. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J. Agric. Food Chem*. **2002**, *50*, 3713–3717.

86. Popov, I; Lewin, G. Antioxidative homeostasis: characterisation by means of chemiluminescent technique. *Meth. Enzymol*. **1999**, *300*, 437–456.

87 Becker, E. M.; Nissen L. R.; Skibsted L. H. Antioxidant evaluation protocols: food quality or health effects. *Eur. Food Res. Technol.* **2004**, *219*, 561–571.

88 Sanchez-Moreno, C. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci. Technol. Int.* **2002**, *8*, 121–137.

89. Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S.

90. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutr. Cancer* **1993b**, *20*, 21–29.

91. Arts, I. C. W.; Hollman, P. C. H.; Feskens, E. J. M.; Bueno de Mesquita, H. B.; Kromhout, D. Catechin intake and associated dietary lifestyle factors in a representative sample of Dutch men and women. *Eur. J. Clin. Nutr.* **2001**, *55*, 76–81.

92. Dragsted, L. O.; Strube, M.; Leth, T. Dietary levels of plant phenols and other non-nutritive components: could they prevent cancer? *Eur. J. Cancer Prev.* **1997**, *6*, 522–528.

93. Kumpulainen, J. T.; Lehtonen, M.; Mattila, P. Trolox equivalent antioxidant capacity of average flavonoids intake in Finland. In: *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*. Kumpulainen, J. T.; Salonen, J. T.; Eds.; Royal Society of Chemistry: Cambridge, U.K., **1999**; pp. 141–150.

94. Wienk, K. J. H.; Marx, J. J. M.; Beynen, A. C. The concept of iron bioavailability and its assessment. *Eur. J. Nutr.* **1999**, *38*, 51–75.

95. Kampman, E.; Arts, I. C. W.; Hollman, P. C. H. Plant foods versus compounds in carcinogenesis; Observational versus experimental human studies. *Int. J. Vitam. Nutr. Res.* **2003**, *73*, 70–78.

96. Manach, C.; Donovan, J. L. Pharmacokinetics and metabolism of dietary flavonoids in humans. *Free Radical Res.* **2004**, *38*, 771–785.

97. Hollman, P. C. H.; de Vries, J.; van Leeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr*. **1995**, *62*, 1276–1282.

98. Gee, J.; DuPont, M. S.; Rhodes, M. J. C.; Johnson, I. T. Quercetin glucosides interact with the intestinal glucose transport pathway. *Free Rad. Biol. Med.* **1998**, *25*, 19–25.

99. Goh, L. M. L.; Barlow, P. J. Flavonoid recovery and stability from *Ginkgo biloba* subjected to a simulated digestion process. *Food Chem.* **2004**, *86*, 195–202.

CHAPTER 2

An Improved, Rapid in Vitro Method to Measure Antioxidant Activity. Application on Selected Flavonoids and Apple juice

ABSTRACT

A rapid in vitro method for measuring antioxidant activity is presented, which enables the evaluation of health claims and the optimization of product development with respect to health protecting compounds. Antioxidant activity is assessed in a system in which lipid peroxidation is induced in male rat liver microsomes by ascorbic acid and $FeSO_4$. This method has been significantly improved by enabling the use of microtiter plates and an ELISA reader. Large numbers of samples can be analyzed with good reproducibility, which is necessary when dealing with microsomes possessing biological variability. An objective mathematical procedure has been developed to translate data obtained from the lipid peroxidation assay into a value describing the antioxidant activity. As an illustration the method has been applied to measure antioxidant activity of individual flavonoids and apple juice.

Key words

Antioxidant activity; flavonoids; assay; apple juice

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2.1. INTRODUCTION

The increasing awareness among consumers about the relation between diet and health is a sign for food producers to pay more attention to the possibilities of health protecting properties in new product development. Product characteristics such as sensory properties (taste, color, texture), microbiological safety, nutritive value, and keepability have always been regarded as the only important quality attributes in food product development. Nowadays interest is growing for compounds that have been considered as nonnutritive, but which may play a physiological role in the human body. These compounds might be important in maintaining human health and are referred to as “bioactive compounds”. Examples are flavonoids, glucosinolates, carotenoids, organosulfides, sterols, and peptides (*1*, *2*).

The concentrations of many bioactive compounds have been analyzed in raw materials (*3*, *4*). However, before consumption, fruits and vegetables in which bioactive compounds are present may undergo different forms of processing. The effect of processing on the concentration and bioactivity of these compounds has not yet been investigated thoroughly. This paper is part of a project that studies the effects of processing on flavonoids in apple and apple products (*5*).

In determining the health protecting capacity of a product, not only the concentration of the components of interest is important, but also the bioavailability and the bioactivity. If healthiness is to be considered as a quality attribute in food product development, it has to be measurable and quantifiable. Therefore, good measurement systems are needed which correspond to a possible functional claim. It is also important to set up a measurement system that is able to quantify bioactivity in a complex matrix, as is the case in food products. This will enable the evaluation of functional claims associated with health foods. Because of the fact that flavonoids as present in apple are strong antioxidants, and may function as antioxidants in preventing aging diseases in humans, we use antioxidant activity as a measure of bioactivity for apple and apple products.

According to the broad definition by Halliwell and Gutteridge (*6*), antioxidants are compounds which, while present in low concentrations compared to those of an oxidizable substrate, prevent or delay that substrate from being oxidized. In the course of lipid peroxidation they can act as oxygen quenchers, radical scavengers

(quenching initial radicals such as hydroxyl radicals as well as quenching intermediate radicals such as peroxy and alkoxy radicals), or metal ion chelators.

A distinction must be made between water-soluble and fat-soluble antioxidants. Examples of water-soluble antioxidants are ascorbic acid (vitamin C) and B-vitamins (e.g., thiamin and riboflavin), while tocopherols (such as vitamin E) and carotenoids (e.g., β-carotene and lycopene) are fat-soluble antioxidants. Antioxidants in the polyphenol and flavonoid groups vary in their hydrophilic–lipophilic properties.

In the literature a lot of methods to assess antioxidant activity of samples can be found (e.g. *7*–*11*). In these methods oxidation is induced in single lipids, mixtures of various lipids, or complex mixtures. Sometimes biological model systems are used to mimic an in vivo situation. These systems may consist of biological membranes, such as liver microsomes, erythrocyte membranes, or liposomes. After induction of oxidation in these systems, antioxidant activity can be assessed by comparing the extent of oxidation after addition of the antioxidant compound of interest with the extent of oxidation occurring in a blank or a reference compound.

Most of these methods are limited to the measurement of pure compounds, single or mixed with other components. Normally these components are dissolved in a liquid solution, which is added to the reaction medium. But in food products antioxidants are present in a complex matrix and the effect of the product matrix on antioxidant activity is not known yet. It is possible that synergism or antagonism between antioxidants or between antioxidants and other compounds present in the product matrix occurs.

To measure antioxidant activity, we chose rat liver microsomes as an oxidative system, because it is close to the in vivo situation where both an aqueousphase and a lipid phase are present. The extent of lipid peroxidation (LPO), after chemical induction by radical formation, is monitored by the thiobarbituric acid (TBA) test. This is a commonly used combination. Microsomes are a complex and not well-defined substrate, they are composed of centrifuged cell plasma membranes and endoplasmatic reticulum, and they are subject to biological variability. They do not only consist of pure membranes; cytostolic enzymes may also be included in the membranes (*7*). Because of the fact that most flavonoids are not completely water-soluble antioxidants, this system with a combination of aqueous and lipid phases seems to provide good possibilities to serve as a very general system to measure antioxidant activity.

Given the fact of the natural variation in the components of the assay and the large variation obtained in the results with some of the existing antioxidant assays there is a need to develop an assay that enables many replications of samples in a fast and convenient way. In this paper we describe the development of such a rapid procedure by adapting an assay based on the LPO of microsomes in such a way that it can easily be performed in microtiter plates. The way in which the antioxidant activity of compounds is expressed is not consistent in the literature. We propose a mathematical method, which enables an objective quantification of this antioxidant activity based upon data covering a wide range of concentration of either a single component or a complex food sample. By using this wide range of concentrations and three or more replications of each concentration, it is possible to reproducibly determine the antioxidant activity of a sample. As an illustration of this improved method, the assessment of the antioxidant activity of several individual flavonoids and of apple juice is given.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals.

Kaempferol, myricetin, quercetin dihydrate, and rutin trihydrate were purchased from Fluka; chlorogenic acid, phloridzin, (±)-catechin, and (–)-epicatechin from Sigma; and quercetin-3-arabinosid, hyperosid, isoquercitrin, quercitrin and ideain chloride from Roth. Quercetin-3-arabinofuranoside and (–)-epigallocatechin were obtained from Apin Chemicals, and reynoutrin was obtained from Plantech. L-(+)Ascorbic acid and iron(II) sulfate heptahydrate were obtained from Merck. The enzyme Rapidase BE Super was supplied by Gist-Brocades. All other chemicals were of analytical or HPLC grade purity.

2.2.2. Sample Preparation.

Flavonoid standards were dissolved in methanol. Two different types of apple juice were prepared from Jonagold apples. Apple pulp was pressed immediately by straight pressing. Pulp-enzymed juice was prepared after the addition of pectolytic enzymes (200 ppm Rapidase BE Super) to apple pulp, which was left for 2 h at room temperature, under continuous stirring before pressing. Another apple juice was bought in an outlet of a nationwide supermarket chain. The apple cultivars that are used in the commercially available apple juice is unknown. Before HPLC analysis

and antioxidant activity determination the apple juice samples were diluted to 50% with methanol.

2.2.3. Quantification of Quercetin Glycosides by HPLC.

Quercetin glycosides were determined in the apple juices by an adaptation of the method described by Lister et al. (*3*). This method was adjusted to the use of a Merck Lichrosorb RP18 (4 x 250 mm, 5 µm) analytical column with a guard column. A Spectra FOCUS scanning UV–Vis detector, a Spectra System P2000 solvent programmer, and a Spectra System AS3000 autosampler from Spectra Physics were used. Integrator software was TSP version 3.0.

Eluents (A) 10% acetic acid in water and (B) acetonitrile were degassed by an All-Tech degassing system. After filtration through a 0.45 µm Millipore filter, samples (100 µL) were injected on the column which was maintained at 35 °C. A linear 20 min solvent gradient from 0 to 21% acetonitrile, with a 10 min hold at the final concentration, was used. The column was returned to the initial solvent composition over 1 min and reequilibrated for 10 min before the next injection. The flow rate was 1.0 mL/min.

Quercetin glycosides were monitored at 350 nm and identified and quantified by comparison with standard solutions of known concentrations, and if necessary by comparison of spectra.

2.2.4. Preparation of Rat Liver Microsomes.

Male Wistar rats (CKP, Wageningen), receiving normal diets and weighing 200–220 g, were killed by decapitation after overnight starvation. Livers were removed and homogenized (1:2, w/v) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 1 mM EDTA and 0.9% NaCl. The homogenate was centrifuged (10000g, 20 min, 4 °C), and the supernatant was collected and centrifuged (100000g, 75 min, 4 °C). The pellet was suspended by pottering (1 g of liver/mL) in the phosphate buffer and centrifuged again (100000g, 60 min, 4 °C). To be stored, the pellet was suspended (0.5 g of liver/mL) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 0.1 mM EDTA and 20% glycerol. Microsomal protein concentrations were determined by the Biuret method with bovine serum albumin used as standard, and the microsomes were diluted with the latter phosphate buffer to 5 mg/mL protein before storage in 1 mL aliquots in liquid nitrogen.

2.2.5. Antioxidant Activity.

Antioxidant activity measurements are based on a method to determine lipid peroxidation in rat liver microsomes (*12*). It was optimized to be able to use microtiter plates, a multichannel pipet and an ELISA reader, which makes it possible to analyze large numbers of samples in a run.

Microsomes (5 mg of protein/mL) were thawed on ice, diluted 5-fold with Tris–HCl buffer (50 mM, pH 7.4), containing 150 mM KCl, and centrifuged (100000g, 60 min, 4 °C). The pellet was resuspended in the Tris buffer and diluted to the concentration needed (final concentration 0.5 mg/mL protein unless otherwise stated).

The microsomes (aliquots of 240 µL) were preincubated in a 48-well plate for 5 min at 37 °C. As a test sample 30 µL of a known concentration of an antioxidant or blank (corresponding with the solvent for the antioxidant, e.g., methanol or water) was added. LPO was induced by adding 15 µL of ascorbic acid (4 mM) and 15 µL of $FeSO_4$ (0.2 mM). After incubation for 60 min at 37 °C the reaction was stopped by addition of 0.5 mL of 0.83% thiobarbituric acid dissolved in TCA–HCl (16.8% w/v trichloroacetic acid in 0.125 N HCl). LPO was assessed by measuring thiobarbituric acid reactive species (TBARS) after the plates were heated for 15 min at 80 °C and subsequent centrifugation (2500 rpm, 15 min). A 250 µL sample of each incubation was transferred to 96-well plates and absorption was read at 540 nm (color) vs 620 nm (turbidity correction) by an ELISA reader.

During the first period of this research, the ELISA reader was equipped with a standard 550 nm filter. Because of the fact that the TBA reaction gives a higher extinction at somewhat lower wavelengths, a filter of 540 nm later on replaced this filter. It was found that measuring at this wavelength increased the accuracy of the method, but that no significant differences in IC_{50} values were obtained. Therefore, results obtained by both filters are given.

2.2.6. Calculations.

The percentage of inhibition produced by a sample at a given concentration can be calculated from the absorbance readings. The percentage of inhibition is expressed as the inhibition of lipid peroxidation of that sample compared to the lipid peroxidation in a blank (eq 1).

$$\%I = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100 \quad (1)$$

%I, the percentage of inhibition; A_{blank}, absorbance of the blank ($A_{540} - A_{620}$); and A_{sample}, absorbance of the sample ($A_{540} - A_{620}$)

The dependence of the percentage inhibition on the concentration of the antioxidant can be described by a sigmoid curve (eq 2). This equation is a general description of a sigmoid curve. The parameters describing the inhibition are fitted to the data using the solver option in Excel. From these parameters, the concentration at which 50% inhibition of lipid peroxidation occurs (IC_{50}) can be calculated (eq 3).

$$\%I = 100 \times \frac{\left(1 - e^{-ac}\right)}{\left(1 + be^{-ac}\right)} \quad (2)$$

$$IC_{50} = \left(\frac{-1}{a}\right) \times \ln \frac{0.5}{(1 + 0.5b)} \quad (3)$$

a and *b*, reaction constants specific for each compound/food; and *c*, concentration (μM, mg/L or mL/L). The IC_{50} value is expressed in the same units as *c*.

The fit error of the calculated IC_{50} can be described by eq 4, corresponding to the statistical method of least squares.

$$\text{fit error (\%)} = \left(\frac{\sum (\%I_{\text{calcd}} - \%I_{\text{measd}})^2}{n}\right)^{1/2} \quad (4)$$

$\%I_{\text{measd}}$, measured percentage of inhibition of a certain antioxidant concentration; $\%I_{\text{calcd}}$, calculated percentage of inhibition of the same antioxidant concentration; *n*, total number of percent inhibition determinations on which the IC_{50} value is based (in the case of six triplicate concentrations, $n = 18$).

2.3. RESULTS AND DISCUSSION

2.3.1. Color Formation in TBARS Reaction.

Determination of the antioxidant activity of a sample or compound is often performed in test tubes or Eppendorfs. It is possible to do more incubations by simultaneously using multititer plates and a multichannel pipet. Faster and more automated absorbance readings can be made using an ELISA reader together with multititer plates. It was possible to obtain absorbance readings which fall within the accuracy

of the instrument by adjusting the concentration of microsomes in the system or by changing the volume of TBA/TCA–HCl which is added to stop the LPO reaction. An incubation in which no antioxidant is added results in maximum TBARS formation, which is represented in the development of pink color. The pink color produced by TBARS is best read at 532 nm (*7*), but it also proved possible to use an ELISA reader with a filter for reading at 550 or 540 nm.

To a methanol blank with a total incubation volume of 0.3 mL and a microsome concentration of 1 mg/mL was added 2.0 mL of 0.42%TBA/TCA–HCl in test tubes. After centrifugation 1 mL was transferred to a cuvette and read by a spectrophotometer at 535–600 nm. This condition resulted in an absorbance reading of 1.2. Adaptations were made that fitted both the well volume and the filter present in the ELISA reader to be able to use 48-wells plates with 1.5 mL wells. By using the same microsome concentration and the same incubation volume, the addition of 1.0 mL of 0.83% TBA/TCA–HCl resulted in an absorbance reading of 0.5 with a 550 nm filter. For the 540 nm filter in the ELISA reader, addition of 0.5 mL of 0.83% TBA/TCA–HCl solution and a microsome concentration of 0.5 mg/mL resulted in absorbance readings of 1.2. By lowering the microsome concentration, less TBARS is formed, which is compensated by the use of a 540 nm filter which gives higher absorbance readings than a 550 nm filter.

2.3.1.1. Effect of Microsomal Concentration.

The amount of lipid material present in the incubation medium (microsomal concentration) also determines the color formation after incubation with a blank or antioxidant. Different microsome concentrations were compared using quercetin as an antioxidant, and the color formation has been monitored by a spectrophotometer and ELISA reader. In Figure 1 the absorbance readings by the ELISA reader are presented. The absorbance readings of the same samples by a spectrophotometer at 540 nm show the same pattern, but at slightly higher absolute values.

Using eq 1 and the data from Figure 1, the percentages of inhibition of the various quercetin concentrations were calculated. Methanol was used as a blank. The percentages of inhibition plotted against the quercetin concentration are presented in Figure 2.

As can be seen in Figure 1 changing the microsome concentration has a considerable effect on the level of absorbance, but this effect is much smaller after

calculation of the corresponding percent inhibition (Figure 2), because they are compared with a blank in which the antioxidant concentration is zero.

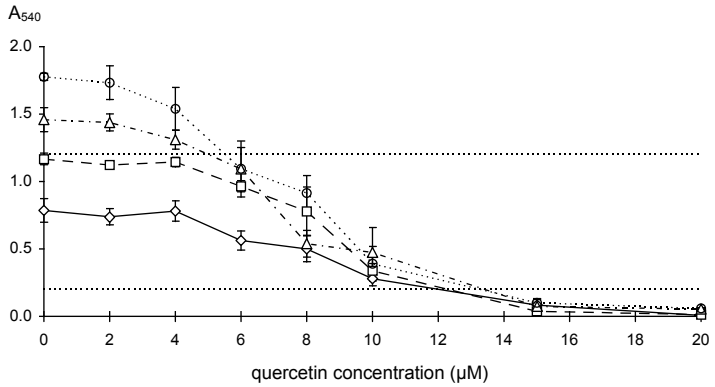

Figure 1. TBARS formation monitored by an ELISA reader. Comparison of color formation produced by microsome concentration 0.3 mg/mL (—◊—), 0.5 mg/mL (- - □- -), 0.7 mg/mL (-˙-Δ-˙-), or 1.0 mg/mL (···○···). Accuracy limits of absorbance measurement (····). Quercetin was used as an antioxidant. A 0.5 mL portion of 0.83% TBA/TCA–HCl was added. Means and SDs of triplicate analysis.

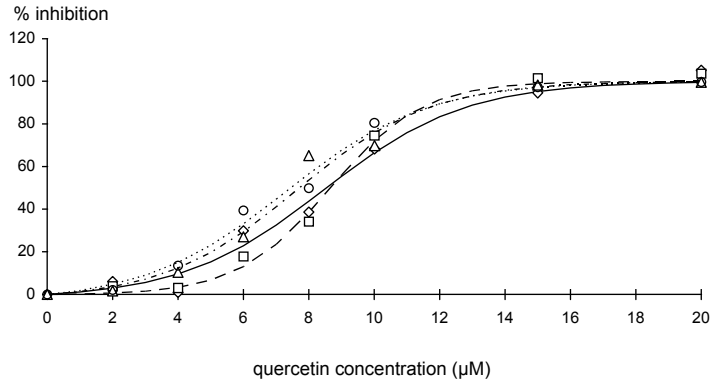

Figure 2. TBARS formation monitored by an ELISA reader. Percentage of inhibition of LPO by quercetin with microsome concentration 0.3 mg/mL (—◊—), 0.5 mg/mL (- - □- -), 0.7 mg/mL (-˙-Δ-˙-), or 1.0 mg/mL (···○···). Quercetin was used as an antioxidant. A 0.5 mL portion of 0.83% TBA/TCA–HCl was added. Means of triplicate analysis. Data from Figure 1.

The parameters *a* and *b*, describing the sigmoidal dependence of the occurring inhibition on the quercetin concentration, were calculated by data fitting using eq 2. From these parameters the concentration at which 50% inhibition of lipid peroxidation occurs (IC_{50}) was calculated as described in eq 3. The resulting IC_{50} values and fit errors are given in Table 1.

Table 1. Calculated IC_{50} (μM) from Parameters *a* and *b* Describing Quercetin Inhibition Curves and Fit Errors, Obtained from Triplicate Determination of Eight Different Quercetin Concentrations[a]

microsome concn (mg/mL)	IC_{50} (μM)	fit error (%)
0.3	8.5	9
0.5	8.7	7
0.7	7.7	9
1.0	7.4	7

[a] Data from Figure 2. An ELISA reader with a 540 nm filter was used. Mean ± SD = 8.1 ± 0.6.

Lowering the microsome concentration has the advantage of the possibility to perform more antioxidant activity measurements with one batch of microsomes. The variation in IC_{50} values caused by changing the microsome concentration is 7.5%. Regression analysis showed that there is no significant trend present in the data (*p* value 0.093), which indicates that within the microsome concentration range tested, IC_{50} values were independent of the microsomal concentration in the test system. For the accuracy and efficacy of using the microsomes, 0.5 mg/mL was chosen as the standard microsome concentration in the assay.

2.3.2. Reproducibility of Antioxidant Activity Determination.

Microsomes were prepared from male rat livers. Because of the fact that the microsomes are of animal origin, they are subject to biological variability. To determine a possible effect of this biological variability on the blanks in the antioxidant activity determinations, in a one year period six different microsome batches were prepared. The protein content of the isolated microsomes was assessed by the Biuret method. The protein estimation of the isolated microsomes (0.5 g of liver/mL) was 7.85 ± 2.60 mg/mL. After isolation and protein content determination, the rat liver microsomes were diluted to 5 mg/mL protein for standardization and stored in liquid nitrogen.

The mean absorbance reading ($A_{550} - A_{620}$) of the methanol blanks in the first four batches was 0.472 ± 0.097. This means a coefficient of variation of 21% in 90 repetitions, collected on 19 experimental days. The mean absorbance reading ($A_{540} - A_{620}$) of the methanol blanks in the last two batches was 1.197 ± 0.158, causing a variation of 13% in 87 repetitions, collected on 9 experimental days. In both cases the variation in absorbance of the blanks within a batch was about 10%.

To see if there is an effect on antioxidant activity determinations caused by possible biological variability of microsomes, in Table 2 the IC_{50} values (in μM) of three different flavonol aglycons are presented.

Table 2. IC_{50} (μM) of Three Flavonol Aglycons (Dissolved in Methanol), Analyzed in Four Different Microsome Batches, Based on Absorbance Readings by an ELISA reader (A_{550}–A_{620})

flavonoid aglycon	microsome isolation	IC_{50} (μM) mean ± SD	n [a]	fit error (%)
myricetin	batch A	8.3 ± 0.8	6	12
	batch B	6.7 ± 1.1	2	12
	batch A and B	7.9 ± 1.1	8	13
quercetin	batch A	10.0 ± 1.2	6	12
	batch B	9.4 ± 1.0	7	11
	batch C	9.8 ± 1.6	3	14
	batch D	9.2 ± 1.2	3	11
	batch A–D	9.6 ± 1.1	19	12
kaempferol	batch B	12.4 ± 0.04	2	12

[a] n number of triplicate determinations of six or seven antioxidant concentrations. Fit errors are obtained from fitting on all data sets together.

Each IC_{50} value was based on triplicate determination of six or seven different antioxidant concentrations, covering a range from no inhibition to full inhibition. Mean IC_{50} values ± standard deviations were calculated per batch microsomes. For quercetin, single factor analysis of variance was performed, and no difference between IC_{50} values obtained in the four batches was indicated (p value 0.763). The variation within batches was larger than the variation between batches. In the case of the two myricetin batches, also no difference between IC_{50} values was observed (p value 0.060). This indicates that it is possible to use different batches of microsomes in antioxidant activity determinations, provided that the rats which are used for microsome isolation have lived on the same diets, and are of the same gender, strain, and age.

The IC_{50} values for quercetin given in Table 2 were derived from Figure 3, using eq 3. As an example, Figure 3 shows how close the inhibition curves of quercetin analyzed in the four different microsome batches overlay. The mean percent inhibition ±

standard deviation points were obtained from 19 repeated determinations. For analysis of standard compounds in this method, a variation of 10–15% in the obtained IC_{50} value was observed.

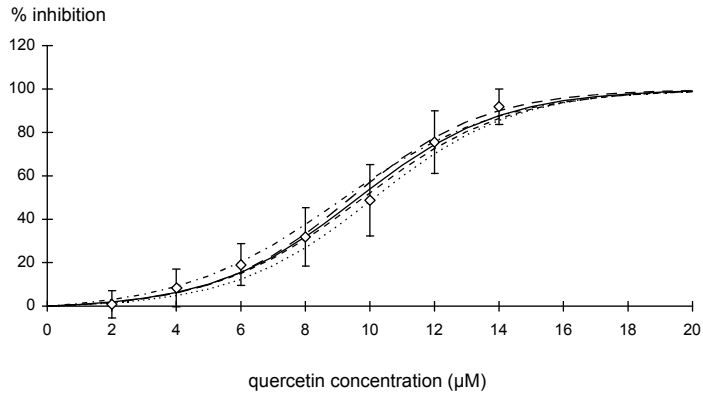

Figure 3. Comparison of the inhibition curves of quercetin analyzed in different microsome batches: mean ± SD (◊), fit batch A (····), fit batch B (- - -), fit batch C (-· -), fit batch D (---), fit all data (—).

In Table 3 the antioxidant activity of selected flavonoid standards is presented. All compounds were dissolved in methanol, unless stated otherwise. Every IC_{50} value was again determined by data fitting on triplicate determinations of six antioxidant concentrations, and the mean IC_{50} ± SD was determined of the replications. The IC_{50} value was also determined by data fitting on all data sets together including the fit error that occurs. Both data fitting on separate data sets and data fitting on combined data sets provide good results.

The quercetin aglycon is a stronger antioxidant than its glycosides. Of the quercetin glycosides the disaccharide rutin is the weakest antioxidant. The other quercetin glycosides possess about the same potency, reynoutrin excluded, which is only a slightly weaker antioxidant than quercetin aglycon. Catechin and epicatechin are less potent antioxidants compared to quercetin, but the catechin gallates, which possess more hydroxyl groups, are stronger than quercetin. The anthocyanidin ideain is a weaker antioxidant than the catechins and quercetin glycosides. From chlorogenic acid (a phenolic acid) the antioxidant activity was determined after the compound was dissolved in methanol or in water. It did not affect the antioxidant capacity of the compound in the test system. The dihydrochalcone phloridzin was the weakest antioxidant tested.

Table 3. IC_{50} (μM) of Quercetin Glycosides, Chlorogenic Acid, Phloridzin, Catechins, and Ideain [a]

flavonoid compound (dissolved in methanol, unless stated otherwise)	IC_{50} (μM) (mean ± SD)	*n*	fit error (%)
Q (quercetin)	9.7 ± 1.9	6	14
Q-3-Ga (hyperin)	16.0 ± 2.8	2	11
Q-3-Gl (isoquercitrin)	17.3 ± 3.6	2	15
Q-3-Ar (guaijaverin, foeniculin)	17.2 ± 2.1	2	9
Q-3-Arf (avicularin)	15.1	1	5
Q-3-Rh (quercitrin)	20.8 ± 2.0	2	8
Q-3-Ru (rutin)	22.3 ± 1.7	3	9
Q-3-Xy (reynoutrin)	11.2	1	4
CA (chlorogenic acid)	124.5 ± 36.0	2	8
CA in water	128.0	1	4
P (phloridzin)	1925.5 ± 199.0	2	10
c (catechin)	15.6	1	8
ec (epicatechin)	12.2 ± 1.9	2	8
egc (epigallocatechin)	8.3	1	4
ecg (epicatechin gallate)	4.8	1	6
egcg (epigallocatechin gallate)	3.5	1	6
Cy-Ga (ideain chloride)	25.5	1	3

[a] Comparison of values based on absorbance readings by an ELISA reader (A_{540}–A_{620}). *n*, number of triplicate determinations of 6 antioxidant concentrations. Fit errors are obtained from fitting on all data sets together. Ar, arabinopyranoside; Arf, arabinofuranoside; Cy, cyanidin; Ga, galactoside; Gl, glucoside; Rh, rhamnoside; Ru, rutinoside; Q, quercetin; and Xy, xyloside.

The IC_{50} values as determined here are in a qualitative way consistent with other methods that are described in the literature. A difficulty for quantitative comparison is the existence of a broad variety of methods and description of the data obtained by those methods. Miller and co-workers (*13*) reported the same order of antioxidant potency for quercetin, rutin, catechin, and epicatechin. But they only observed a factor of 2 difference between chlorogenic acid and phloridzin, with phloridzin as the less antioxidative compound. They used the Trolox equivalent antioxidant capacity value, which gives the millimolar concentration of a Trolox solution having the same antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation. In this assay the antioxidants' capacity to scavenge the $ABTS^{\bullet+}$ radical

in an aqueous phase is measured. Vinson and co-workers, (*14*, *15*) also find the same order in antioxidant activity for quercetin and rutin. But in their test system epicatechin is more active than quercetin, and chlorogenic acid and cyanidin are almost as active as quercetin. Their test system for antioxidant activity was lipoprotein, and cupric ion was used as oxidant. The differences mentioned are probably caused by the hydrophilic or lipophilic properties of the different test systems and the polarity of the antioxidant itself. Therefore, it is very important to test the standard compounds of interest and the food products in which they are present in one and the same antioxidant test system. This enables the use of such an assay in product development.

2.3.3. Possible Complications in the Assay Caused by the Food Matrix.

The purpose of this research is to measure antioxidant activity in food products to be able to evaluate health claims and the optimization of product development with respect to health protecting compounds. For that reason it is important to know the behavior of other compounds that are present in the food matrix in the antioxidant activity assay. Macronutrients present in apple juice are carbohydrates (123 g/L) and up to 0.5 g/L protein (*16*). The main organic acids in apple juice are chlorogenic acid (up to 85 mg/L) and phloridzin (up to 67 mg/L), with concentrations depending on the production process. Caffeic acid, *p*-coumaric acid and ferulic acid can be present in lower concentrations (up to 11 mg/L) (*17*).

In Figure 4 absorption spectra of a blank and a commercially available apple juice sample after microsomal incubation and TBARS formation are given.

The blank shows a pink color after 80 min of incubation at 37 °C, and the apple juice sample after the same 80 min of incubation shows an orange color. The same apple juice sample after 0 min of incubation shows a yellow color after the TBARS reaction. The yellow color was also observed upon heating of apple juice with TBA (without the presence of microsomes). The yellow color is caused by the reaction of sugars that are present in the apple juice with TBA. Wilbur and co-workers (*18*) also found a yellow color produced by a reaction between TBA and sugars, with a maximum absorption at 450 nm. In the apple juice sample that had been incubated with the microsomes, TBARS was formed, but the present sugars also reacted with TBA, so the yellow color together with the pink color produced an orange appearance.

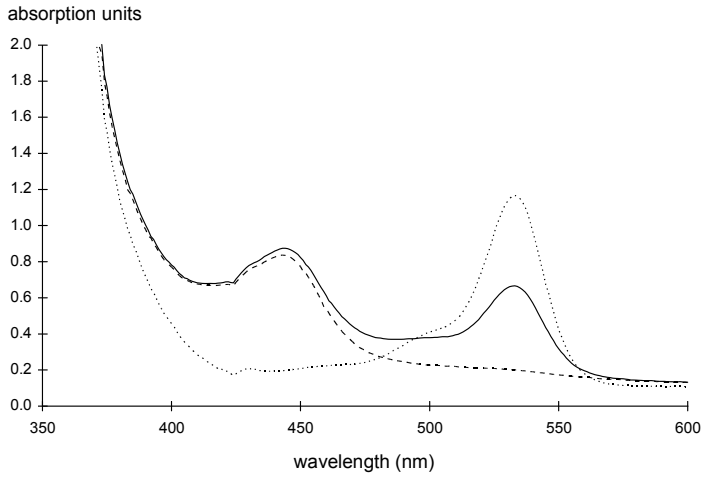

Figure 4. Absorption spectra after TBARS formation monitored by a spectrophotometer: a blank showing a pink color after 80 min of microsomal incubation at 37 °C (···), a commercially available apple juice after 0 min of incubation showing a yellow color (---), and the same apple juice after 80 min of incubation showing an orange color (—).

From Figure 4 the conclusion can be drawn that the yellow color has its absorption maximum at 443 nm and does not influence the measurement of the pink color produced by TBARS at 535 nm. Antioxidant activity measurement of apple juice is possible with this method, but when other products are measured in this assay, good controls (choice of blanks and testing of the product in the TBA test in the absence of microsomes) should be taken into account to be sure that no interference of protein, lipids, organic acids, or colored compounds originating from the product itself with the reaction products of the assay occurs.

2.3.4. Antioxidant Activity of Apple Juice Samples.

In Table 4 IC_{50} values and the quercetin-glycoside content of differently produced apple juices are shown. A commercially available apple juice is compared to Jonagold apple juice prepared by straight pressing and Jonagold apple juice that is prepared by pulp enzyming. Pulp enzyming is often used in industry to obtain a higher juice yield and to facilitate the pressing.

The three different apple juices all have a low quercetin glycoside content, compared to that present in apple, which is 36 ± 19 mg/kg (expressed as quercetin aglycon) (*4*). Recalculated to quercetin glycosides (using a mean molecular weight of 445.37 for the quercetin glycosides), this would be 53 ± 28 mg/kg.

Table 4. IC_{50} Values and Quercetin Glycoside Content of Differently Produced Apple Juices[a]

	straight pressing (n=4)	pulp enzymed juice (n=2)	commercially available juice (n=3)
[Q-gly] in juice (mg/kg)	6.9 ± 1.6	5.7 ± 0.6	3.5 ± 0.1
IC_{50} of juice (g of fresh juice/L)	57 ± 38	130	59 ± 22

[a] Fit errors < 10%

Apple juice prepared by straight pressing and the commercially available juice have the same antioxidant activity. Apple juice prepared by pulp enzyming has a lower antioxidant activity compared to the other two. The IC_{50} value of the pulp-enzymed juice is obtained by extrapolation (by the data solver) because at the highest concentration possible to test in the antioxidant assay (52 g of fresh juice/L assay for this sample) only 11.4 ± 3.6 percent inhibition was measured. In this case it was not possible to have antioxidant concentrations that covered a range from no inhibition to full inhibition of LPO.

During the stirring that takes place in the pulp-enzyming process, enzymatic oxidation of compounds (other than quercetin glycosides) occurs, which is the possible cause of the decreased antioxidant activity of pulp-enzymed juice.

The concentration of quercetin glycosides that is present in apple indicates the existence of a big variability (coefficient of variation 53%) in the raw material. The concentration of quercetin glycosides in the apple juices is about 10 times lower. The variation obtained in the antioxidant activity determination is also big (38% and 67% for the commercially available juice and the straight pressed juice, respectively). But the fit errors for the individual antioxidant activity determinations each were lower than 10%.

The possibility to analyze antioxidant activity of differently processed apple juices enables the choice for the product with the highest activity and to optimize apple juice processing with respect to the antioxidant activity of the product.

2.4. CONCLUSIONS

In these experiments antioxidant activity was determined by measuring the inhibition of LPO by a range of six different concentrations of a certain antioxidant. For each concentration the percent inhibition was measured after 60 min of incubation. The

concentrations were chosen in such a way that the lowest concentration provided no inhibition of LPO and the highest concentration gave full inhibition of LPO. From these data it was possible to calculate the IC_{50} value by an objective mathematical procedure. This makes it possible to evaluate antioxidant activity in a quantitative way, which is not feasible when the inhibition of LPO by a certain concentration of an antioxidant is compared in time courses. In that case only qualitative conclusions can be drawn.

The reproducibility of this method is good, the variation in IC_{50} values that are obtained when standard antioxidant compounds are applied is 10–15%. Within the microsome concentration range tested, IC_{50} values were independent of the microsomal concentration in the test system. However, it is advised to use a standardized microsome concentration (0.5 mg/mL) in the assay. The use of different batches of microsomal isolates did not show an effect on the antioxidant activity determinations.

Antioxidant activity measurement of a sugar-rich food product such as apple juice is possible with this method, but when other products are measured in this assay, good controls should be taken into account to be sure that no interference of proteins, lipids, organic acids, or colored compounds originating from the product itself with the reaction products of the assay occurs.

If interest lies in studying underlying mechanisms regarding antioxidant activity, it is necessary to compare radical-generating systems different from those described in this method, and to test the difference in behavior of antioxidants and products in such assays.

The possibility to compare differently processed food products in one method, such as shown here for apple juices, with regard to their antioxidant activity enables the choice for the product with the highest activity and the optimization of processing with respect to the antioxidant activity of the product.

2.5. ACKNOWLEDGMENT

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2.6. LITERATURE CITED

1. Steinmetz, K. A.; Potter, J. D. Vegetables, fruit, and cancer prevention: a review. *J. Am. Diet. Assoc.* **1996**, *96*, 1027–1039.
2. Tanaka, T. Cancer chemoprevention by natural products (review). *Oncol. Rep.* **1994**, *1*, 1139–1155.
3. Lister, C. E.; Lancaster, J. E.; Sutton, K. H. Developmental changes in the concentration and composition of flavonoids in skin of a red and green apple cultivar. *J. Sci. Food Agric.* **1994**, *64*, 155–161.
4. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in The Netherlands. *J. Agric. Food Chem.* **1992**, *40*, 2379–2383.
5. Van der Sluis, A. A.; Dekker, M.; Jongen, W. M. F. Effect of processing on content and antioxidant activity of flavonoids in apple juice. In *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*; Kumpulainen, J. T.; Salonen, J. T., Eds; Royal Society of Chemistry: Cambridge, U.K., **1999**; pp 209–211.
6. Halliwell, B.; Gutteridge, J. M. C. *Free radicals in biology and medicine*, 2nd ed.; Clarendon Press: Oxford, **1989**.
7. Gutteridge, J. M. C. Lipid peroxidation: some problems and concepts. In *Oxygen radicals and tissue injury*, Proceedings of an Upjohn Symposium; Halliwell, B., Ed.; Federation of American Societies for Experimental Biology: Maryland. **1988**. pp 9–19.
8. Heinonen, I. M.; Lehtonen, P. J.; Hopia, A. I. Antioxidant activity of berry and fruit wines and liquors. *J. Food Sci.* **1998**, *46*, 25–31.
9. Miller, N. J.; Rice-Evans, C. A.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407–412.
10. Vinson, J. A.; Hontz, B. A. Phenol antioxidant index: comparative antioxidant effectiveness of red and white wines. *J. Agric. Food Chem.* **1995**, *43*, 401–403.
11. Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Barclay, L. R. C.; Locke, S. J. The relative contribution of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochim. Biophys. Acta* **1987**, *924*, 408–419.
12. Haenen, G. R. M. M. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. *FEBS Lett.* **1983**, *159*, 24–28.
13. Miller, N. J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem.* **1995**, *43*, 1794–1801.
14. Vinson, J. A.; Jang, J.; Dabbagh, Y. A.; Serry, M. M.; Cai, S. Plant polyphenols exhibit lipoprotein-bound antioxidant activity using an in vitro oxidation model for heart disease. *J. Agric. Food Chem.* **1995**, *43*, 2798–2799.
15. Vinson, J. A.; Dabbagh, Y. A.; Serry, M. M.; Jang, J. Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease. *J. Agric. Food Chem.* **1995**, *43*, 2800–2802.
16. Schobinger, U.; Dousse, R.; Dürr, P.; Tanner, H. Vergleichende Untersuchungen von Presssaft, Diffusionssaft und Tresterextrakt aus Äpfeln. *Fluess. Obst* **1978**, *45*, 210–216.
17. Schols, H. A.; In ’t Veld, P. H.; van Deelen, W.; Voragen, A. G. J. The effect of the manufacturing method on the characteristics of apple juice. *Z. Lebensm.-Unters.-Forsch.* **1991**, *192*, 142–148.
18. Wilbur, K. M.; Bernheim, F.; Shapiro, O. W. The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acid by various agents. *Arch. Biochem.* **1949**, *24*, 305–313.

CHAPTER 3

Activity and Concentration of Polyphenolic Antioxidants in Apple: Effect of Cultivar, Harvest Year, and Storage Conditions

ABSTRACT

Consumers' increasing interest in the relationship between diet and health is a sign for food producers to pay more attention to potential health-protecting compounds in new product development and food processing. From a production chain perspective the choice of the raw material that is used is important for the health-protecting potential of the end product. Four apple cultivars (Jonagold, Golden Delicious, Cox's Orange, and Elstar), which can be used as fresh apples or in processed apple products, were compared with regard to flavonol, catechins, phloridzin, and chlorogenic acid concentrations and antioxidant activity. Jonagold apples possessed the highest flavonoid concentration and the highest antioxidant activity. To study seasonal differences, apples from three different harvest years were analyzed, but in three cultivars no effect on flavonoid concentration and antioxidant activity was observed. Long-term storage, both at refrigerator temperature and under controlled atmosphere conditions, was found not to influence flavonoid concentration or antioxidant activity.

Key words

Antioxidant activity; flavonoids; storage; apples; cultivar; harvest

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3.1. INTRODUCTION

Flavonoids are secondary plant metabolites present in fruits and vegetables. They belong to the group known as polyphenolics, and generally they occur in plants as glycosides. Over 6400 different structures have been identified, and this number continues to increase (*1*).

In epidemiological research some flavonoids are associated with protection against aging diseases (*2*). This may be ascribed to their action as antioxidants. Formation of oxygen radicals is supposed to play a key role in the development of cancer and coronary heart disease. Free radicals may attack biomolecules, such as lipids, proteins, or DNA, which can be prevented by antioxidants.

In the Dutch diet important sources of flavonoids are tea, onions, and apples (*3*). The most important flavonoids present in apple and apple products are flavanols or catechins, flavonols, and anthocyanidins (*4*). Flavonols are mainly present as quercetin glycosides; cyanidin galactoside is the most common anthocyanin, and (–)-epicatechin is the predominant form of the catechins. In contrast with flavonols and anthocyanidins, catechins are not glycosylated. They appear in monomeric form as well as in oligomeric form (procyanidins) (*5*). Furthermore, dihydrochalcones (e.g., phloridzin) and phenolic acids (in particular chlorogenic acid) are present in apple (*5*).

The dietary intake of flavonoids has been estimated as 1 gram/day (6). More recently, new analysis techniques became available and Dutch flavonol and flavone intake was estimated on 23 mg/day (*7*). In the latter research catechins and anthocyanidins were excluded. This might cause an underestimation of the total flavonoid intake. Finnish flavonoid intake (catechins, flavonols, flavones, and flavanones) was estimated to be 55.2 mg/day, with anthocyanidins excluded (*8*).

To be a bioactive compound of interest, not only should the intake be sufficiently high but also the bioavailability (the absorption into the body) must be at a sufficient level to obtain active levels within the body. Polyphenolic antioxidants are absorbed in the human body to some extent; for example 52% of the quercetin glycosides present in onions (*9*) and 33% of chlorogenic acid present in a supplement (*10*) are absorbed. Upon absorption of quercetin glycosides the compounds are hydrolyzed to quercetin and subsequently converted to quercetin glucuronides and sulfates in the human body (*11*).

Food producers are increasingly interested in developing new products with an increased level of certain health-protecting compounds to address the increasing interest of consumers in the relationship between diet and health. For this development not only it is relevant to know which health-protecting compounds are present in raw materials and in what concentrations, but also their bioactivity is of importance. Before consumption, fruits and vegetables in which bioactive compounds are present may undergo different forms of processing, which might affect the concentration and bioactivity of the health-protecting compounds in the product. From a production chain perspective all of the steps will have an effect on the final level and activity of bioactive compounds in the final product (*12*). Important aspects within the production chain of apple are the choice of the raw material [cultivar, growing conditions (climate, soil type, use of fertilizers), seasonal differences, and harvest and storage conditions], industrial processing, packaging, storage of the final product and consumer processing. Until now studies have mainly focused on the influence of these factors on the polyphenolic composition of apples (*13*–*16*) and not on their bioactivity.

This paper is part of a project that studies the effects of storage and processing on flavonoids and chlorogenic acid in apple and apple products (*17*). Polyphenolic compounds as present in apple are strong antioxidants (*18*) and may function as such in preventing aging diseases; therefore, the antioxidant activity of apples was used as a measure of their bioactivity (*19*).

The biological variability within a certain cultivar of apple is investigated, and on the basis of this information a sampling protocol has been developed. Four apple cultivars (Jonagold, Golden Delicious, Cox's Orange, and Elstar) were compared with regard to polyphenolic composition and antioxidant activity.

To study potential seasonal differences in polyphenolic composition and antioxidant activity, apples from three different harvest years were analyzed.

To investigate the effect of apple storage, controlled atmosphere (CA) storage and cold storage were tested.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals.

Kaempferol, myricetin, quercetin dihydrate, and rutin trihydrate were purchased from Fluka; chlorogenic acid, phloridzin, (±)-catechin, and (–)-epicatechin were from Sigma; and quercetin 3-arabinoside, hyperoside, isoquercitrin, quercitrin, and ideain chloride were from Roth. Quercetin 3-arabinofuranoside was obtained from Apin chemicals and reynoutrin from Plantech. L-(+)Ascorbic acid and iron(II) sulphate heptahydrate were obtained from Merck. All other chemicals were of analytical or HPLC grade purity.

3.2.2. Apple Cultivars and Harvest and storage Conditions.

In this research the two main apple cultivars grown in The Netherlands were used (Jonagold and Elstar) as were two less important apple cultivars (Golden Delicious and Cox's Orange). Apples were harvested from commercial orchards in three growing seasons, 1996–1998, to investigate seasonal variability. Each year the apples were collected from the same beds in the same orchards. Fruit trees were planted in a four-row system, called a "Zeeuws bed", with a density of ~3500 trees/ha, except for Jonagold harvest 1998, when apples were obtained from trees from a different orchard, planted in single rows (3300 trees/ha).

The day of harvest was predicted using variety-specific models (*20*) that predict the optimal harvest date for Jonagold apples for long-term storage in controlled atmosphere. This means that at the time of harvest apples have not reached the stage of complete maturity suitable for immediate consumption.

Fruits were picked at one time, from the outer layer, avoiding the tops and bottoms of the trees. Apples from the inner layer of the tree were not used because their total flavonoid concentration is much lower due to lack of light (*21*). Trees at the border of the orchard were avoided as well.

Applied CA storage conditions for Jonagold, Golden Delicious, and Elstar were 1.5 °C, 1.2% O_2, and 2.5% CO_2. Cox's Orange apples were stored at 4.0 °C, 1.2% O_2, and 0.7% CO_2.

In cold storage all apple cultivars were kept at 4 °C. The effect of combined storage (cold storage after a storage period of approximately 13 or 46 weeks in CA conditions) was determined as well.

Apples were stored in 12 kg boxes. Samples were taken regularly (four to seven times) throughout the storage period, until the apples lost their quality as assessed by loss of firmness or development of brown spots.

3.2.3. Sample Preparation.

Flavonoid and chlorogenic acid standards were dissolved in methanol. Apple samples were taken as follows. Four apples (unless stated otherwise) were chosen at random and in duplicate. After cleaning with water, stalks were removed and the complete apples were cut into pieces by knife and subsequently ground to a fine powder (with an ATO-MSE mix) under liquid nitrogen to prevent oxidation. The frozen samples were stored immediately at –20 °C until lyophilization. Dry weight was determined from the sample weight before and after lyophilization. Lyophilized apple samples were stored at –20 °C until analyzed.

To determine the distribution of flavonoids and chlorogenic acid within an apple, apples were peeled with a potato knife; a thin layer of apple flesh remained adhered to the peel. Therefore, the peel can be considered as the epidermic zone of the apples.

To establish the effect of oxidation time on the flavonoids and chlorogenic acid in the apple samples before extraction, apples were sliced in a food processor and left at room temperature for 0–30 min before they were ground under liquid nitrogen.

Apple samples were extracted before HPLC analysis and antioxidant activity determination. Extraction conditions were optimized in order to be able to use the same extract in both determinations. Lyophilized sample (0.5 g) was extracted with 10 mL of methanol (unless stated otherwise) and sonicated for 30 min followed by 10 min of centrifugation at 2500 rpm. The supernatant was filtered through a 0.45-µm CA filter (Schleiger and Schuell).

3.2.4. Quantification of Flavonoids by HPLC.

Quercetin glycosides, catechins, chlorogenic acid, phloridzin, and cyanidin galactoside were determined in the apple samples by an adaptation of the method described by Lister et al. (*4*). This method was adjusted for the use of a Merck Lichrosorb RP18 (4 x 250 mm, 5 µm) analytical column with guard column. A Spectra Focus scanning UV–VIS detector, a Spectra System P2000 solvent programmer, and a Spectra System AS3000 autosampler from Spectra Physics were used. Integrator

software was TSP version 3.0. Eluents, solvent gradient, flow rate, and column conditions were described before (*19*).

The individual compounds were identified and quantified by comparison with standard solutions of known concentrations and, if necessary, by comparison of spectra. Quercetin, its glycosides, and chlorogenic acid were monitored at 350 nm, phloridzin and the catechins were monitored at 280 nm, and cyanidin galactoside was monitored at 525 nm. The coefficients of variation for the slope of the calibration curves of the various compounds (16 replications) were as follows: chlorogenic acid, 2%; Q-3-galactoside, 5%; Q-3-rutinoside, 15%; Q-3-glucoside, 3%; Q-3-arabinoside, 5%; Q-3-rhamnoside, 16%; quercetin, 6%; catechin, 10%; epicatechin, 13%; phloridzin, 14%.

3.2.5. Antioxidant Activity Determination.

Preparation of rat liver microsomes for antioxidant activity determination was performed as described earlier (*19*). After isolation, microsomal protein concentration, determined by Biuret assay with bovine serum albumin used as standard, was 7.2 mg/mL. Microsomes were diluted with phosphate buffer to 5 mg/mL protein before storage in 1 mL aliquots in liquid nitrogen.

Antioxidant activity was determined by measuring the inhibition of lipid peroxidation in rat liver microsomes by the apple samples (*19*). In the assay microsomal protein concentration was 0.5 mg/mL. Lipid peroxidation (LPO) was induced by adding ascorbic acid (final concentration = 0.2 mM in the assay) and FeSO4 (final concentration = 0.01 mM in the assay). Antioxidant samples were 10 times diluted by administration to the assay. LPO was assessed by measuring thiobarbituric acid reactive species (TBARS) after heating, and absorption was read at 540 nm (color) versus 620 nm (turbidity correction) by an ELISA reader. The mean absorbance reading ($A_{540} - A_{620}$) ± standard deviation of the methanol blanks was 1.172 ± 0.129 (in 59 repetitions collected on 5 experimental days).

The concentration of the antioxidant sample at which 50% inhibition of LPO occurs (IC_{50}) was calculated from triplicate determination of six different antioxidant concentrations ranging from no to full inhibition of LPO.

3.2.6. Statistical Analysis.

Statistical analysis of the data was performed on the original data by one-way analysis of variance (ANOVA) or regression analysis, with significance level α = 0.05 using the statistical package from Microsoft Excel.

3.3. RESULTS AND DISCUSSION

3.3.1. Effect of Sample Taking.

3.3.1.1. Effect of Extraction Solution.

Extraction conditions were optimized in order to be able to use the same extract for HPLC analysis and antioxidant activity determination. The following extraction solutions were compared: water, 50% aqueous methanol, and 100% methanol (with or without 15% acetic acid). Hertog and co-workers (*3*) reported that 50% aqueous methanol was most efficient for flavonoid extraction. Lister et al. (*4*) used 100% methanol as extraction solution for proanthocyanidins and 15% acetic acid in methanol for the extraction of flavonols and anthocyanins.

Table 1. Flavonoid and Chlorogenic Acid Concentration (Milligrams per Kilogram of Fresh Weight) of a Jonagold Apple Sample Extracted by Four Different Extraction Solutions[a]

compound	water	50%MeOH	100%MeOH	15%HAc/MeOH
Q-3-Ga	11 ± 2 [a]	30 ± 2 [b]	27 ± 3 [b]	28 ± 4 [b]
Q-3-Ru	0 ± 0 [a]	0 ± 0 [b]	0 ± 0 [b]	0 ± 0 [b]
Q-3-Gl	2 ± 1 [a]	6 ± 1 [b]	6 ± 0 [b]	6 ± 1 [b]
Q-3-Xy	3 ± 1 [a]	12 ± 3 [b]	12 ± 1 [b]	12 ± 3 [b]
Q-3-Ar	5 ± 1 [a]	23 ± 4 [b]	26 ± 3 [b]	24 ± 6 [b]
Q-3-Rh	11 ± 1 [a]	28 ± 8 [b]	29 ± 8 [b]	30 ± 10 [b]
catechin	5 ± 3 [a]	16 ± 1 [b]	17 ± 2 [b]	14 ± 3 [b]
epicatechin	16 ± 5 [a]	93 ± 14 [b]	121 ± 7 [c]	129 ± 14 [c]
Cy-Ga	1 ± 1 [a]	9 ± 1 [b]	11 ± 1 [b]	11 ± 1 [b]
phloridzin	9 ± 2 [a]	25 ± 6 [b]	31 ± 2 [b]	34 ± 5 [b]
chlorogenic acid	61 ± 17 [a]	173 ± 7 [b]	195 ± 11 [c]	219 ± 26 [c]

[a] Extractions were performed in quadruplicate (mean ± SD). Values within a row having the same letter are not different at the 5% level.

Table 1 shows that water is not suitable for flavonoid extraction; this is the case for all of the compounds analyzed. Epicatechin and chlorogenic acid are better extracted by 100% methanol (with or without 15% acetic acid) than by 50% aqueous methanol. The extraction solution of 100% methanol (with or without 15% acetic acid) gives reproducible good yields, and no difference in extraction efficiency was observed in all compounds. For antioxidant activity determination it is better not to add acetic acid to the test system. Therefore, 100% methanol was chosen as extraction solution for HPLC analysis and antioxidant activity determination.

3.3.1.2. Effect of Oxidation Time before Extraction.

During processing, apple samples brown very quickly by enzymatic oxidation of polyphenols. We have studied the effects of exposure to air during the sample treatment on the level of the components of interest. As expected, Figure 1 shows significant decreases in the levels of total catechins (–28%) and chlorogenic acid (–25%), which are known substrates for polyphenol oxidase (22). Phloridzin and cyanidin galactoside concentrations were not affected by oxidation. Figure 1 also shows that quercetin glycosides are not substrates for polyphenol oxidase. There seems to be a slight but significant increase (17%) in the total quercetin glycoside concentration.

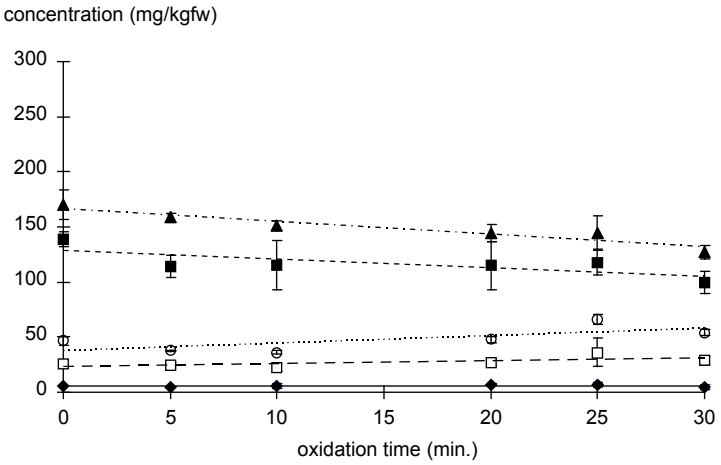

Figure 1. Effect of oxidation time (min) on flavonoid and chlorogenic acid concentrations (mg/kg of fw) of Jonagold apple, harvest 1997: Cy-Ga (—♦—), Phloridzin (- - □- -), Chlorogenic acid (-˙ -˙ ▲-˙ -˙), total Q-gly (···○···), total catechins (---■---).Triplicate extractions were performed.

In Figure 2 the effect of oxidation time on dry weight and antioxidant activity of Jonagold apple is depicted. The IC_{50} value shows a slight but significant increase (13%), which means that the antioxidant activity slightly lowers with increasing oxidation time. Therefore, it is important to ensure that no oxidation occurs during sample taking. The sample should not thaw during this process but remain frozen until lyophilization.

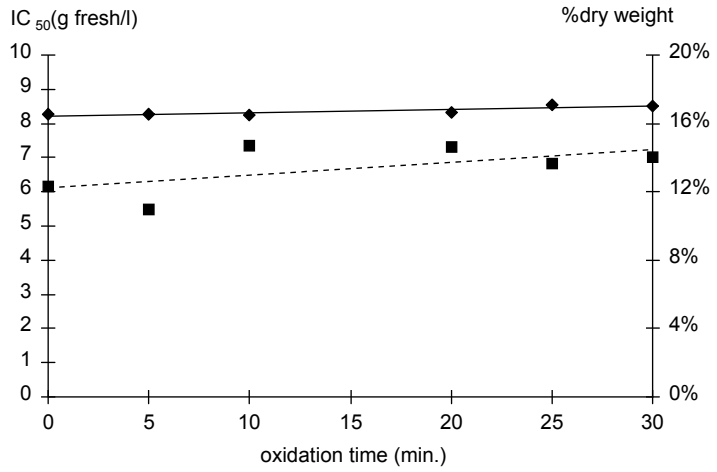

Figure 2. Effect of oxidation time (min) on antioxidant activity (g of fw/L) and percent dry weight of Jonagold apple, harvest 1997: antioxidant activity (---■---), %dry weight (—♦—).

3.3.1.3. Variation in Flavonoid and Chlorogenic Acid Concentration within an Apple.

Flavonoids are not equally distributed throughout the apple. Table 2 shows that quercetin glycosides were almost exclusively found in the peel, but low concentrations were detected in the flesh, which is consistent with the findings of others (*14*, *15*). Phloridzin was present in both flesh and peel, although with a higher concentration in the peel. Chlorogenic acid was equally distributed over peel and flesh ($p = 0.583$). Phloridzin and chlorogenic acid distributions within an apple confirm the findings of Burda et al. (*23*). Catechins were not analyzed in this part of our study, but it has been reported that the epicatechin concentration was higher in the skin than in the flesh (*23*). In all apple samples quercetin aglycon was absent.

Table 2. Distribution of Quercetin Glycosides, Phloridzin, and Chlorogenic Acid (Milligrams per Kilogram of Fresh Weight) in Jonagold Apple Peel and Flesh, Harvest 1996

compound	peel	flesh
Q-3-Ga	126 ± 22	0 ± 0
Q-3-Gl	20 ± 2	0 ± 0
Q-3-Xy	57 ± 7	1 ± 1
Q-3-Ar	159 ± 21	2 ± 2
Q-3-Rh	145 ± 7	7 ± 3
phloridzin	66 ± 3	14 ± 5
chlorogenic acid	148 ± 16	170 ± 43

3.3.1.4. Variation in Flavonoid and Chlorogenic Acid Concentration and Antioxidant Activity between Individual Apples.

To determine the variation in flavonoid and chlorogenic acid concentration and antioxidant activity between individual apples, four Jonagold apples were analyzed separately. Flavonoid concentrations are presented in Table 3. The variation in compound concentration between individual apples was 10–30%, depending on the compound. The largest variations were found in epicatechin and phloridzin concentrations. The same variation was observed in Elstar and Golden Delicious apples (data not shown). In Table 3 the dry weight and the antioxidant activity expressed as IC_{50} (the concentration of the antioxidant sample at which 50% inhibition of lipid peroxidation occurs) of the individual Jonagold apples are also presented. The variation in dry weight among the four Jonagold apples was 8%, and the variation in IC_{50} value was 17%.

3.3.1.5. Number of Apples in a Sample.

Because of the observed variation in flavonoid and chlorogenic acid concentration between individual apples, it is better to compose samples of more than one apple. Jonagold apple samples composed of three, four or five apples were analyzed. Flavonoid and chlorogenic acid concentrations and dry weights are given in Table 3. Not much difference in the concentration of these compounds was found. Therefore, we chose duplicate sample taking with samples composed of four apples. In duplicate sample taking of four apples a variation of 10–30% existed, which is considered to be the biological variation.

Table 3: Flavonoid and Chlorogenic Acid Concentration (Milligrams per Kilogram of Fresh Weight), Percent Dry Weight and Antioxidant Activity of Four Individual Jonagold Apples and Jonagold Apple Samples Composed of Three or More Apples (Mean ± SD)

compound	1	2	3	4	mean	three apples	four apples	five apples	mean
Q-3-Ga	20	10	29	22	22 ± 7	33 ± 2	31 ± 10	21 ± 3	29 ± 9
Q-3-Ru	3	1	2	2	2 ± 1	in Q-3-Glu	in Q-3-Glu		in Q-3-Glu
Q-3-Gl	4	2	4	3	3 ± 1	7 ± 1	5 ± 1		6 ± 1
Q-3-Xy	9	7	14	11	11 ± 3	14 ± 3	11 ± 2	13 ± 2	12 ± 2
Q-3-Ar	29	20	41	30	32 ± 8	36 ± 3	35 ± 5	33 ± 5	35 ± 5
Q-3-Rh	38	30	53	37	41 ± 10	32 ± 0	30 ± 5	29 ± 3	31 ± 4
epicatechin	76	99	128	81	99 ± 26	nd	nd	nd	nd
Cy-Ga	4	4	7	5	5 ± 3	nd	nd	nd	nd
phloridzin	13	28	24	15	20 ± 6	47 ± 4	56 ± 33	74 ± 26	57 ± 27
chlorogenic acid	126	176	200	167	173 ± 27	241 ± 25	240 ± 24	322 ± 3	256 ± 40
% dry wt	17.6	15.9	18.0	19.4	18.1 ± 1.4	15.8 ± 0.4	16.1 ± 1.1	19.9 ± 1.8	17.0 ± 2.1
IC_{50} (g of fw/L)	6.4	7.5	4.9	6.9	6.4 ± 1.1	nd	nd	nd	nd

nd: not determined

We used whole apples in a sample, not just the peel where higher flavonoid concentrations are found, which has been done by many authors (*4*, *13*, *24*). Later in the project complete apples were processed, and for comparison purposes it was therefore necessary to analyze the concentration in the complete apple.

3.3.2. Seasonal Variability.

Fruits of four apple cultivars were collected during three years to study the effect of seasonal variation on flavonoid concentration and antioxidant activity. Table 4 describes the means of the flavonoid concentration of the three harvest seasons.

Table 4. Concentration of Flavonoids and Chlorogenic Acid (Milligrams per Kilogram of Fresh Weight), Percent Dry weight and Antioxidant Activity of Four Apple Cultivars [a]

compound	Jonagold	Golden Delicious	Cox's Orange	Elstar
total Q-glycosides [b]	98 ±16	67 ±16	54 ±15	60 ± 4
total catechins [c]	197 ±17	173 ±26	143 ±59	162 ± 2
Cy-Ga [d]	8 ± 2	2 ± 0	4 ± 2	3 ± 0
phloridzin	28 ±13	35 ±16	14 ± 9	26 ±15
chlorogenic acid	201 ±15	171 ±18	69 ±25	70 ± 4
% dry weight	17.4 ± 0.4	17.1 ± 0.3	16.7 ± 0.4	16.8 ± 0.5
IC_{50} (g of fw/L) [d]	5.8 ± 0.3	8.0 ± 0.5	7.2 ± 1.6	6.3 ± 0.5

[a] Mean (± SD) of three harvest years (1996, 1997 and 1998). [b] The group total Q-glycosides is composed of Q-3-Ga, Q-3-Ru, Q-3-Gl, Q-3-Xy, Q-3-Ar and Q-3-Rh. [c] The group total catechins consists of catechin and epicatechin. [d] Mean (± SD) of two harvest years (1997 and 1998)

In Jonagold apples the total quercetin glycoside concentration did not significantly differ over the three harvest years, although the total quercetin glycoside concentrations between the years 1996 and 1998 were significantly different ($p = 0.022$) and was highest in harvest year 1996. Total catechin, cyanidin galactoside, and chlorogenic acid concentrations were the same in the analyzed harvest years. Phloridzin concentration was highest in the 1998 harvest ($p = 0.004$).

In Golden Delicious apples, concentrations of chlorogenic acid, total catechins, and cyanidin galactoside were the same at harvest time over the three years. Some variation in total quercetin glycoside concentration ($p = 0.041$) and phloridzin concentration ($p = 0.050$) was measured.

Cox's Orange apples seemed to be most sensitive to seasonal variation. Seasonal variation in chlorogenic acid and total catechin concentrations was significant ($p = 0.018$ and 0.043). For total quercetin glycoside, cyanidin galactoside, and phloridzin the significance was between the 5 and 10% levels ($p = 0.062$, 0.056, and 0.068, respectively).

Seasonal variation in total quercetin glycoside, cyanidin galactoside, chlorogenic acid, and total catechin concentrations of Elstar apples was not significant and therefore lower than the observed biological variation. However, seasonal variation was observed for phloridzin concentration in Elstar ($p = 0.006$).

The conclusions found for Jonagold and Elstar apples are similar to the findings for apple peels reported by Awad and de Jager (*25*), except that we observed seasonal variation in phloridzin concentration instead of cyanidin galactoside concentration.

Table 4 also gives the antioxidant activity of the four apple cultivars from the 1997 and 1998 harvests. In the four analyzed apple cultivars no seasonal effect on antioxidant activity was observed. Jonagold apples possessed the highest antioxidant activity, followed by Elstar and Cox's Orange. Golden Delicious apples showed the lowest antioxidant activity.

3.3.3. Varietal Differences and Quercetin Glycoside or Catechin Profiles.

Table 4 shows that Jonagold had the highest quercetin glycoside concentration, followed by Golden Delicious, Elstar, and Cox's Orange at a 30–40% lower level. The same pattern was found in total catechin concentration, with a 10–20% lower total catechin concentration.

Quercetin glycoside concentrations are within the range described for eight different apple cultivars by Price and coworkers (*15*) [40–110 mg/kg of fresh weight after recalculation to quercetin glycosides], but Cox's Orange quercetin total glycoside content was lower than reported by them. In all cultivars, total catechin was higher than reported by Arts et al. (*26*). Varietal differences in chlorogenic acid are also high [30–430 mg/kgfw (*27*)], and our findings are within this range.

In Table 5 the percentage of individual quercetin glycosides and catechins of their corresponding totals in the four cultivars is given.

In Jonagold and Golden Delicious apples these quercetin glycoside profiles were similar; therefore, it is not possible to distinguish between these cultivars on the basis of their quercetin glycoside profile. In both cultivars Q-rhamnoside was the most

abundant quercetin glycoside present, followed by Q-arabinoside and Q-galactoside. Q-xyloside, Q-glucoside, and Q-rutinoside had the lowest contributions (in decreasing order).

Table 5. Quercetin Glycoside and Catechin Profiles in Four Apple Cultivars[a]

	mean % of total quercetin glycosides			
	Jonagold	Golden Delicious	Cox's Orange	Elstar
Q-3-Ga	25 ± 3	23 ± 8	32 ± 4	33 ± 3
Q-3-Ru	1 ± 1	1 ± 1	2 ± 0	2 ± 0
Q-3-Gl	5 ± 2	7 ± 1	7 ± 2	10 ± 2
Q-3-Xy	11 ± 2	11 ± 3	10 ± 3	11 ± 2
Q-3-Ar	28 ± 1	27 ± 2	34 ± 2	31 ± 1
Q-3-Rh	30 ± 3	31 ± 3	15 ± 1	13 ± 2
	mean % of total catechins			
	Jonagold	Golden Delicious	Cox's Orange	Elstar
catechin	7 ± 1	6 ± 3	7 ± 3	7 ± 4
epicatechin	93 ± 1	94 ± 3	93 ± 3	93 ± 4

[a] Mean (± SD) of three harvest years (1996, 1997 and 1998).

The quercetin glycoside profiles of Cox's Orange and Elstar apples were also similar to each other but differed substantially from those of Jonagold and Golden Delicious. In these cultivars Q-galactoside was the most abundant quercetin glycoside, followed by Q-arabinoside and Q-rhamnoside. Q-xyloside, Q-glucoside, and Q-rutinoside again had the lowest contributions (in decreasing order). All four apple cultivars showed very similar catechin profiles, with epicatechin as the predominant compound.

3.3.4. Storage.

3.3.4.1. CA storage.

Storage at CA conditions did not have a significant influence on the total quercetin glycoside, phloridzin, and cyanidin galactoside concentrations in all apple cultivars (Figure 3). Chlorogenic acid and total catechin concentrations decreases were significant in Jonagold apples. The observed decreases were not very high (18 and 40%, respectively), and after 52 weeks of storage, still substantial amounts were

present. In Golden Delicious apples a small decrease in total catechin concentration was observed as well, but changes in chlorogenic acid concentration were not significant.

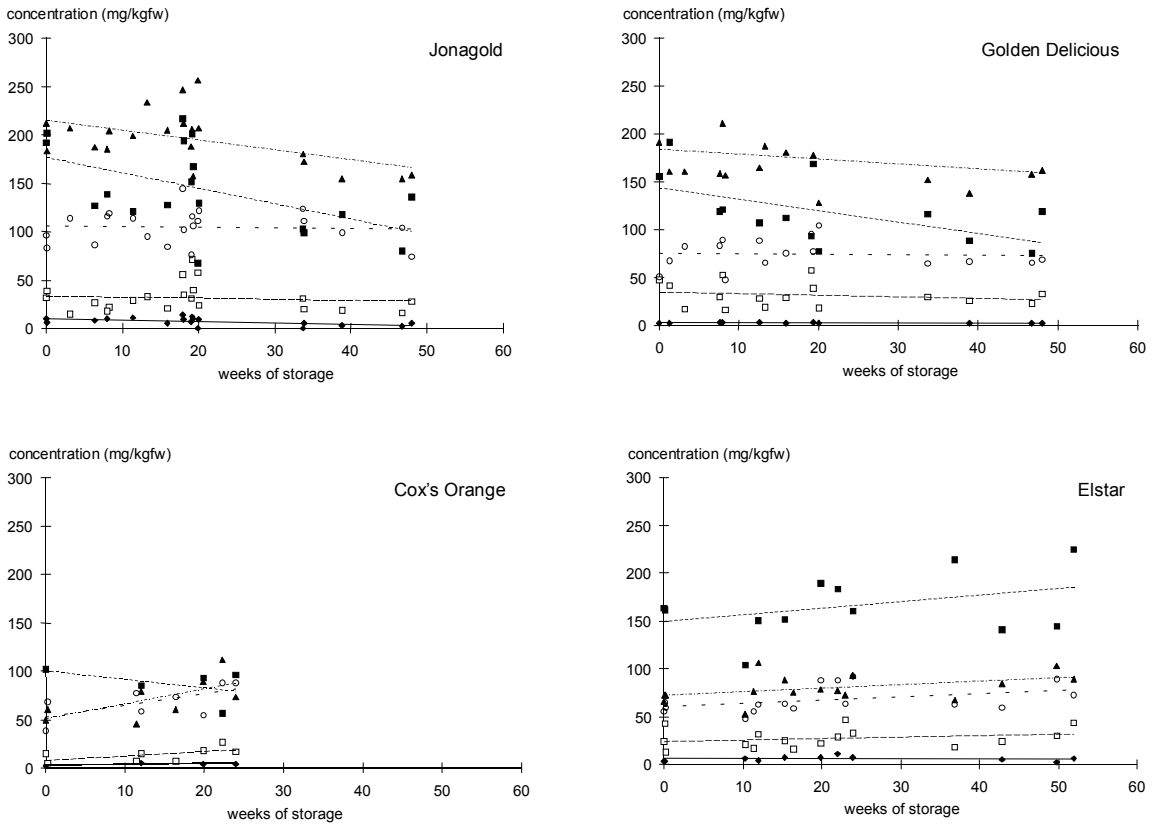

Figure 3. Flavonoid and chlorogenic acid concentrations (mg/kg of fw) of four apple cultivars (harvests 1996, 1997, and 1998) during storage in CA: Cy-Ga (—♦—), Phloridzin (- - □- -), Chlorogenic acid (-˙ -˙ ▲-˙ -˙), total Q-gly (···○···), total catechins (---■---). Apple samples were taken in duplicate (SD = 15–20%).

In Cox's Orange and Elstar apples significant differences were observed in chlorogenic acid concentrations ($p = 0.03$ and 0.039, respectively, for the 3-year data), but in both cultivars this was observed in only one of the three years (1996 and 1998, respectively). Total catechin concentration remained fairly stable in Cox's Orange apples, and in Elstar apples small but significant changes were observed only in storage year 1997. The storage time of Cox's Orange apples (24 weeks) was much shorter than of the other three cultivars (48–52 weeks), as they began to develop brown spots and softened earlier.

Flavonoids appeared to be quite stable compounds during CA storage, as was observed by Awad and de Jager (*25*). They reported finding no significant changes upon 30 weeks of storage under regular and CA conditions.

3.3.4.2. Cold Storage.

Cold storage was only possible for a shorter time compared to the storage at CA conditions. Figure 4 shows that cold storage did not have a significant effect on the total quercetin glycoside, phloridzin, and cyanidin galactoside concentrations in the four cultivars, corresponding to what was observed during CA storage. The chlorogenic acid concentration also did not significantly change during the 25 week period of cold storage. In total catechin concentrations, however, small but significant differences were observed in Golden Delicious (for only the year 1998), Cox's Orange (1997), and Elstar apples.

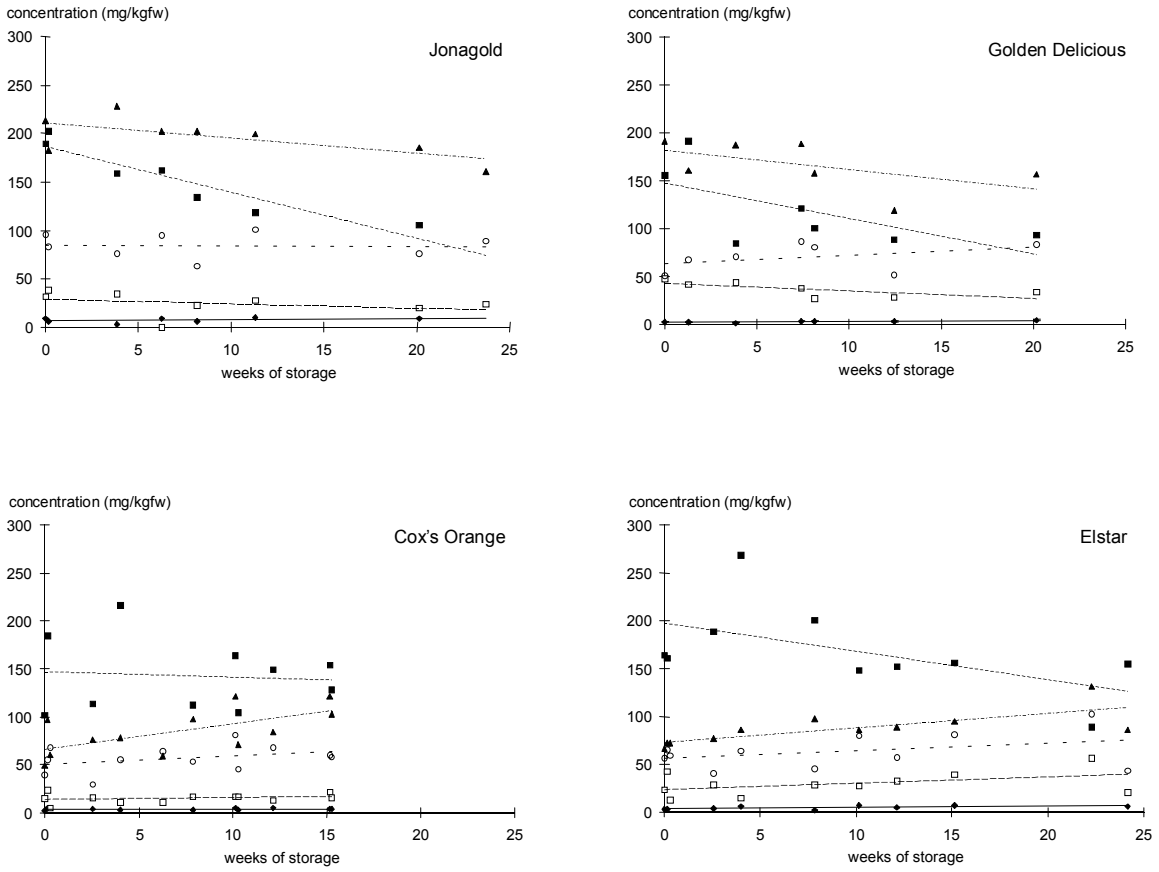

Figure 4. Flavonoid and chlorogenic acid concentrations (mg/kg of fw) of four apple cultivars (harvests 1996, 1997 and 1998) during cold storage: Cy-Ga (—♦—), Phloridzin (- - □- -), Chlorogenic acid (-˙ -˙ ▲-˙ -˙), total Q-gly (···○···), total catechins (---■---). Apple samples were taken in duplicate (SD = 15–20%).

3.3.4.3. Antioxidant Activity during Apple Storage.

IC_{50} values during storage of Jonagold, Golden Delicious, Cox's Orange, and Elstar apples for 1997 and 1998 are given in Figure 5. Cold storage or storage at CA conditions did not affect the antioxidant activity of the apple samples. The same differences in antioxidant potency among the four different cultivars were observed, as was described in Table 4.

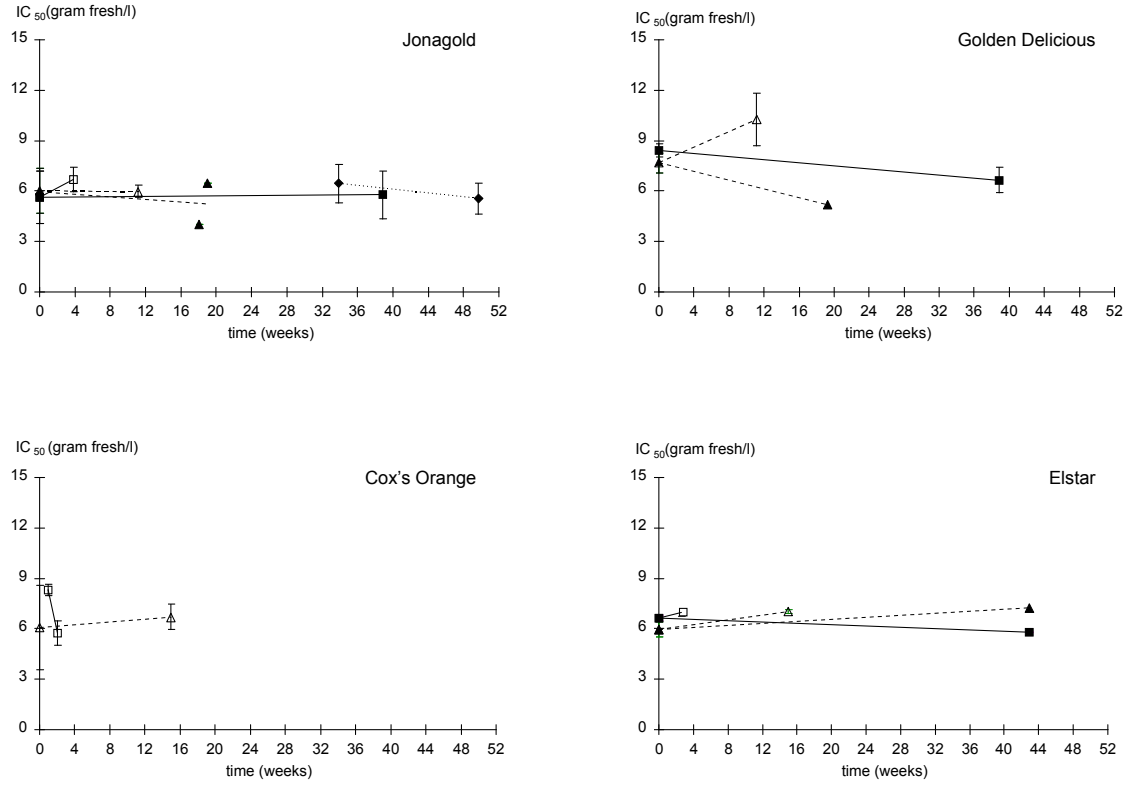

Figure 5. Antioxidant activity (g of fw/L) of four apple cultivars during storage in CA (solid symbols) and cold storage (open symbols): apples from harvest 1996 (···♦···), harvest 1997 (—■—), and harvest 1998 (---▲---). Samples were taken at least in duplicate (mean ± SD). Each IC_{50} value was based on triplicate determination of six different antioxidant concentrations ranging from no to full inhibition of lipid peroxidation.

In Table 6 mean concentrations of all apple samples collected during the three year (1996, 1997, and 1998) storage experiments (CA and cold storage) are given per cultivar. The sum of quercetin glycosides concentration shows that this value does not correlate with the order found in antioxidant activity differences. Jonagold possessed the highest quercetin glycoside concentration, followed by Golden

Delicious. The lowest quercetin glycoside concentration was found in Cox's Orange and Elstar apples.

The total catechin concentration also did not show a correlation with the antioxidant activity of the various cultivars. Elstar had the highest total catechin concentration, followed by Jonagold. Golden Delicious and Cox's Orange had the lowest.

Table 6. Comparison of Composition and Antioxidant Activities of Four Apple Cultivars [a]

cultivar	total Q-glycosides (mg/kg of fw)	total catechins (mg/kg of fw)	sum all compounds analyzed (mg/kg of fw)	IC_{50} (g of fw/L)
Jonagold	95 ± 11	145 ± 37	467 ± 86	5.8 ± 0.8
Golden Delicious.	67 ± 11	121 ± 29	385 ±108	7.6 ± 1.9
Cox's Orange	64 ± 12	106 ± 47	265 ± 98	6.7 ± 1.1
Elstar	63 ± 12	152 ± 42	326 ±100	6.6 ± 0.6

[a] Mean (± SD) of all apple samples during the three year (1996, 1997 and 1998) storage experiments (CA and cold storage).

The sum of all analyzed compounds showed the following order: Jonagold > Golden Delicious > Elstar > Cox's Orange. Because Jonagold apples possessed the highest antioxidant activity, followed by Elstar, Cox's Orange, and then Golden Delicious apples, other compounds must be present in apples that contribute to the antioxidant activity, such as procyanidins or vitamins, the presence of which has been reported (*28*–*30*). The existence of synergistic effects between compounds might be another explanation.

3.3.5. Implications for Product Development.

If food producers want to pay more attention to possible health-protecting compounds in food processing and new product development, the choice of the raw material that is used is important. Of the four tested cultivars Jonagold apples possessed the highest antioxidant activity; therefore, this cultivar might be the most interesting choice of raw material to use in a product. Elstar apple antioxidant activity was lower, followed by Cox's Orange and Golden Delicious. No correlation between flavonoid concentration and antioxidant activity was found.

After choosing the apple cultivar with the highest antioxidant activity in the raw material, the producer has to take care that as much as possible of the active compounds remains in the product. The effects of storage have shown to be minor. For further processing to, for instance, apple juice, the producer should find the optimal way of processing to preserve as much as possible of these compounds and activity in the juice. This will be a subject of further research.

3.4. ABBREVIATIONS USED

Q-3-Ga, quercetin galactoside or hyperin; Q-3-Ru, quercetin rutinoside or rutin; Q-3-Gl, quercetin glucoside or isoquercitrin; Q-3-Xy, quercetin xyloside or reynoutrin; Q-3-Ar, quercetin arabinoside or avicularin; Q-3-Rh, quercetin rhamnoside or quercitrin; Cy-Ga, cyanidin galactoside or ideain.

3.5. ACKNOWLEDGMENT

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3.6. LITERATURE CITED

1. Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochem*. **2000**, *55*, 481–504.
2. Hertog, M. G. L.; Freskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007–1011.
3. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J. Agric. Food Chem*. **1992**, *40*, 2379-2383.
4. Lister, C. E.; Lancaster, J. E.; Sutton, K. H. Developmental changes in the concentration and composition of flavonoids in skin of a red and green apple cultivar. *J. Sci. Food Agric*. **1994**, *64*, 155–161.
5. Macheix, J. J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*. CRC Press: Boca Raton, FL, **1990**; pp 68–71, 113.
6. Kühnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet*. **1976**, *24*, 117–191.
7. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutr. Cancer* **1993**, *20*, 21–29.

8. Kumpulainen, J. T.; Lehtonen, M.; Mattila, P. Trolox equivalent antioxidant capacity of average flavonoids intake in Finland. In *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*. Kumpulainen, J. T.; Salonen, J. T.; Eds.; Royal Society of Chemistry: Cambridge, U.K., **1999**; pp 141–150.

9. Hollman, P. C. H.; de Vries, J.; van Leeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr*. **1995**, *62*, 1276–1282.

10. Olthof M. R.; Hollman P. C. H.; Katan M. B. Chlorogenic acid and caffeic acid are absorbed in humans. *J. Nutr*., **2001**, *131*, 66–71.

11. Day, A. J.; Bao, Y. P.; Morgan, M. R. A.; Williamson, G. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Biol. Med*. **2000**, *29*, 1234–1243.

12. Dekker, M.; Verkerk, R.; Jongen W. M. F. Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. *Trends Food Sci. Technol*. **2000**, *11*, 174–181.

13. Awad, M. A.; de Jager, A.; van Westing, L. M. Flavonoid and chlorogenic acid levels in apple fruit: characterisation of variation. *Sci. Hortic*. **2000**, *83*, 249–263.

14. Pérez-Ilzarbe, J.; Hernández, T.; Estrella, I. Phenolic compounds in apples: varietal differences. *Z. Lebensm. Unters. Forsch*. **1991**, *192*, 551–554.

15. Price, K. R.; Prosser, T.; Richetin, A. M. F.; Rhodes, M. J. C. A comparison of the flavonol content and composition in dessert, cooking and cider-making apples; distribution within the fruit and effect of juicing. *Food Chem*. **1999**, *66*, 489–494.

16. Spanos, G. A.; Wrolstad, R. E. Phenolics of apple, pear, and white grape juices and their changes with processing and storage - a review. *J. Agric. Food Chem.* **1992**, *40*, 1478–1487.

17. Van der Sluis, A. A.; Dekker, M.; Jongen, W. M. F. Effect of processing on content and antioxidant activity of flavonoids in apple juice. In *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*. Kumpulainen, J. T.; Salonen, J. T.; Eds; Royal Society of Chemistry: Cambridge, U.K., **1999**; pp 209–211.

18. Miller, N. J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem*. **1995**, *43*, 1794–1801.

19. Van der Sluis, A. A.; Dekker, M.; Verkerk, R.; Jongen W. M. F. An improved, rapid in vitro method to measure antioxidant activity; application on selected flavonoids and apple juice. *J. Agric. Food Chem*. **2000**, *48*, 4116–4122.

20. De Jager, A.; Roelofs, F. P. M. M. Optimum harvest date of apples for prolonged storage. *Annual Report 1995*; Fruit Research Station: Randwijk, The Netherlands, **1996**; pp 84–86.

21. Awad, M. A.; Wagenmakers, P. S.; de Jager, A. Effect of light environment on flavonoid and chlorogenic acid levels in the skin of 'Jonagold' apples. *Sci. Hortic*. **2001**, *88*, 289–298.

22. Van Buren, J.; de Vos, L.; Pilnik, W. Polyphenols in Golden Delicious apple juice in relation to method of preparation. *J. Agric. Food Chem*. **1976**, *24*, 448–451.

23. Burda, S.; Oleszek, W.; Lee, C. Y. Phenolic compounds and their changes in apples during maturation and cold storage. *J. Agric. Food Chem*. **1990**, *38*, 945–948.

24. Dick, A. J.; Redden, P. R.; DeMarco, A. C.; Lidster, P. D.; Grindley, T. B. Flavonoid glycosides of Spartan apple peel. *J. Agric. Food Chem*. **1987**, *35*, 529–531.

25. Awad, M. A.; de Jager, A. Flavonoid and chlorogenic acid concentrations in skin of 'Jonagold' and 'Elstar' apples during and after regular and ultra low oxygen storage. *Postharvest Biol. Technol*. **2000**, *20*, 15–24.

26. Arts, I. C. W.; van de Putte, B.; Hollman, P. C. H. Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, Vegetables, Staple foods, and processed foods. *J. Agric. Food Chem*. **2000**, *48*, 1746–1751.

27. Podsedek, A.; Wilska-Jeszka, J.; Anders, B.; Markowski, J. Compositional characterisation of some apple varieties. *Eur. Food Res. Technol*. **2000**, *210*, 268–272.

28. Guyot, S.; Doco, T.; Souquet, J.-M.; Moutounet, M.; Drilleau, J.-F. Characterization of highly polymerized procyanidins in cider apple (Malus Sylvestris var. Kermerrien) skin and pulp. *Phytochemistry* **1997**, *44*, 351–357.

29. Mayr, U.; Treutter, D.; Santos-Buelga, C.; Bauer, H.; Feucht, W. Developmental changes in the phenol concentrations of 'Golden Delicious' apple fruits and leaves. *Phytochemistry* **1995**, *38*, 1151–1155.

30. Lee, C. Y.; Mattick, L. R. Composition and nutritive value of apple products. In: *Processed Apple Products*; Downing, D. L., Ed.; AVI (Van Nostrand Rheinhold): New York, **1989**; p 314.

CHAPTER 4

Activity and Concentration of Polyphenolic Antioxidants in Apple Juice. 1. Effect of Existing Production Methods

ABSTRACT

Apples are an important source of flavonoids in the human diet. The effect of processing apples into juice on polyphenolic antioxidant content and activity is described. Raw juice obtained from Jonagold apples by pulping and straight pressing or after pulp enzyming had an antioxidant activity that was only 10 and 3%, respectively, of the activity of the fresh apples. The levels of flavonoids and chlorogenic acid in the juice were reduced to between 50% (chlorogenic acid) and 3% (catechins). Most of the antioxidants were retained in the pomace rather than being transferred into the juice. Apparently, most of the antioxidant compounds are absorbed to the solid matter of the pomace. In apple juice, 45% of the total measured antioxidant activity could be ascribed to the analyzed antioxidants. For three apple cultivars tested (Elstar, Golden Delicious, and Jonagold), the processing methods had similar effects. The results indicate that processing can have a major impact on the bioactivity of products.

Key words

Antioxidant activity; quercetin glycosides; catechins; phloridzin; anthocyanins; chlorogenic acid; processing; apple juice; cultivar

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4.1. INTRODUCTION

There is a growing interest in compounds in food which possess a possible health-protecting capacity and which were previously regarded as non-nutrients. Those compounds, if derived from plants, are described as phytochemicals. They hardly contribute to the nutritional value of the product but might play an important role in maintaining human health. Flavonoids are an example of these compounds, and in epidemiological studies inverse relations with aging diseases such as coronary heart diseases and cancer have been described (*1*–*3*). This is ascribed to their function as antioxidants, or in modulating enzyme activity (*4*).

To satisfy a growing demand by consumers for products with a high content of bioactive components, it is necessary to know the influence of the various stages in the food production chain on the presence of these compounds in the product. Cultivation methods (*5*), choice of raw material (*6*), industrial processing, storage (*7*), distribution, and final processing by the consumer may all affect the final concentrations and the bioactivity of the product (*8*). Knowledge of these aspects will provide the food processor with information that can be used in product optimization with respect to health-protecting compounds. This should be performed in a way that will not affect traditional quality aspects, such as color and taste.

The three most important groups of flavonoids present in apple and apple products are flavanols or catechins, flavonols, and anthocyanins (*9*), with the main representatives (–)-epicatechin, quercetin glycosides, and cyanidin galactoside, respectively. These compounds all belong to the group “polyphenolics”, together with procyanidins, which consist of oligomeric and polymeric catechins (*10*), phloridzin and phloretin xyloglucoside (dihydrochalcones), and chlorogenic acid and *p*-coumaroylquinic acid (phenolic acids), which are also present in apple (*11*). Besides their contribution to potential health benefits, flavonoids contribute to the color and taste of apples. Their (bio)chemical and physical properties are important in processing.

Catechins are colorless and oxidation sensitive. Normally they occur as aglycons, but sometimes they are esterified with gallic acid (*12*). However, galloylated catechins have not been detected in apple (*13*). Catechins are, together with chlorogenic acid, good substrates for the enzyme polyphenoloxidase (*11*, *14*). This enzyme plays a

role in the browning of fruits caused by damage, such as cutting. In the presence of oxygen, these substrates form brown complexes.

Flavonols are usually present in the plant as glycosides and are colorless or light yellow in color (*12*). They are not substrates for polyphenoloxidase, because of the steric hindrance of the sugars bound to the C3 atom in the flavonol skeleton (*15*).

Anthocyanins are glycosylated or acylated anthocyanidins and are more water-soluble than flavonols and flavanols. They are important plant pigments and contribute to the purple and red colors of flowers and fruit. They accumulate in the vacuoles of epidermal and subepidermal cells (*16*). Anthocyanins are unstable during chemical and physical processing. Their color is strongly pH-dependent (*17*).

After processing of apples to apple juice, a low flavonoid concentration is found in the juice. In commercially available apple juice, only 2.5 mg/L quercetin was detected (*18*), while in apples the concentration was 36 ± 19 mg/kg (analyzed as aglycon) (*19*). To know whether this loss in flavonoid content is due to degradation of these compounds during processing or to other factors, it is necessary to look more precisely into the apple juice production process. The basic processing method for apple juice consists of the following steps (*20*): apple fruit selection (on the basis of cultivar, maturity, and quality), washing and inspection, crushing, milling, or slicing to pulp, pressing (or extraction) to raw juice, clarification/filtration, pasteurization, and packaging.

To obtain a higher juice yield, enzymatic treatment of the pulp before pressing is often applied (*21*). Pectolytic enzymes are used to increase the pressability by degrading pectins in the cell walls. Before addition of pectolytic enzymes, the pulp can be aerated to allow oxidation of polyphenols. This may prevent inhibition of pectolytic enzymes by these compounds.

It is likely that the industrial practice of enzyming and oxidizing polyphenols may affect the antioxidant activity of the juice, as the polyphenols are important contributors to antioxidant activity. It is known that the polyphenol concentration indeed is affected by these treatments. Enzymed versus straight pressed Golden Delicious apple juice prepared by Schols et al. (*22*) showed a reduction in the level of epicatechin, catechin, phloridzin, and chlorogenic acid. In apple juices from three different cultivars prepared by pulp enzyming, Spanos et al. (*7*) found no quercetin glycosides, a low catechin concentration (0–4 mg/L), 1–9 mg/L epicatechin, 5–12 mg/L phloridzin, and 17–59 mg/L chlorogenic acid, depending upon the cultivar.

Others reported the presence of traces of quercetin glycosides in apple juice following commercial scale pressing (*23*) and in apple juice prepared in a domestic juice processor (*24*). In commercially available apple juice, no catechin or epicatechin was detected (*25*).

The objective of the present study was to investigate the effects of the various steps of existing production methods on flavonoid content and antioxidant activity of apple juice. Straight pressing of apple pulp is compared with enzyme treatment of the pulp before pressing. Three different apple cultivars that can be used in apple juice production were tested: Jonagold, Golden Delicious and Elstar.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals.

Kaempferol, myricetin, quercetin dihydrate, and rutin trihydrate were purchased from Fluka Chemicals (Zwijndrecht, The Netherlands); chlorogenic acid, phloridzin, (±)-catechin, and (–)-epicatechin were from Sigma Chemicals (Zwijndrecht, The Netherlands); and quercetin 3-arabinoside, hyperoside, isoquercitrin, quercitrin, and ideain chloride were from Carl Roth GmbH and Co. (Karlsruhe, Germany). Quercetin 3-arabinofuranoside was obtained from Apin Chemicals (Oxfordshire, United Kingdom) and reynoutrin from Plantech (Reading, United Kingdom). L-(+)Ascorbic acid and iron(II) sulfate heptahydrate were obtained from Merck (Darmstadt, Germany). The enzyme Rapidase BE Super was supplied by Gist-Brocades International B.V. (Delft, The Netherlands). All other chemicals were of analytical or HPLC grade purity.

4.2.2. Apple Cultivars and Harvest and Storage Conditions.

Three main apple cultivars grown in The Netherlands were used: Jonagold, Elstar, and Golden Delicious. Apples were harvested from commercial orchards in 1998, which were the same as described in Van der Sluis et al. (*6*).

The day of harvest was predicted using variety-specific models (*26*) that predict the optimal harvest date for Jonagold apples for long-term storage in controlled atmosphere (CA). This means that at the time of harvest, apples have not reached the stage of complete maturity suitable for immediate consumption.

Fruits from all apple cultivars were picked at one time, from the outer layer of the trees, avoiding the tops and bottoms. Apples from the inner layer of the tree were not

used because their total flavonoid concentration is much lower due to lower light levels (*5*). Trees at the border of the orchard were avoided as well.

Applied CA storage conditions for Jonagold, Elstar and Golden Delicious were 1.5 °C, 1.2% O_2, and 2.5% CO_2. After 3.5 months of storage, the apples were transported to Matforsk, Norway, for processing of juice.

4.2.3. Sample Preparation.

Flavonoid standards were dissolved in methanol. Fresh apple samples were taken as follows. Four apples were chosen at random. After cleaning with water and removal of stalks, the whole apples were cut to pieces with a knife and subsequently ground under liquid nitrogen in order to prevent oxidation. Duplicate samples were taken from each four-apple sample. At various stages during the apple juice production, duplicate samples were taken and also ground under liquid nitrogen. All frozen samples were stored immediately at –20 °C until lyophilization. Dry weight was determined from the sample weight before and after lyophilization. Lyophilized samples were stored at –20 °C in the dark until analyzed.

All samples were extracted before HPLC analysis and antioxidant activity determination. Lyophilized sample (0.5 g) was extracted with 10 mL of methanol, while juice samples were diluted to 50% with methanol. The samples were sonicated for 30 min, followed by 10 min of centrifugation at 2500 rpm. The supernatant was filtered through a 0.45-µm filter.

4.2.4. Quantification of Flavonoids by HPLC.

Quercetin glycosides, catechins, chlorogenic acid, phloridzin, and cyanidin galactoside were determined in the apple samples by HPLC as described by Van der Sluis et al. (*6*).

The individual compounds were identified and quantified by comparison with standard solutions of known concentrations and, if necessary, by comparison of spectra. Quercetin, its glycosides, and chlorogenic acid were monitored at 350 nm, phloridzin and the catechins were monitored at 280 nm, and cyanidin galactoside was monitored at 525 nm.

Quercetin glycosides were analyzed separately and presented as group “total Q-glycosides”, which consists of the compounds Q-3-Ga, Q-3-Ru, Q-3-Gl, Q-3-Xy, Q-3-Ar, and Q-3-Rh (see Abbreviations Used). The catechins were also analyzed

separately and presented as group “total catechins”, which consists of the compounds catechin and epicatechin. Quercetin in aglycon form was not detected in any of the apple samples.

4.2.5. Antioxidant Activity Determination.

Antioxidant activity was determined by an in vitro method in which lipid peroxidation (LPO) is induced in rat liver microsomes (*27*). The reaction is initiated by Fe^{2+} (10 µM) and ascorbic acid (200 µM). Reaction products are measured spectrophotometrically by the thiobarbituric assay ($A_{540–620}$). Inhibition of LPO is an indication of the antioxidant activity. The mean absorbance reading ($A_{540–620}$) ± standard deviation of the blanks was 1.026 ± 0.145 (in 70 repetitions collected on 6 experimental days).

The antioxidant concentration at which 50% inhibition of LPO occurs (IC_{50}) was calculated from triplicate determination of six different antioxidant concentrations ranging from no (0%) to full (100%) inhibition of LPO.

4.2.6. Apple Juice Production.

Different types of apple juice were prepared. Starting weights of about 25 kg were used. Apples were cleaned by washing, stalks were removed and the fruit was cut in two, all during a 30-min period. Apple pulp was prepared by quick slicing in a dicing machine (BL-1000, Eillert B.V., The Netherlands), this took about 15 min. Two apple pulp particle sizes were prepared (3 x 3 x 10 mm versus 3 x 3 x 3 mm). Straight pressed apple juice was prepared by immediate pressing of apple pulp. Pulp-enzymed juice was prepared after addition of pectolytic enzymes (200 ppm Rapidase BE Super) to apple pulp (particle size 3 x 3 x 10 mm) and leaving the mixture for 2 h at room temperature with continuous stirring before pressing. Apple pulp was pressed in a Bucher-Guyer juice press (three times pressing to 160 bar). The pressing process lasted for about 80 min for the straight pressing juice and 50 min for the pulp-enzymed juice.

During apple juice production, samples of the apple fractions (fresh apple, pulp, pomace, and raw juice) were taken. All fractions were weighed, and mass balances were composed. From the mass balances, standardized mass balances were calculated in which the weight of the apple fractions was corrected for sample taking. No correction was made for losses that remained in the equipment during the

production. The degree Brix of the obtained raw apple juices was determined, and in the standardized mass balances the raw juices were adjusted with water to 12 °Brix. This degree Brix level is higher than the 10.2 °Brix minimum value set for single strength apple juice not from concentrate (*28*).

4.2.7. Calculations.

4.2.7.1. Mass Balances.

Mass balances and compound mass balances were calculated for all processing steps described in Figure 1. An overall mass balance (eq 1a) describes the effect of washing and grinding of fresh apples to pulp. Compound mass balances were used for the 10 different flavonoids and chlorogenic acid: they were composed of the standardized weights of the apple fractions and the concentrations of those compounds present in the fractions using eq 1b.

The effect of washing and grinding:

standardized mass balance: $m_{\mathrm{f}} = m_{\mathrm{p}} + m_{\mathrm{l}}$ (kg) (1a)

compound mass balance: $m_{\mathrm{f},i} \times c_{\mathrm{f},i} = m_{\mathrm{p},i} \times c_{\mathrm{p},i} + m_{\mathrm{l},i} \times c_{\mathrm{l},i}$ (mg) (1b)

with m the weight of apple fraction (kg) and c the concentration of compound i (i = 1–11) in the apple fraction (mg/kg of fw). Apple fractions are represented as f, fresh apple; p, pulp or ground apple; and l, loss.

The effect of pressing can be described by eqs 2a and 2b:

standardized mass balance: $m_{\mathrm{p}} = m_{\mathrm{pc}} + m_{\mathrm{srj}} + m_{\mathrm{l}}$ (kg) (2a)

compound mass balance: $m_{\mathrm{p},i} \times c_{\mathrm{p},i} = m_{\mathrm{pc},i} \times c_{\mathrm{pc},i} + m_{\mathrm{srj},i} \times c_{\mathrm{srj},i} + m_{\mathrm{l},i} \times c_{\mathrm{l},i}$ (mg) (2b)

Apple fractions are represented as p, pulp or ground apple; pc, pomace; srj, standardized raw juice (12 °Brix); and l, loss.

Equations 3a and 3b describe the standardization of raw juice to a chosen degree Brix:

standardized mass balance: $m_{\mathrm{rj}} + m_{\mathrm{w}} = m_{\mathrm{srj}}$ (kg) (3a)

compound mass balance: $m_{\mathrm{rj},i} \times c_{\mathrm{rj},i} + m_{\mathrm{w},i} \times c_{\mathrm{w},i} = m_{\mathrm{srj},i} \times c_{\mathrm{srj},i}$ (mg) (3b)

Apple fractions are represented as rj, raw juice; w, water; and srj, standardized raw juice.

4.2.7.2. Antioxidant Activity.

The antioxidant activity of the apple samples is derived from the IC_{50} value (in grams of fw per liter), which gives the concentration of the antioxidant sample (an individual compound or food extract) at which 50% inhibition of lipid peroxidation occurs. The measured (or actual) antioxidant activity of an apple sample is expressed as the dilution factor that gives 50% inhibition ($DF_{50,sample}$) according to eq 4:

$$DF_{50,sample} = \frac{1000}{IC_{50,sample}} \quad (4)$$

where $IC_{50,sample}$ is the IC_{50} value of the apple sample (g of fw/L).

From the concentrations of compounds in a food product and the IC_{50} values of these individual compounds, it is theoretically possible to calculate the antioxidant activity of that product. Assumptions are that no synergistic, antagonistic, or other matrix effects play a role and that all compounds with antioxidant activity in the food product are known and detectable (*29*). To predict the antioxidant activity of the apple fractions from its composition, eq 5 and the IC_{50} values of 11 standard components as given by Van der Sluis et al. (*27*), recalculated to milligrams per liter, were used.
The calculated (or predicted) antioxidant activity of a mixture of known antioxidants is described by eq 5. The ratio in eq 5 equals the calculated dilution factor of the mixture of components to give 50% inhibition of lipid peroxidation ($DF_{50,mixture}$). In the ideal situation, where the mentioned assumptions are valid, the measured $DF_{50,sample}$ (eq 4) equals the calculated value (eq 5).

$$\frac{\sum_{i=1}^{n} C_i}{IC_{50,mixture}} = \sum_{i=1}^{n} \frac{C_i}{IC_{50,i}} = DF_{50,mixture} \quad (5)$$

where C_i is the concentration of component i (mg/kg of fw) and $IC_{50,i}$ is the IC_{50} value of component i (mg/L).

Antioxidant activity values of raw juices with values of $DF_{50,sample}$ below 30 are within the detection limit of the method. The values are obtained by extrapolation in the assay in the case where it was not possible to compose a concentration range that provided a range from 0% to 100% inhibition in the LPO assay. Therefore, these values might be less accurate. For lyophilized samples, this detection limit was 70.

4.2.8. Statistical Analysis.

Statistical analysis of the data was performed on the means of duplicate analytical determinations by one-way Analysis of Variance (ANOVA), with significance level α= 0.05 using the statistical package from Microsoft Excel.

4.3. RESULTS AND DISCUSSION

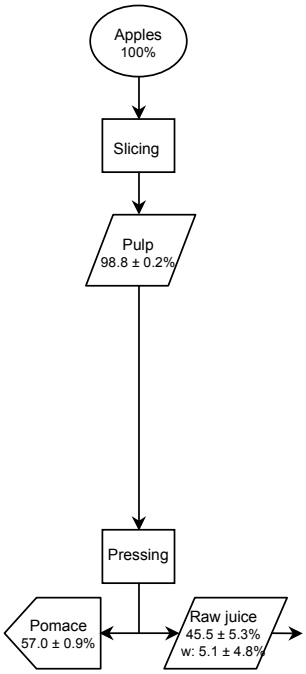
4.3.1. Existing Juice Production Methods: Straight Pressing and Pulp Enzyming.

Straight pressed apple juice was prepared following the processing scheme described in Figure 1a. This figure shows the standardized weights (in % related to the fresh apple) of the apple fractions during processing. The initial degree Brix values of the juices were between 14.2 and 15.0, and the raw juices were adjusted to 12 °Brix for standardization purposes by adding water. The addition of standardization water causes the sum of the weights of the pomace and the standardized raw juice to be more than 100%.

Due to the low apple juice yield obtained by straight pressing of apple pulp (46% for Jonagold apples in a Bucher-Guyer press), pectolytic enzymes are often applied in industrial apple juice production to facilitate pressing and to increase yield. The processing scheme and standardized weights for the production of pulp-enzymed apple juice are given in Figure 1b. The juice yield increased to 80% for Jonagold apples when the pectolytic enzymes were applied. An alternative way to increase juice yield is to reduce the particle size of the pulp before pressing. In the present study, the juice yield increased from 45.5% to 59.2% when the particle size of the pulp was reduced from 3 x 3 x 10 to 3 x 3 x 3 mm. It is possible to increase the juice yield further by adding water to the pomace and applying a second pressing cycle (*22*).

The use of equations 1a–3a enabled the calculation of the losses that occurred in apple juice production. The losses in the various apple fractions caused by material remaining in the equipment can be calculated from Figure 1. Slicing caused a loss of 1% of the apple starting weight, pressing a loss of about 3% (when no correction for the degree Brix values of the juices is made). In the present processing trial, these losses were small compared to the biological variation of flavonoid and chlorogenic acid concentration in apples (10–30%, Table 1).

(a) Straight pressing (*n*=3)



(b) Pulp enzyming (*n*=1)

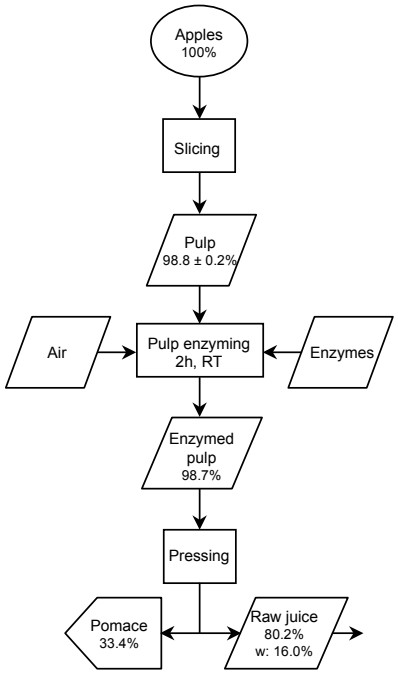


Figure 1. Processing schemes and standardized weights of apple fractions during Jonagold apple juice production. Comparison of straight pressing (a) and pulp enzyming (b). All percentages are related to the fresh apple fraction. Apple pulp particle size, 3 x 3 x 10 mm; w, amount of water in the raw juice that was added to standardize the obtained juice to 12 °Brix.

To compare the two apple juice production methods with respect to their ability to give juice with high bioactivity, levels and activity of antioxidants, data for pulps, pomaces and juices are presented in Table 1.

4.3.1.1. Apple Pulp.

Slicing of the apples did not affect chlorogenic acid and flavonoid (cyanidin galactoside, phloridzin, total quercetin glycosides, total catechins) concentration or antioxidant activity (Table 1). Pulp enzyming caused decreases in phloridzin (31%), chlorogenic acid (44%), and total catechin (58%) concentrations. These compounds are oxidation sensitive and are known substrates for polyphenoloxidase. In the 2 h of pulp enzyming with continuous stirring, oxidation occurs. Cyanidin galactoside and total quercetin glycoside concentration were not affected by the extended oxidation period during enzyming, most likely because these compounds are not substrates for polyphenoloxidase (*15*, *17*).

Table 1. Mass Balances, Concentrations of Flavonoids and Chlorogenic Acid (Milligrams per Kilogram of Fresh Weight), and Antioxidant Activities of Apple Fractions during Processing of Jonagold Apple Juice: Straight Pressing versus Pulp Enzyming

			straight pressing		pulp enzyming		
Jonagold	apples (n = 5)	pulp (3 x 3 x 10 mm) (n = 4)	pomace (n = 3)	standardized raw juice (n = 3)	enzymed pulp (n = 1)	pomace (n = 1)	standardized raw juice (n = 1)
	mean ± SD	mean ± SD	mean ± SD	mean ± SD			
standardized mass balance (kg)	25.0 ± 0	24.7 ± 0.0	14.2 ± 0.2	11.4 ± 1.3	24.7	8.4	20.1
included added water (kg)				1.3 ± 1.1			4.0
concentrations (mg/kg of fw)							
Cy-Ga	10 ± 3	8 ± 2	14 ± 2	3 ± 0	6	11	2
phloridzin	46 ± 17	36 ± 7	63 ± 11	4 ± 1	25	46	2
chlorogenic acid	202 ± 33	178 ± 16	170 ± 20	133 ± 15	100	87	63
total Q-glycosides	109 ± 25	103 ± 17	179 ± 30	13 ± 1	98	186	11
total catechins	186 ± 26	176 ± 13	173 ± 19	16 ± 1	74	105	6
measured activity: $1000/IC_{50}$ (L/mg of fw)	202.3 ± 67.6[a]	195.0 [b]	203.9 [b]	21.2 ± 18.4[a]	-	-	6.2 [b]
calculated activity: Σ (C / IC_{50})	72.7 ± 11.3	68.4 ± 5.8	78.0 ± 9.7	9.5 ± 1.2	36.9	57.8	4.7
explained activity: calculated/measured	36%	35%	38%	45%			76%

[a] n = 2. [b] n = 1

The calculated antioxidant activity of enzymed pulp, based upon the analyzed antioxidants and using equation 5, was lower than that of the untreated pulp, due to the loss in total catechins and chlorogenic acid. Phloridzin does not contribute to the antioxidant activity with the method applied (*6*).

4.3.1.2. Pomace.

The concentrations of all analyzed compounds were higher in the pomaces than in the corresponding juices (Table 1). Similarly, Price et al. (*23*) reported higher levels of quercetin glycosides in pomace than in juice. Lu and Foo (*30*) reported the presence of phloretin-2'-xyloglucoside and 3-hydroxyphloridzin in apple pomace from undescribed origin, in concentrations of 12% and 19% of that of phloridzin respectively. They concluded that apple pomace contained a high level of polyphenols which could be commercially exploited.

Comparisons of both pomaces in the present study showed the same pattern as found in the apple pulp from which they were produced. The pomace from the pulp enzyming process was lower in phloridzin, chlorogenic acid, and total catechin concentration, while cyanidin galactoside and total quercetin glycoside concentration were unchanged in both pomaces. Due to the lower levels of chlorogenic acid and total catechins, the calculated antioxidant activity was approximately 25% lower for the pulp enzyming process than for the straight pressing process. The compound concentrations in the pomaces cannot be directly compared to their concentrations in the corresponding pulps, because pressing causes a split of the pulp into two fractions: the pomace and the raw juice. The use of compound mass balances prevents this problem.

4.3.1.3. Raw Apple Juice.

Chlorogenic acid was found to have the highest concentration in the juice compared with the other compounds analyzed (Table 1). This indicates that during pressing only a small fraction of the analyzed compounds other than chlorogenic acid is extracted into the juice. The differences in water solubility between the different flavonoid groups and the chlorogenic acid might contribute to this observation. Chlorogenic acid is the most water-soluble compound, which explains the highest yield for this compound in the juice. Furthermore, flavonols and dehydrochalcones

are mainly located in the “solid” parts of the fruits (such as the skin and seeds), which complicates their extraction.

In both raw juices, the levels of flavonoids and chlorogenic acid were reduced 2–30-fold when compared to those in the fresh apple fraction (depending on the different groups studied: quercetin glycosides 10-fold lower, phloridzin 10–20-fold lower, anthocyanins 5 times lower, and chlorogenic acid 2 times lower). The most striking reduction (30-fold) was that of the catechins in the juice obtained with enzyme treatment and aeration. Simultaneously, an extensive loss of antioxidant activity was observed. For both raw juices, the measured and calculated antioxidant activities were only a small fraction of that of the fresh apples they were produced from. The juice obtained by straight pressing or by incorporating an enzymatic treatment and oxidation step had a measured antioxidant activity that was only 10% and 3% of the original activity of fresh apples, respectively. The measured antioxidant activity of the raw juices was within the lower detection limits of the analytical method.

Flavonoid concentrations and antioxidant activity of apple juice prepared from 3 x 3 x 3 mm apple pulp did not significantly differ from those of juice prepared from the larger sized apple pulp (for which data are given in Table 1). Therefore, lowering the apple particle size did not improve the apple juice production method with respect to retaining polyphenolic antioxidant content and activity.

4.3.1.4. Mass Balances during Juice Production.

The compound mass balances of components present in the various apple fractions of both treatments are presented in Figure 2. Equations 1b–3b were used for calculations. Chlorogenic acid was equally distributed between the pomace and the juice, while quercetin glycosides, total catechins, phloridzin, and cyanidin galactoside remained preferentially in the pomace. This indicates that, for most of the analyzed antioxidant components in apples, adsorption to the solid matter of the pomace is favored. Renard and co-workers (*31*) reported that procyanidins are able to bind to the cell wall matrix, whereas hydroxycinnamic acids and epicatechin are not.

Catechins are the most vulnerable compounds during apple juice production. The compound mass balance shows that the total amount of catechins in raw juice and pomace was 43% lower than the amount present in fresh apple or pulp (80% lower in the case of pulp enzyming). This loss may be explained by the oxidation sensitivity of the catechins. On the other hand, all quercetin glycosides could be traced back in the

mass balance. The amount of quercetin glycosides present in the raw juice and pomace together was equal to the amount present in the fresh apple or in the pulp. In both apple juice production methods, slicing and pressing did not affect the quercetin glycosides. The pressing only caused a “partitioning” of these compounds between the pomace and the juice, in such a way that 93% of the quercetin glycosides was present in the pomace (from straight pressing). Phloridzin behaved similarly to the quercetin glycosides, and more than 77% of the initial level remained in the pomace.

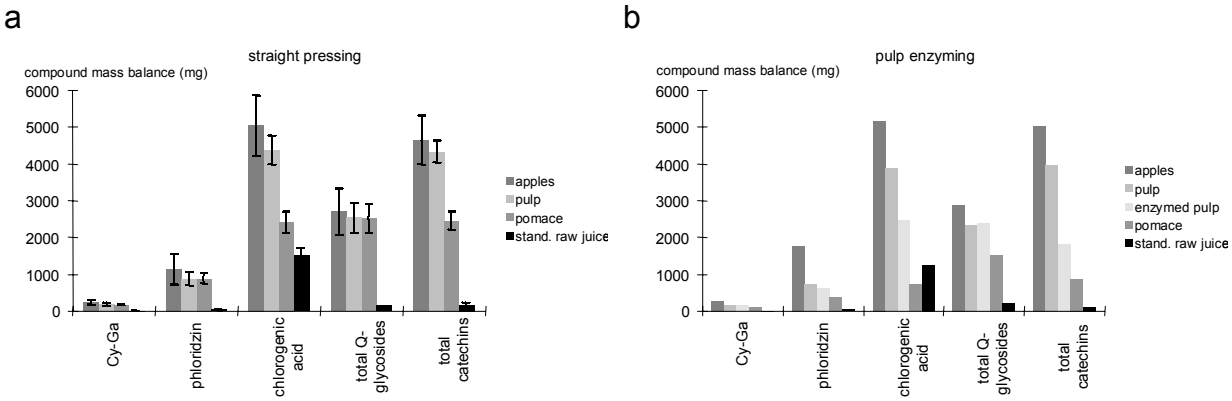


Figure 2. Compound mass balances of apple fractions in Jonagold apple juice production, straight pressing (n=3) versus pulp enzyming (n=1). Starting weight was 25 kg.

The level of cyanidin galactoside was low in the apples and thus also in the various fractions from the juice processing, compared with the other polyphenolics analyzed. No significant losses occurred throughout the juice processing. In the straight pressing process, about 10% of the cyanidin galactoside occurred in the raw juice, while 80% remained in the pomace.

The use of compound mass balances was extremely useful in locating processing steps that affect the concentrations of compounds contributing to the antioxidant activity of the product. Its use revealed that, besides the partitioning phenomenon that caused losses during the production, another process, most probably oxidation (especially of the catechins), resulted in considerable losses of the antioxidant compounds of the apples.

4.3.1.5. Contribution of Compounds to the Measured Antioxidant Activity.

Comparison of the measured antioxidant activity with the calculated antioxidant activity using eqs 4 and 5 (Table 1), showed that only about 35% of the antioxidant

activity in fresh apples, pulp, and pomace and 45% in the raw juice can be ascribed to the analyzed components. In Figure 3, the relative contribution of each analyzed flavonoid and of chlorogenic acid to the measured antioxidant activity of the various fractions in the apple juice production is given. For all fractions, the group of “total catechins” was the most important contributor to the antioxidant activity. In straight pressed apple juice, the contribution to the measured antioxidant activity was 25% in the fresh apples, the pulp, and the pomace, and in the raw juice the contribution was 30%. The group “total quercetin glycosides” was the second most important contributor to the measured antioxidant activity, with 6%, 8%, and 12% in the fresh apples, the pulp, and the pomace, respectively. In the raw juice, however, chlorogenic acid was the second most important contributor, with 21% of the measured antioxidant activity, followed by total quercetin glycosides, with 12%. The contribution of cyanidin galactoside in all apple fractions was below 2%. Phloridzin did not contribute at all to the measured antioxidant activity in any of the apple fractions, due to the low antioxidant activity of phloridzin in the assay (*27*).

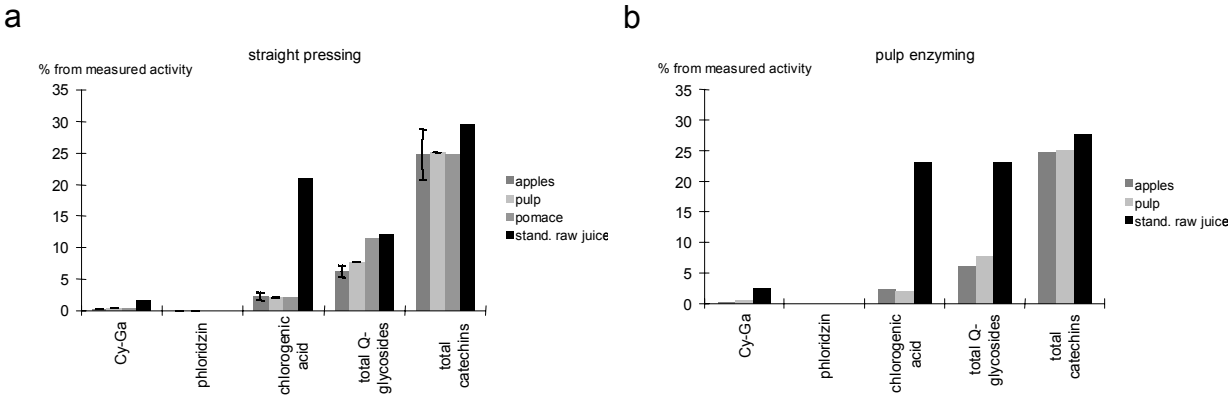


Figure 3. Calculated contribution of flavonoids and chlorogenic acid to the measured antioxidant activity of apple fractions in Jonagold apple juice production, straight pressing (n=2) versus pulp enzyming (n=1).

The pulp after enzyming and the pomace were not analyzed for their antioxidant activity, but the results of the other fractions from the processing of pulp-enzymed apple juice were similar to those seen for straight pressed apple juice. The only difference occurred in the raw juice, where there was a higher (76%) total explained antioxidant activity (Table 1). The contribution of the group “total quercetin

glycosides" was higher than that in straight pressed apple juice. This is explained by the higher decrease in the catechins by this method, as was discussed before.

To summarize these findings: the contribution of analyzed compounds to the measured antioxidant activity of fresh Jonagold apple, apple pulp, and pomace was total catechins > quercetin glycosides > chlorogenic acid > cyanidin galactoside >> phloridzin. In both raw juices, the contribution was total catechins > chlorogenic acid > quercetin glycosides > cyanidin galactoside >> phloridzin.

Comparison of antioxidant activity data of apple juice is, to a certain extent, possible with the findings of Miller et al. (*32*). They analyzed total antioxidant activity of apple juice from a not further specified production process, but with no added vitamin C. In their method, 51.4% of the total antioxidant activity was explained by analyzed compounds, with chlorogenic acid (32%) and phloridzin and phloretin glycosides (11%) as the most important contributors to the total antioxidant activity. In contrast to our results, quercetin glycosides were not detected in that study, and epicatechin was detected only in a negligible amount. Also, their use of a test system based on radical scavenging ability ($ABTS^{\bullet+}$) instead of the capacity to inhibit LPO may explain the discrepancies seen between the two studies. It has been suggested that antioxidant activity may differ between these assays, since they are based on different chemical and physical principles for monitoring oxidation (*33*). Phloridzin clearly shows differences in reactivity in both assays.

4.3.1.6. Antioxidant Activity Unaccounted for.

A large proportion of the measured activity could not be ascribed to the analyzed antioxidants in the present study. The explanation for this difference could be the occurrence of synergism between the various antioxidant components. Further, the presence of compounds other than those analyzed might contribute to the antioxidant activity in apples and their products. These compounds could be procyanidins, which possess good antioxidant properties (*34*) and whose presence has been reported in apples (*35*–*37*) and in pomace (*38*). Other possibilities are carotenoids (*39*) and vitamins (*40*).

In orange juice and black currant drink, 5% and 24%, respectively, of the antioxidant activity remained unaccounted for (*41*). In these juices, added vitamin C was the main contributor to the measured antioxidant activity. In the apple juices of the present study, no vitamin C was added. Miller et al. (*32*) reported that the contribution

of ascorbic acid to the measured antioxidant activity of apple juice was only 1%. Similarly, Gardner et al. (*42*) reported that the contribution of total carotenoid content to the antioxidant activity of various fruit juices (apple juice included) was negligible.

To resolve the characteristics of the compounds that are providing extra antioxidant activity, the correlation of the unaccounted antioxidant activity with the calculated activity of all analyzed antioxidants in the fractions in Jonagold apple juice produced by straight pressing in the present study was determined. As can be seen in Figure 4a, a high correlation was obtained for total catechins (R^2 = 0.994). The correlation between explained and unaccounted antioxidant activity from total quercetin glycosides (Figure 4b, R^2 = 0.753) and cyanidin galactoside (Figure 4c, R^2 = 0.716) was less profound. Low correlation was found with chlorogenic acid activity (Figure 4d, R^2 = 0.797).

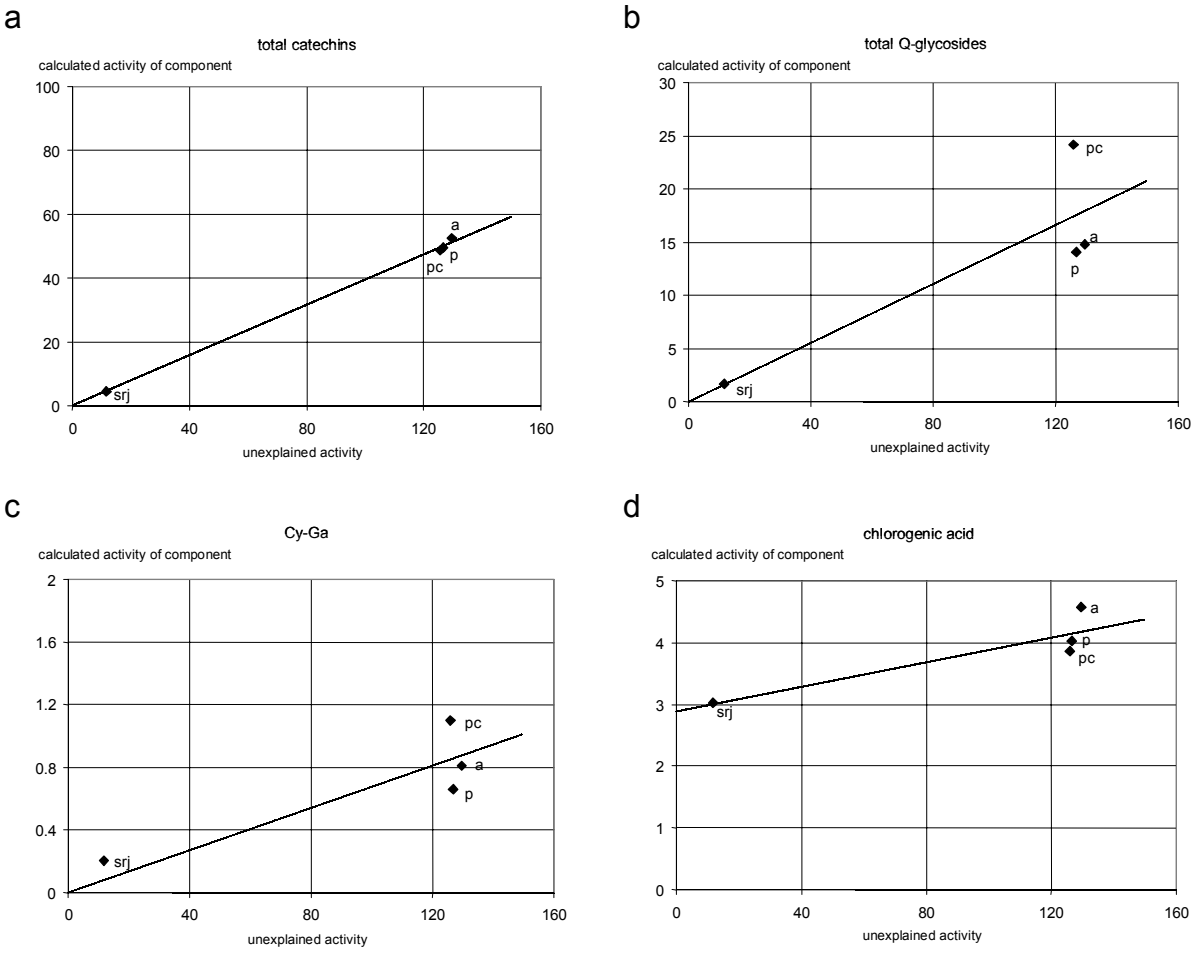


Figure 4. Correlation of the unaccounted antioxidant activity with the calculated activity of selected components of apple fractions in Jonagold apple juice produced by straight pressing. a= fresh apple, p= apple pulp, pc= pomace, srj= standardized raw juice

The high correlation for the explained and unaccounted antioxidant activity for the catechins indicates that the unknown antioxidant compound(s) behaved quite similarly to the catechins in the juice production process. Rice-Evans and Miller (*43*) suggested that unaccounted antioxidant activity presumably is derived from unidentified polyphenols and phenolic acids as well as polymers formed from them. The results of the present study may thus add more specificity to this explanation by suggesting that the unidentified compounds could be polymeric catechins, such as procyanidins (*38*).

These findings demonstrate that it is necessary to measure the antioxidant activity of potentially important intermediate fractions during processing with the purpose of locating processing steps that affect unanalyzed compounds that are important for the antioxidant activity of a product. This information can be used for product and process optimization.

4.3.2. Choice of Apple Cultivar in Apple Juice Production.

Three apple cultivars (Elstar, Golden Delicious, and Jonagold), which all can be used as starting material for apple juice, were compared. In Table 2, the flavonoid and chlorogenic acid concentrations of the raw apple juices as well as the juice yield from the three apple cultivars are given. Elstar apples seemed to give a lower juice yield than Golden Delicious and Jonagold apples, but the difference was not significant.

Table 2. Concentrations of Flavonoids and Chlorogenic Acid (Milligrams per Kilogram of Fresh Weight) and Juice Yields of Raw Apple Juices Produced by Straight Pressing, from Three Apple Cultivars

compound	Elstar (*n* = 1)	Golden Delicious (*n* = 2)	Jonagold (*n* = 3)
Cy-Ga	4	2 ± 1	3 ± 0
phloridzin	6	3 ± 1	4 ± 1
chlorogenic acid	48	17 ± 14	133 ± 15
total Q-glycosides	8	7 ± 2	13 ± 1
total catechins	33	5 ± 0	16 ± 3
juice yield (%) (12 °Brix)	34	42 ± 2	46 ± 5
included water (%)	3	4 ± 5	5 ± 4

The juices prepared from the three cultivars had low concentrations of all analyzed compounds compared to the concentrations in the fresh apples. This confirms the finding that flavonoids preferably remain in the pomace and are hardly extracted into the juice. The concentrations of polyphenolic compounds in Elstar and Golden Delicious apples have been described previously (*6*). The low flavonoid and chlorogenic acid contents of the raw apple juices indicate that the apple juice production method itself appears to have a greater influence on the concentration of these compounds than the variation between cultivars.

Figure 5 shows the antioxidant activity of the various apple fractions occurring in juice manufacture from these cultivars. The measured antioxidant activity of the raw apples and their juices, as well as the antioxidant activity calculated from the apples' composition, are given.

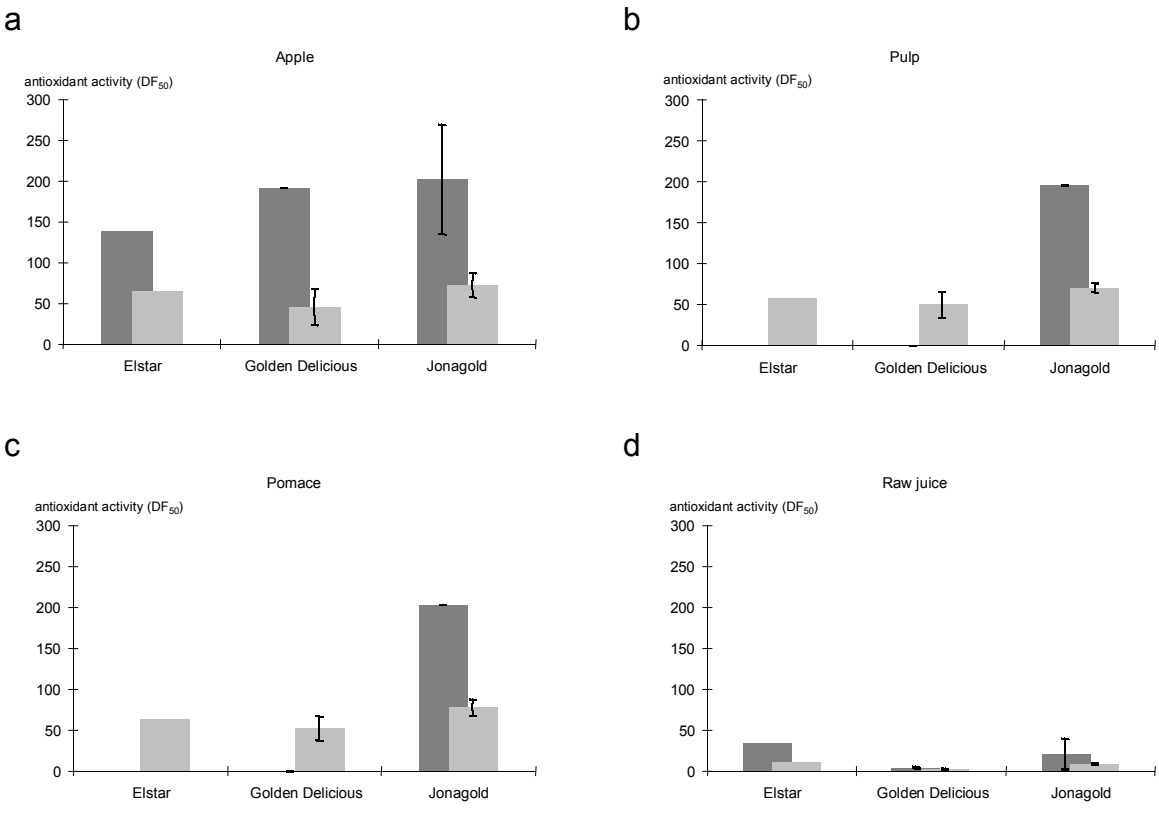


Figure 5. Antioxidant activity of various apple fractions during juice production (straight pressing). Comparison of three apple cultivars used as starting material. Dark gray: measured antioxidant activity. Gray: antioxidant activity calculated from composition.

There were no significant differences in antioxidant activity between the three cultivars for any of the apple fractions tested. This is also valid for the calculated antioxidant activity of the four apple fractions, with the exception of the calculated antioxidant activity of raw juice produced from Golden Delicious apples, which was lower. The measured antioxidant activity of the raw juices was only 17%, 6%, and 13%, respectively, of the initial activity of fresh Elstar, Golden Delicious, and Jonagold apples.

4.3.3. Possible Enhancement of Antioxidant Activity of Apple Juice.

The results of the present study clearly show that processing can have a major impact on the potential health benefits of a product. Therefore, studying the effects of processing methods on both levels of active compounds and biological activity of the different fractions occurring during processing will provide tools for finding the most efficient steps to improve a product with respect to its healthiness.

The fact that flavonoids and other compounds that contribute to antioxidant activity preferentially remain in the pomace offers a possibility for apple juice optimization with respect to flavonoid content and antioxidant activity. These compounds are not deteriorated or lost during juice manufacture, but remain in the pomace, which normally is a waste from the apple juice production. Thus, it is a challenging option to search for methods in which the flavonoids may be extracted from the pomace and later added to the final apple juice.

4.4. ABBREVIATIONS USED

Q-3-Ga, quercetin galactoside or hyperin; Q-3-Ru, quercetin rutinoside or rutin; Q-3-Gl, quercetin glucoside or isoquercitrin; Q-3-Xy, quercetin xyloside or reynoutrin; Q-3-Ar, quercetin arabinoside or avicularin; Q-3-Rh, quercetin rhamnoside or quercitrin; Cy-Ga, cyanidin galactoside or ideain.

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4.6. LITERATURE CITED

1. Middleton, E. Jr.; Kandaswami, C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In *The Flavonoids, Advances in Research since 1986*; Harborne, J. B., Ed.; Chapman and Hall: London; **1994**; pp 619–652.
2. Hertog, M. G. L.; Freskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007–1011.
3. Hollman, P. C. H. Evidence for health benefits of plant phenols: local or systemic effects? *J. Sci. Food Agric.* **2001**, *81*, 842–852.
4. Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985–1012.
5. Awad, M. A.; Wagenmakers, P. S.; de Jager, A. Effect of light environment on flavonoid and chlorogenic acid levels in the skin of 'Jonagold' apples. *Sci. Hortic. (Amsterdam)* **2001**, *88*, 289–298.
6. Van der Sluis, A. A.; Dekker, M.; de Jager, A.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple; effect of cultivar; harvest year and storage conditions. *J. Agric. Food Chem.* **2001**, *49*, 3606–3613.
7. Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of processing and storage on the phenolic composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572–1579.
8. Dekker, M.; Verkerk. R.; Jongen, W. M. F. Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. *Trends Food Sci. Technol.* **2000**, *11*, 174–181.
9. Lister, C. E.; Lancaster, J. E.; Sutton, K. H. Developmental changes in the concentration and composition of flavonoids in skin of a red and green apple cultivar. *J. Sci. Food Agric.* **1994**, *64*, 155–161.
10. Nicolas, J. J.; Richard Forget, F. C.; Goupy, P. M.; Amiot, M. J.; Aubert, S. Y. Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci.* **1994**, *34*, 109–157.
11. Macheix, J.-J.; Fleuriet, A.; Billot, J. *Fruit phenolics*; CRC Press: Boca Raton, FL, **1990.**
12. Kühnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet.* **1976**, *24*, 117–191.
13. Arts, I.; van de Putte, B.; Hollman, P. C. H. Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. *J. Agric. Food Chem*. **2000**, *48*, 1746–1751.
14. Goupy, P.; Amiot, M. J.; Richard-Forget, F.; Duprat, F.; Aubert, S.; Nicolas, J. Enzymatic browning of model solutions and apple phenolic extracts by apple polyphenoloxidase. *J. Food Sci.* **1995**, *60*, 497–501, 505.
15. Van Buren, J.; de Vos, L.; Pilnik, W. Polyphenols in Golden Delicious apple juice in relation to method of preparation. *J. Agric. Food Chem.* **1976**, *24*, 448–451.

16. Strack, D.; Wray, V. The anthocyanins. In *The Flavonoids; Advances in Research since 1986*; Harborne, J. B., Ed.; Chapman and Hall: London, **1994**; pp 1-22.

17. Clifford, M. N. Anthocyanins — nature, occurrence and dietary burden. *J. Sci. Food Agric.* **2000**, *80*, 1063–1072.

18. Hertog, M. G. L.; Hollman, P. C. H.; van de Putte, B. Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *J. Agric. Food Chem.* **1993**, *41*, 1242–1246.

19. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J. Agric. Food Chem.* **1992**, *40*, 2379–2383.

20. Bump, V. L. Apple pressing and juice extraction. In *Processed apple products*; Downing, D. L., Ed.; AVI (Van Nostrand Rheinhold): New York, **1989**; p 54.

21. Kilara, A. Enzymes and their uses in the processed apple industry: a review. *Process Biochem.* **1982**, *17*, 35–41.

22. Schols, H. A.; In 't Veld, P. H.; van Deelen, W.; Voragen, A. G. J. The effect of the manufacturing method on the characteristics of apple juice. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 142–148.

23. Price, K. R.; Prosser, T.; Richetin, A. M. F.; Rhodes, M. J. C. A comparison of the flavonol content and composition in dessert, cooking and cider-making apples; distribution within the fruit and effect of juicing. *Food Chem*. **1999**, *66*, 489–494.

24. Pérez-Ilzarbe, J.; Hernández, T.; Estrella, I. Phenolic compounds in apples: varietal differences. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 551–554.

25. Arts, I.; van de Putte, B.; Hollman, P. C. H. Catechin contents of foods commonly consumed in the Netherlands. 2. Tea, wine, fruit juices and chocolate milk. *J. Agric. Food Chem.* **2000**, *48*, 1752–1757.

26. De Jager, A.; Roelofs F. P. M. M. Optimum harvest date of apples for prolonged storage. *Annual Report 1995*. Fruit Research Station: Wilhelminadorp, The Netherlands, **1996**; pp 84–86.

27. Van der Sluis, A. A.; Dekker, M.; Verkerk, R.; Jongen, W. M. F. An improved, rapid *in vitro* method to measure antioxidant activity; application on selected flavonoids and apple juice. *J. Agric. Food Chem.* **2000**, *48*, 4116–4122.

28. Codex Alimentarius Commission. *Proposed Draft Codex General Standard for Fruit Juices and Nectars*; Report of the first session of the *ad hoc* codex intergovernmental task force on fruit and vegetable juices. Brasilia, Brazil, 18–22 Sept 2000; Joint office of FAO and WHO: Rome, **2000**; p 18.

29. Dekker, M.; Van der Sluis, A. A.; Verkerk, R.; Jongen, W. M. F. Effects of processing on the level and bioactivity of antioxidants in food products. In *AgriFood Quality II, Quality Management of Fruits and Vegetables*; Hägg, M., Ahvenainen, R., Evers, A. M., Tiilikkala, K., Eds.; Royal Society of Chemistry: Cambridge; **1999**; pp 17–21.

30. Lu, Y. R.; Foo, L. Y. Identification and quantification of major polyphenols in apple pomace. *Food Chem.* **1997**, *59*, 187–194.

31. Renard, C. M. G. C.; Baron, A.; Guyot, S.; Drilleau, J. F. Interactions between apple cell walls and native apple polyphenols: quantification and some consequences. *Int. J. Biol. Macromol.* **2001**, *29*, 115–125.

32. Miller, N. J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem*. **1995**, *43*, 1794–1801.

33. Schwarz, K.; Bertelsen, G.; Nissen, L. R.; Gardner, P. T.; Heinonen, M. I.; Hopia, A.; Huynh-Ba, T.; Lambelet, P.; McPhail, D.; Skibsted, L. H.; Tijburg, L. Investigation of plant extracts for the protection of processed foods against lipid peroxidation. Comparison of antioxidant assays based

on radical scavenging, lipid peroxidation and analysis of the principal antioxidant compounds. *Eur. Food Res. Technol.* **2001**, *212*, 319–328.

34. Lotito, S. B.; Actis Goretta, L.; Renart, M. L.; Caligiuri, M.; Rein, D.; Schmitz, H. H.; Steinberg, F. M.; Keen, C. L.; Fraga, C. G. Influence of oligomer chain length on the antioxidant activity of procyanidins. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 945–951
35. Guyot, S.; Doco, T.; Souquet, J.-M.; Moutounet, M.; Drilleau, J.-F. Characterization of highly polymerized procyanidins in cider apple (Malus Sylvestris var. Kermerrien) skin and pulp. *Phytochemistry* **1997**, *44*, 351–357.
36. Mayr, U.; Treutter, D.; Santos-Buelga, C.; Bauer, H.; Feucht, W. Developmental changes in the phenol concentrations of 'Golden Delicious' apple fruits and leaves. *Phytochemistry* **1995**, *38*, 1151–1155.
37. Suàrez Vallés B.; Santamaría Victorero, J.; Mangas Alonso, J. J.; Gomis, D. B. High-Performance Liquid Chromatography of the neutral phenolic compounds of low molecular weight in apple juice. *J. Agric. Food Chem.* **1994**, *42*, 2732–2736.
38. Foo, L. Y.; Lu, Y. R. Isolation and identification of procyanidins in apple pomace. *Food Chem.* **1999**, *64*, 511–518
39. Lancaster, J. E. Regulation of skin color in apples. *Crit. Rev. Plant Sci.* **1992**, *10*, 487–502.
40. Lee, C. Y.; Mattick, L. R. Composition and nutritive value of apple products. In *Processed apple products;* Downing, D. L., Ed.; AVI (Van Nostrand Rheinhold): New York, **1989**; p 314.
41. Miller, N. J.; Rice-Evans, C. A. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem*. **1997**, *60*, 331–337.
42. Gardner, P. T.; White, T. A. C.; McPhail, D. B.; Duthie, G. G. The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. *Food Chem*. **2000**, *68*, 471–474.
43. Rice-Evans, C. A.; Miller, N. J. Antioxidant activities of flavonoids as bioactive components of food. *Biochem. Soc. Trans.* **1996**, *24*, 790–795.

CHAPTER 5

Activity and Concentration of Polyphenolic Antioxidants in Apple Juice. 2. Effect of Novel Production Methods

ABSTRACT

There is a great interest in food components that possess possible health-protecting properties, as is the case with flavonoids. Previous research showed that conventional apple juice processing resulted in juices poor in flavonoids and with a low antioxidant activity. This paper shows that it is possible to improve flavonoid content in juice and its antioxidant activity by applying an alcoholic extraction either on the pulp or on the pomace. The levels of flavonoids and chlorogenic acid in enriched juice were between 1.4 (chlorogenic acid) and 9 (quercetin glycosides) times higher than in conventional apple juice. In enriched juice the antioxidant activity was 5 times higher than in conventional apple juice, with 52% of the antioxidant activity of the originating fruits present. The novel processing method had similar effects for three apple cultivars tested (Elstar, Golden Delicious, and Jonagold). The taste and color of enriched juice were different from those of conventional juice.

Key words

Antioxidant activity; quercetin glycosides; catechins; phloridzin; anthocyanins; chlorogenic acid; processing; optimization; apple juice

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5.1. INTRODUCTION

There is growing interest in food components that possess possible health-protecting properties, such as flavonoids, which are polyphenolic antioxidants. Epidemiological studies showed inverse relationships between some of these compounds and aging diseases such as coronary heart diseases and cancer (*1*, *2*). This is ascribed to their function as antioxidants or by modulation of enzyme activity (*3*). Consumption of 700 mL of apple juice increased plasma antioxidant activity in human volunteers (*4*).

Being secondary plant metabolites, flavonoids are present in fruits and vegetables. In apple and apple products the most important groups of polyphenolic antioxidants present are flavonols (with quercetin glycosides as the main representative), flavanols (or catechins) and their oligo- and polymers, and anthocyanins *(5)*. Furthermore, dihydrochalcones (e.g., phloridzin) and phenolic acids (such as hydroxycinnamic acids and, in particular, chlorogenic acid) are present in apple *(6)*.

Before consumption, fruits and vegetables may undergo different forms of processing. Cultivation methods, industrial processing, storage, distribution, and final processing by the consumer all will affect the final concentration of flavonoids in the product and their bioactivity *(7)*. Therefore, it is important for a food processor to have insight in these factors, because it provides information that can be used in product and process design and optimization.

Previous work showed that processing apples into juice results in big losses of flavonoids. Conventional apple juice production (straight pressing of apple pulp or pressing after pulp enzyming) resulted in a juice poor in flavonoids and with only 3–10% of the antioxidant activity of the fruit they were produced from *(8)*. Several possibilities exist within the juice production chain to enhance the flavonoid content of apple juice: by choice of the raw material or production methods and processing conditions or by adding additional flavonoids from other sources to the juice.

Using conventional cross-breeding or even by genetic modification, it seems to be possible to enhance the flavonoid content of apples. Examples of genetic manipulation of antioxidants in plant foods are described, but in the case of flavonoids the interrelation of the flavonoid classes makes it difficult to predict the outcome of overexpressing enzymes in the biosynthetic pathway, which is an option for the manipulation *(9)*. However, Muir et al. *(10)* succeeded in producing transgenic tomato lines that contained up to 78-fold more flavonols in the peel, mainly quercetin-

3-rutinoside. The tomato flesh did not accumulate significant amounts. Furthermore, the concentration of flavonoids in apple fruit skin can be increased by optimizing fertilization in the orchard, especially that of nitrogen *(11)*.

The possibility of improving the flavonoid content of apple juice by adjustments in production and processing methods seems to be easier and faster to implement than changes in the raw material itself. Effects of various production methods on the flavonoid content of apple juice are to some extent already described in the literature. Spanos and co-workers *(12)* compared the effect of straight pressing and diffusion extraction at various temperatures on the content of quercetin glycosides, catechin, epicatechin, and phloridzin in apple juice produced from Red Delicious apples. They reported a 3–5-fold increase in catechin, epicatechin, and phloridzin content, an almost 2-fold increase in chlorogenic acid content, and an increase from 0 mg/L quercetin glycosides in apple juice obtained by straight pressing to 38 mg/L in apple juice obtained by diffusion extraction at 73 °C *(12)*. In diffusion extraction the elevated temperatures inactivate the enzyme polyphenol oxidase. Oxidation products are hardly formed, and a larger part of the polyphenols originally present in the apple is found in the juice *(13)*. Disadvantages of this method are that deviation in aroma and taste may occur and that the juice is more susceptible to browning *(13)*.

Schols and co-workers *(14)* showed that it was possible to increase the catechin, epicatechin, phloridzin, and chlorogenic acid concentration of apple juice produced from Golden Delicious apples by liquefaction (300 ppm of pectolytic enzyme, 4 h, 45 °C) of the apple pulp. The effect on quercetin glycosides was not determined.

The fact that in conventional apple juice production methods >80% of the quercetin remains in the pomace suggests that the production process may be optimized *(15)*. At any time the effect of changes in production and processing methods on other quality factors (such as taste, color, and keepability), production efficiency, economic feasibility, and consumer acceptance should be taken into account. In addition, restrictions imposed by food laws regarding the final product should be obeyed.

We explore the possibility of performing a specific extraction on the apple pulp or on the pomace. In flavonoid analysis alcoholic extraction is often used when plant samples are prepared for analysis *(16)*. Flavonoids have to be removed from the matrix in which they are present, which is exactly the same objective in flavonoid-rich apple juice production.

In this paper the design of a process to obtain an apple juice with an enhanced content of polyphenolic antioxidants is presented. Enriched apple juice was produced by performing an extra extraction on pomaces obtained in conventional juice production methods. Pomaces obtained after straight pressing of apple pulp and after enzyme treatment of the pulp before pressing were compared as starting materials. The effects of pomace extraction on polyphenolic antioxidant content as well as antioxidant activity were assessed. Three different apple cultivars were tested: Jonagold, Golden Delicious, and Elstar. Antioxidant activity determined in a microsomal oxidation assay was used as a measure of bioactivity for apple and apple products. The activity of the analyzed compounds in the juice was calculated and compared to the measured one.

5.2. MATERIALS AND METHODS

5.2.1. Materials.

Chemicals, apple cultivars (Jonagold, Golden Delicious, and Elstar), harvest year (1998, if not stated otherwise), harvest conditions, and storage conditions were the same as described in Van der Sluis et al. *(8)*.

5.2.2. Methods.

Sampling and sample preparation, extraction before HPLC analysis, and antioxidant activity determination are described in Van der Sluis et al. *(8)*. Extracted pomace samples were lyophilized, juice samples were not. The same extract was used for both HPLC analysis and antioxidant activity determination.

Quantification of flavonoids by HPLC and HPLC equipment are described earlier *(17)*. Quercetin glycosides were analyzed separately and presented as the group 'total Q-glycosides', which consists of the compounds Q-3-Ga, Q-3-Ru, Q-3-Gl, Q-3-Xy, Q-3-Ar, and Q-3-Rh. The catechins were also analyzed separately and presented as the group 'total catechins', which consists of the compounds catechin and epicatechin. Quercetin in aglycon form was not detected in any of the apple samples.

The antioxidant concentration at which 50% inhibition of lipid peroxidation occurs (IC_{50}) was calculated from triplicate determination of six different antioxidant concentrations ranging from no to full inhibition of lipid peroxidation, which was assessed by measuring thiobarbituric acid reactive species (TBARS) after heating.

Absorption was read at 540 nm (color) versus 620 nm (turbidity correction) by an ELISA reader *(18)*.

5.2.3. Apple Juice Enrichment.

5.2.3.1. Pulp Extraction.

Alcoholic pulp extraction was performed at small scale (starting weights of fresh apples of ~1.5 kg). Jonagold apples (harvest year 1997) were cleaned by washing, stalks were removed, and the fruits were cut in four pieces. Apple pulp was prepared by quick slicing in a domestic food processor (Braun). Alcohol (methanol, ethanol, or propanol) was added in a 1:1 proportion (on weight basis) to the pulp, which was then extracted for 90 min in a 30 °C water bath and stirred now and then. Extracted apple pulp was pressed in a hydraulic manual press using a cheesecloth saturated with water. The obtained juice was concentrated to 75 °Brix using a rotary evaporator and diluted to 12 °Brix with water.

5.2.3.2. Pomace Extraction.

Pomace extraction was performed at pilot plant scale (starting weights of fresh apples of ~25 kg) and at small scale (starting weights of fresh apples of ~1.5 kg).

At pilot plant scale pomaces and raw juices as produced in Van der Sluis et al. *(8)* were used as basic material, with the same variables: straight pressing (A) versus pulp enzyming (B); and apple pulp particle size of 3 x 3 x 10 mm (A1) versus 3 x 3 x 3 mm (A2). Unless stated otherwise, A1 was the standard procedure.

Pomaces were extracted in a stirring tank with ethanol (4 h at room temperature) in 1:1 proportion and under continuous stirring. Extracted pomaces were carefully pressed in a Bucher–Guyer juice press. From each batch a juice containing ethanol was obtained, and the ethanol was removed using a rotary evaporator by concentration to 50–60 °Brix. In this concentrate no ethanol could be detected by smelling. The obtained concentrate was diluted with water to 12 °Brix, and this diluted extract was added to the earlier produced raw juice.

During enriched apple juice production, samples were taken of the most important fractions that occurred in the process chain (extracted pomace, concentrated extract, diluted extract, and enriched juice). All fractions were weighed, and mass balances were composed (see below under Calculations). Mass balances were standardized

by correcting for sample taking (weight). No correction was made for losses that remained in the equipment during the production. The °Brix values of the obtained concentrated extract, diluted extract, and enriched juice were determined and in the standardized mass balances adjusted with water to 50, 12, and 12 °Brix, respectively.

5.2.4. Sensory Evaluations.

Potential differences in taste between the obtained conventional and enriched juice were evaluated using the triangle difference test. The members of an untrained 12-member panel were each presented with two triangles, each consisting of three samples. Panelists were asked to indicate if any sample differed in taste from the other two. The color of the juices was masked by a cover of aluminum foil and by using red light. For a panel of 12 members, each examining two triangles, differences between the taste of conventional and enriched juice would be significant at the 5, 1, and 0.1% levels if the odd sample was identified 13, 15, and 17 times, respectively *(19)*.

The colors of raw juice and enriched apple juice were compared using a Tricolor LMF3 spectrophotometer in order to determine the *L**, *a**, and *b** values.

5.2.5. Calculations.

5.2.5.1. Mass Balances.

Mass balances and compound mass balances were calculated for all of the processing steps described in Figure 1. The balances of the starting material (apples, pulps, pomaces, and raw juices) were described earlier *(8)*. An overall mass balance (eq 1a) describes the effect of pomace extraction. Compound mass balances were used for the 10 different flavonoids and chlorogenic acid; they were composed of the standardized weights of the apple fractions and the concentrations of those compounds present in the fractions using eq 1b.

Effect of pomace extraction

standardized mass balance: $$m_{\mathrm{pc}} + m_{\mathrm{e}} = m_{\mathrm{epc}} + m_{\mathrm{ej}} + m_{\mathrm{l}} \quad \text{(kg)} \quad \text{(1a)}$$

compound mass balance:

$$m_{\mathrm{pc},i} \times c_{\mathrm{pc},i} + m_{\mathrm{e},i} \times c_{\mathrm{e},i} = m_{\mathrm{epc},i} \times c_{\mathrm{epc},i} + m_{\mathrm{ej},i} \times c_{\mathrm{ej},i} + m_{\mathrm{l},i} \times c_{\mathrm{l},i} \quad \text{(mg)} \quad \text{(1b)}$$

with *m* is the weight of apple fraction (kg) and *c* the concentration of compound *i* (*i* = 1–11) in the apple fraction (mg/kg of fresh weight). Fractions are represented as subscripts pc, pomace; epc, extracted pomace; ej, ethanolic juice; e, ethanol; and l, loss. $C_{(e),i} = 0$.

The production of a flavonoid-rich concentrate from the ethanolic juice by rotary evaporation is described by

standardized mass balance: $m_{ej} = m_{co} + m_{(w+e)} + m_l$ (kg) (2a)

compound mass balance:

$$m_{ej,i} \times c_{ej,i} = m_{co,i} \times c_{co,i} + m_{(w+e),i} \times c_{(w+e),i} + m_{l,i} \times c_{l,i} \quad \text{(mg)} \quad \text{(2b)}$$

Fractions are represented as subscripts co, concentrate (50 °Brix), and w+e, water containing ethanol.

The obtained concentrate is further processed to a diluted extract according to

standardized mass balance: $m_{co} + m_w = m_{de} + m_l$ (kg) (3a)

compound mass balance:

$$m_{co,i} \times c_{co,i} + m_{w,i} \times c_{w,i} = m_{de,i} \times c_{de,i} + m_{l,i} \times c_{l,i} \quad \text{(mg)} \quad \text{(3b)}$$

Fractions are represented as subscripts de, diluted extract (12 °Brix), and w, water. $C_{w,i} = 0$.

The production of an enriched juice is described by

standardized mass balance: $m_{de} + m_{srj} = m_{serj} + m_l$ (kg) (4a)

compound mass balance:

$$m_{de,i} \times c_{de,i} + m_{srj,i} \times c_{srj,i} = m_{serj,i} \times c_{serj,i} + m_{l,i} \times c_{l,i} \quad \text{(mg)} \quad \text{(4b)}$$

Apple fractions are represented as subscripts serj, standardized enriched raw juice (12 °Brix), and srj, standardized raw juice (12 °Brix).

5.2.5.2. Antioxidant Activity.

The calculated (or predicted) antioxidant activity of a mixture of known antioxidants and the measured antioxidant activity of an apple sample were derived as described earlier *(8)*. To predict the antioxidant activity of the apple fractions from its composition, the IC_{50} values of 11 standard components as given by Van der Sluis et al. *(18)*, were recalculated to milligrams per liter.

5.2.6. Statistical Analysis.

Statistical analysis was performed on the original data by one-way analysis of variance for juice yields and by two-way analysis of variance with replications for concentrations, with significance level α= 0.05 using the statistical package from Microsoft Excel. Data are represented as mean ± standard deviation (SD).

5.3. RESULTS AND DISCUSSION

5.3.1. Novel Apple Juice Production Methods.

To optimize flavonoid content and antioxidant activity of apple juice, adjustments in production methods were tested. Various solvents such as methanol, ethanol, and propanol were compared for their capacity to remove flavonoids from the matrix in which they are present. In flavonoid analysis these solvents are often used, perhaps partly diluted with water (*20*, *21*).

5.3.1.1. Pulp Extraction.

At first the solvents were applied directly on the pulp, as shown in the process scheme described in Figure 1a. Using ethanol, the juice yield was 88 $\pm$ 8%, and the concentration of quercetin glycosides in the juice was 65 ± 32 mg/kg of fresh weight (*n*=2). With methanol (*n*=5) and propanol (*n*=2) these values were not significantly different (data not shown). Comparison with conventionally produced apple juice (juice yield 46 $\pm$ 5% and quercetin glycoside concentration 13 ± 1 mg/kg of fresh weight) *(8)* shows that it was possible to improve flavonoid content in apple juice and the juice yield considerably by applying an alcoholic extraction on the apple pulp. In food production methanol and propanol can be used as extractants if the residual level is below 10 mg/kg *(22)*, and ethanol can be used if its use leads to the presence of residues in only technically unavoidable amounts that present no danger to human health (*22*, *23*). Therefore, ethanol was chosen to be used in further apple juice experiments.

5.3.1.2. Pomace Extraction.

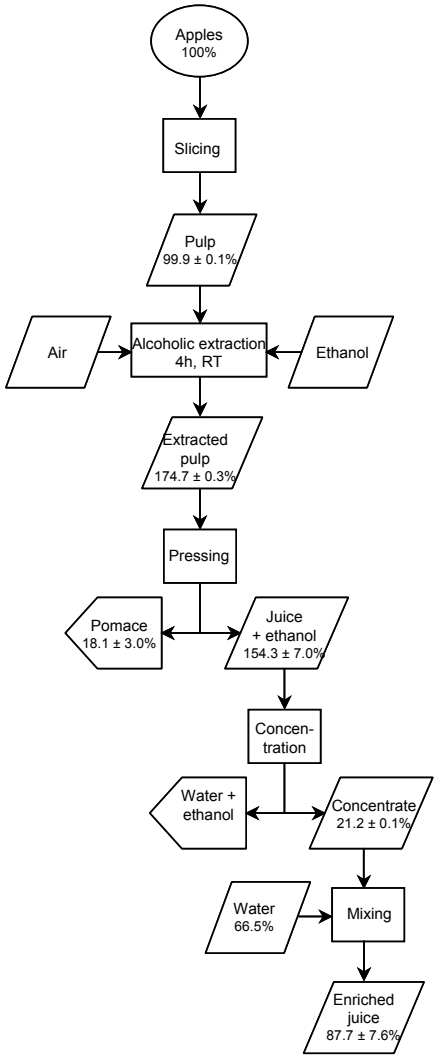
It is more effective to perform an alcoholic extraction on the pomace, due to the high concentration of flavonoids that remains there. Because of the reduced weight, less alcohol is needed in pomace extraction. A flavonoid-rich solution can be created, from which the alcohol can be removed. This extract can be concentrated and added

to the conventionally produced apple juice, at any desired stage and level, and other potential processes in which a loss of flavonoids might occur can be avoided.

Pomace extraction was performed following the process scheme described in Figure 1b. This figure shows the standardized weights (in percentage related to the fresh apple) of the apple fractions during processing.

(a) Pulp extraction

(*n*=2, small scale, starting weight 1.5 kg)



(b) Pomace extraction (code A1)

(*n*=2, pilot plant scale, starting weight 25 kg)

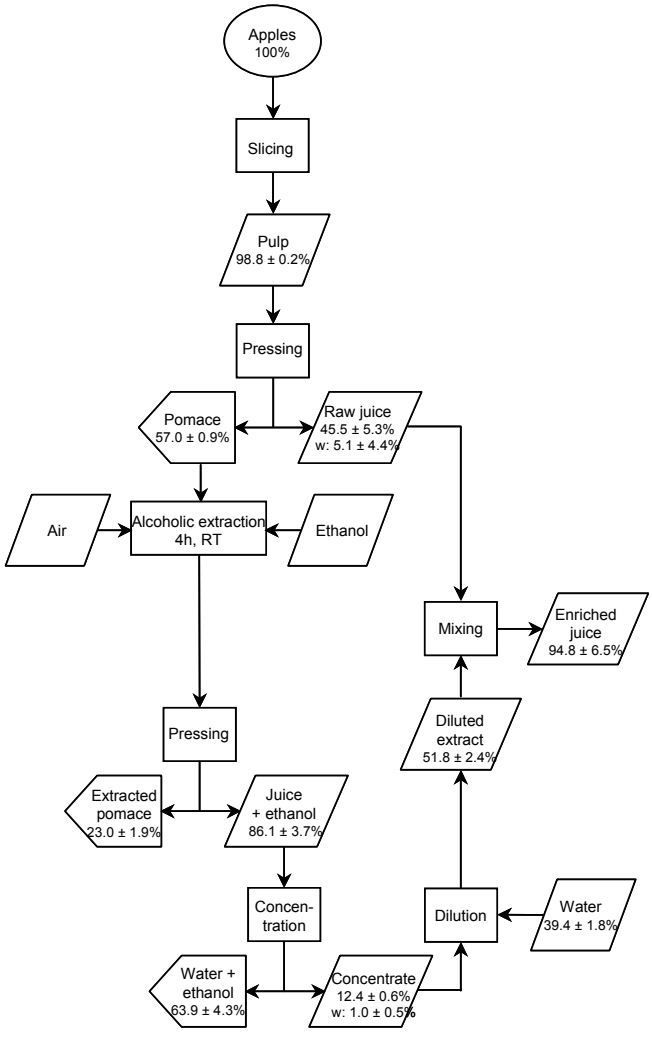


Figure 1. Process schemes and standardized weights of apple fractions during flavonoid-rich apple juice production; comparison of ethanolic pulp extraction (a) and pomace extraction (b). All percentages are related to the fresh apple fraction. w, amount of water that was added for standardization purposes; A1, pomace and raw juice obtained by straight pressing (particle size of pulp was 3 x 3 x 10 mm). Values are mean ± SD.

The first stages in this process scheme have been described before *(8)*, and whenever comparisons are made with conventionally produced pomaces and raw juices, the reader is referred to that paper. After pomace extraction, the enriched juice yield was 95 ± 6% (Figure 1b), which is again considerably higher than in conventional production. After extraction of pomaces A2 (from smaller sized pulp) and B (from pulp enzyming), the juice yields were 105 and 108% respectively, which indicates that for the juice yield it does not matter what type of preparation the pomace and raw juice are subjected to. It also indicates that pulp enzyming will not be necessary for obtaining a higher juice yield. Due to standardization to 12°Brix, the juice yields can be >100%. For comparison, the pomace was extracted with water, which gave a final juice yield of 83 ± 0% (*n*=2).

In Table 1 the mass balances, concentrations of flavonoids and chlorogenic acid, and the antioxidant activity of apple fractions during enriched apple juice production from Jonagold apples are presented. Pomaces were obtained after straight pressing or pulp enzyming and were then used for extraction with ethanol. The levels of flavonoids and chlorogenic acid in the enriched juices obtained from pomace A1 were all higher than in the corresponding raw juices described earlier *(8)*. Chlorogenic acid concentration was 1.4 times higher; cyanidin galactoside, 2.8; total catechins, 4.1; phloridzin, 8.9; and quercetin glycosides, 9.0 times higher than in conventional apple juice. Comparable results were obtained for enriched juices from pomaces A2 (data not shown) and B. However, the levels of all analyzed compounds were lower in enriched juice from pomace B compared to those in juice from pomace A1, caused by the lower levels that were present in pomace B itself.

This indicates that it is possible to enhance the polyphenolic antioxidant content of apple juice by performing an alcoholic extraction on the pomace and to use this extract to enrich the earlier obtained raw juice. Lu and Foo *(24)* also suggested that apple pomace be exploited commercially as a source for polyphenols, because it contains high levels of quercetin glycosides, phloridzin, and epicatechin (in total 6.5 g/kg of dry matter). Wolfe and Liu *(25)* succeeded in producing a value-added food ingredient out of apple peel, because of its high antioxidant content.

Table 1. Mass Balances (Kilograms), Concentrations of Flavonoids and Chlorogenic Acid (Milligrams per Kilogram of Fresh Weight), and Antioxidant Activity of Apple Fractions during Jonagold Apple Juice Production (Extraction of Pomaces A1 and B with Ethanol)

			straight pressing (A1) [a]			pulp enzyming (B) [a]		
Jonagold	apples (n = 5)		extracted pomace (n = 2)	standardized concentrated extract (n = 2)	standardized enriched juice (n = 2)	extracted pomace (n = 1)	standardized concentrated extract (n = 1)	standardized enriched juice (n = 1)
	mean	SD	mean SD	mean SD	mean SD			
standardized mass balance (kg)	25.0	± 0	5.8 ± 0.5	3.1 ± 0.1	23.7 ± 1.6	4.8	1.7	26.9
included added water (kg)				0.2 ± 0.1			-1.2	
concentrations (mg/kg of fw)								
Cy-Ga	10	± 3	6 ± 1	44 ± 0	7 ± 1	8	36	6
phloridzin	46	± 17	18 ± 0	207 ± 14	35 ± 3	30	261	25
chlorogenic acid	202	± 33	55 ± 6	869 ± 64	180 ± 1	52	589	99
total Q-glycosides	109	± 25	89 ± 0	768 ± 34	117 ± 2	128	1068	96
total catechins	186	± 26	59 ± 0	432 ± 21	67 ± 7	58	317	40
measured activity: $1000/IC_{50}$ (L/mg of fw)	202.3	± 67.6 [b]	87.2 [c]	- -	104.3 ± 3.0 [b]	-	-	49.1 [c]
calculated activity: $\Sigma\ (C/IC_{50})$	72.7	± 11.3	30.4 ± 0.2	248.2 ± 11.7	39.5 ± 1.7	35.6	249.8	27.0
explained activity: calculated/measured	36%		35%		38%			55%

[a] A1, pomace and raw juice obtained by straight pressing (particle size of pulp was 3 x 3 x 10 mm). B, pomace and raw juice obtained by pulp enzyming.

[b] n=2. [c] n=1.

After alcoholic pomace extraction, not all of the analyzed compounds were extracted completely from the pomace. The resulting extracted pomaces still contained 50% of the concentration of quercetin glycosides present in the original pomace; for cyanidin galactoside, total catechins, chlorogenic acid, and phloridzin, these values were 44, 34, 33, and 29%, respectively.

(a) Mass Balances in Pomace Extraction.

The use of eqs 1a–4a enabled the calculation of the losses that occurred in the enriched juice production. The losses in the various processing steps caused by material remaining in the equipment can be calculated from Figure 1. Pressing of the extracted pomace caused a loss of 5% of the apples starting weight, which was small compared to the biological variation of flavonoid and chlorogenic acid concentration in apples (10–30%, from Table 1). Concentrating in order to remove the alcohol caused a weight loss of ~30%, which is quite large but not unexpected using rotary evaporation. Dilution of the concentrate to 12 °Brix and mixture of the diluted extract with the raw juice to obtain the enriched juice caused a loss of 3%. When the apple juice productions were performed on a small scale, the losses were relatively higher; however, it is expected that on an industrial scale, the losses will be much smaller and that the concentration procedure can be optimized.

The compound mass balances (in milligrams) of components present in the various apple fractions in enriched apple juice production (from straight pressing or pulp enzyming) are presented in Figure 2. Enriched apple juices were prepared by ethanolic pomace extraction. Standardized mass balance and the concentrations as mentioned in Table 1 were used as input. The total amount of all the analyzed compounds present in the extracted pomaces (A1 and B) was very low compared to the amount present in the original apples or in the pomaces prior to the extraction. In the pomace extracted after straight pressing (Figure 2a) only 19% of the amount of quercetin glycosides, 14% of the cyanidin galactoside, 7% of the total catechins, 6% of the chlorogenic acid, and 9% of the phloridzin that were present in the originating apples were found. In the enriched juice 102% of the amount of total Q-glycosides, 84% of the chlorogenic acid, 71% of the phloridzin, 67% of the cyanidin galactoside, and 34% of the total catechins that were present in the originating apples were detected. This indicates that the analyzed antioxidant components present in the pomace were extracted by ethanol from the pomace into the enriched juice.

Furthermore, due to the low weight of the extracted pomace (which was ~20% of the weight of the originating apples) and the use of compound mass balances, it can be seen that only low amounts of the components of interest remain there. This makes the need for optimization of the pomace extraction less than what might have been concluded from looking at the concentrations of these components only.

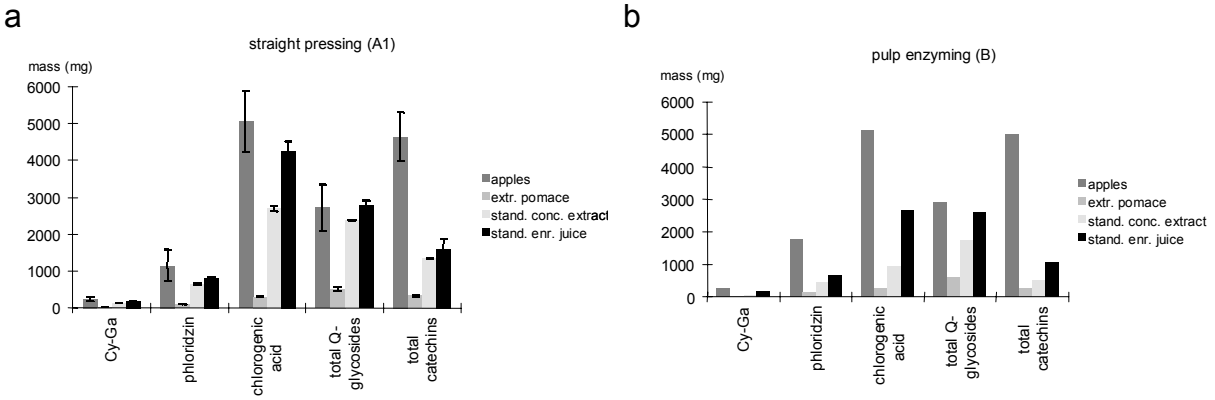


Figure 2. Compound mass balances of apple fractions in Jonagold apple juice production, prepared by ethanolic pomace extraction after straight pressing (panel a, mean ± SD, *n* = 2) or pulp enzyming (panel b, *n* = 1). Each set of four bars shows (left to right) apples, extracted pomace, standardized concentrated extract, and standardized enriched juice.

(b) Antioxidant Activity and Contribution of Compounds.

Table 1 shows that the antioxidant activity of the extracted pomace was 43% of the activity of the pomace before extraction as well as of the activity of the fresh apples, which showed the same activity. The standardized enriched apple juice had an antioxidant activity that was 4.9 times higher than that of the corresponding raw juice. This indicates that compounds that possess antioxidant activity indeed could be extracted from the pomace and transferred into the juice, but apparently the extraction still was not complete.

Enriched juice obtained from Jonagold apples from pomaces A1 and B had antioxidant activities that were 52 and 24%, respectively, of the activity of the fresh apples. Therefore, it can be concluded that the pulp-enzyming procedure significantly lowered the antioxidant activity of the juice.

Figure 3 shows the calculated contribution of flavonoids and chlorogenic acid to the measured antioxidant activity of the various fractions occurring in enriched apple juice production. Ethanolic pomace extractions performed on pomaces A1 (from 3 x 3

x 10 mm sized pulp) and B (from pulp enzyming) were compared. The contributions of the analyzed compounds to the antioxidant activity of the extracted pomace A1 and its corresponding enriched juice were 35 and 38% respectively. Of these compounds the group of “total catechins” was the most important with contributions of 19 and 18%, respectively. The group “total quercetin glycosides” was the second most important contributor to the measured antioxidant activity, at 14 and 15%, respectively. Chlorogenic acid, cyanidin galactoside, and phloridzin hardly contributed to the measured antioxidant activity. After pulp enzyming and ethanolic pomace extraction (B), the contribution of the analyzed compounds to the antioxidant activity of the enriched juice was 55%, with a contribution of total quercetin glycosides of 26% and of total catechins of 23%. The findings considering the contribution of the analyzed compounds to the measured antioxidant activity of the apple fractions occurring in enriched apple juice production correspond to the findings described earlier *(8)*.

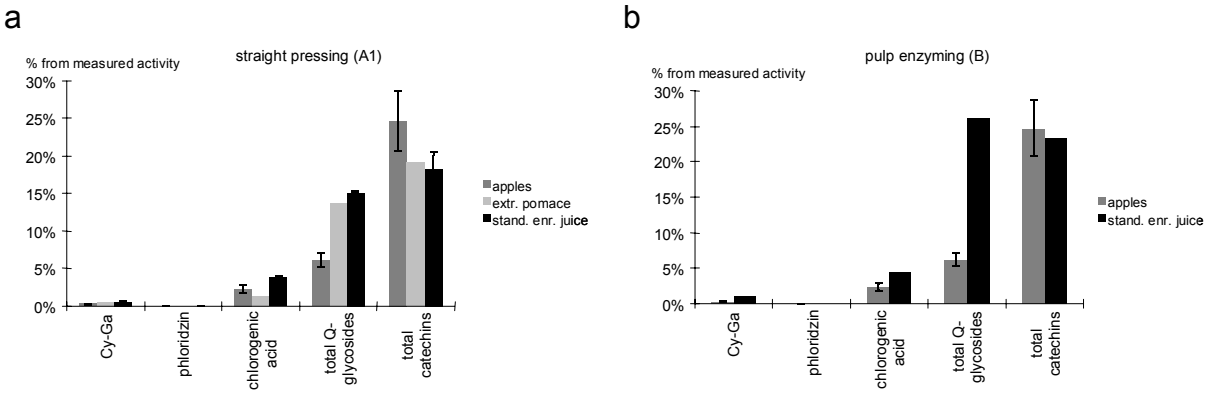


Figure 3. Calculated contribution of flavonoids and chlorogenic acid to the measured antioxidant activity of apple fractions in Jonagold apple juice production, prepared by ethanolic pomace extraction after straight pressing (panel a, mean ± SD, *n* = 2) or pulp enzyming (panel b, *n* = 1).). In panel a, each set of three bars shows (left to right) apples, extracted pomace, and standardized enriched juice. In panel b, each set of two bars shows (left to right) apples and standardized enriched juice.

(c) Unaccounted for Antioxidant Activity.

As was the case in the previous study *(8)*, a large proportion of the measured activity could not be ascribed to the analyzed antioxidants. One possible explanation for this difference could be the occurrence of synergism between the various antioxidant components. To see if interaction between the analyzed antioxidants existed,

mixtures composed of standard compounds in the same concentrations as found in enriched apple juice samples were tested for their antioxidant activity. The enriched apple juices used for these experiments showed antioxidant activity values (expressed as DF_{50}) of 105.2 ± 12.8 (*n*=3), and had calculated antioxidant activity values of 20.1 ± 2.4 (*n*=3). The mixtures composed of standard compounds in the same concentrations as found in the enriched apple juice showed antioxidant activity values of 15.2 ± 1.9 (*n*=3), which was not significantly different from the calculated antioxidant activity (*p*=0.052). Thus, no interaction was observed between the analyzed antioxidants.

A second hypothesis for the observed discrepancy is the presence of compounds other than those analyzed that contribute to the antioxidant activity of extracted pomaces and enriched juices. These compounds could be procyanidins, carotenoids, and/or vitamins *(8)*. In the apple fractions occurring in conventional apple juice production a high correlation between the unexplained and calculated antioxidant activities of catechins was shown *(8)*, indicating that the unknown antioxidant compound(s) behaved quite similarly to catechins. Therefore, the correlation of the unexplained antioxidant activity with the calculated activity of catechins and of the other analyzed antioxidants present in the extracted pomace and enriched juice were also determined.

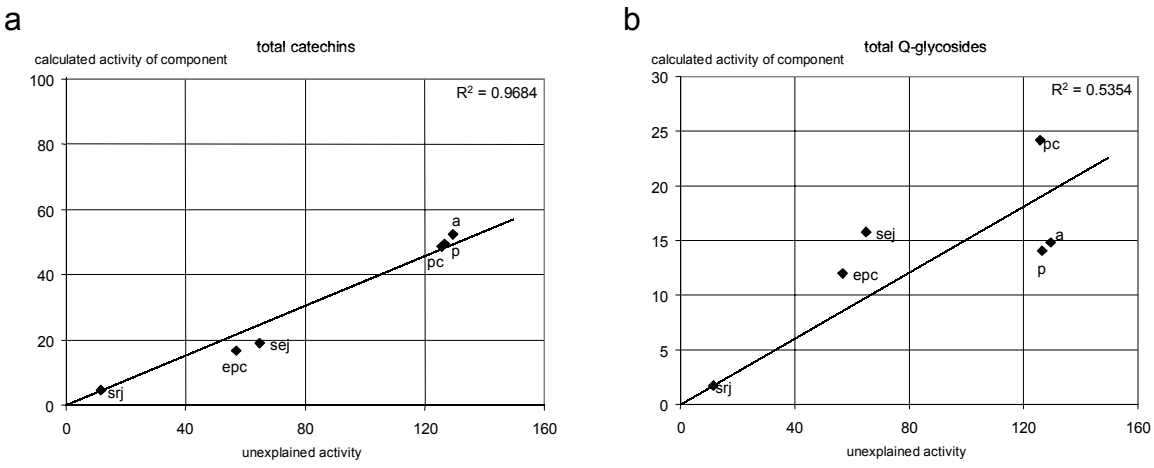


Figure 4. Correlation of the unexplained antioxidant activity with the calculated antioxidant activity of selected components of apple fractions in standardized enriched juice from pomace A1. Jonagold apples were used. Lines are trend lines. Unexplained activity is measured antioxidant activity minus calculated antioxidant activity. Symbols with letters are defined as follows: a, fresh apple; p, apple pulp; pc, pomace; srj, standardized raw juice; epc, extracted pomace; sej, standardized enriched juice.

Values for the fractions occurring earlier in the production process were added as well *(8)*. Figure 4a shows that a high correlation was again obtained for total catechins (R^2 = 0.968). The correlation between unexplained antioxidant activity and calculated antioxidant activity of total quercetin glycosides (Figure 4b, R^2 = 0.535) and of cyanidin galactoside (R^2 = 0.686) was less profound. Low correlation was found for chlorogenic acid activity (R^2 = 0.344). This indicates that in the enriched apple juice production process the unknown antioxidant compound(s) behaved quite similarly to the group of "total catechins". Foo and Lu *(26)* reported the presence of procyanidins (epicatechin polymers) in apple pomace, and most probably these compounds are extracted into the juice during alcoholic pomace extraction.

5.3.2. Choice of Apple Cultivar in Enriched Apple Juice Production.

Pomaces from three different apple cultivars (Elstar, Golden Delicious, and Jonagold) were used as starting material for the production of enriched apple juice. Table 2 shows the concentration of flavonoids and chlorogenic acid together with the yields of the concentrates and enriched juices produced from pomaces A1, which were extracted with ethanol. From all three cultivars enriched juice yield was at least 2 times higher than conventionally produced juice yield *(8)*.

The concentration of the analyzed compounds was considerably enhanced as well. The differences in compound concentrations resulted from differences in levels already existing in the fresh apples. Jonagold was the apple cultivar with highest concentration of analyzed compounds, with a total of 532 mg /kg of fresh weight, followed by Golden Delicious and Elstar with total concentrations of 448 and 321 mg /kg of fresh weight, respectively *(17)*. In the produced concentrates and in the enriched juices from Jonagold apples total concentrations were highest as well. Therefore, the proposed novel process of pomace extraction can be used for different apple cultivars with similar effects.

In Figure 5 the antioxidant activities of apple and enriched juice prepared by ethanolic pomace extraction (A1) are shown. The measured antioxidant activity and the antioxidant activity calculated from the samples composition are given. The measured antioxidant activity of enriched Jonagold juice was significantly higher (p = 0.007) than that produced from Elstar and Golden Delicious apples. The measured antioxidant activities of the enriched juices were 51, 16, and 52%, respectively, of the initial activity of fresh Elstar, Golden Delicious, and Jonagold apples. The measured

antioxidant activities of the enriched juices from Elstar, Golden Delicious, and Jonagold apples were 2, 7, and 5 times higher than that of the conventionally produced juices described earlier *(8)*.

Table 2. Concentration of Flavonoids and Chlorogenic Acid (Milligrams per Kilogram of Fresh Weight) and Yields of Concentrates and Flavonoid-rich Apple Juices (Pomaces A1 Were Extracted with Ethanol); Comparison of Three Apple Cultivars [a]

	Elstar (*n*=1)	Golden Delicious (*n*=2)	Jonagold (*n*=2)
concentrate			
Cy-Ga	31	21 ± 9	44 ± 0
phloridzin	154	183 ± 0	207 ± 14
chlorogenic acid	330	426 ± 69	869 ± 64
total Q-glycosides	487	491 ±173	768 ± 34
total catechins	443	286 ± 67	432 ± 21
concentrate yield (%) (50 °Brix)	14	15 ± 1	12 ± 1
enriched juice			
Cy-Ga	7	3 ± 0	7 ± 1
phloridzin	30	22 ± 6	35 ± 3
chlorogenic acid	69	58 ± 21	180 ± 1
total Q-glycosides	89	69 ± 35	117 ± 2
total catechins	81	27 ± 16	67 ± 7
juice yield (%) (12 °Brix)	92	104 ± 8	95 ± 6

[a] Harvest 1998. Pomace and raw juice (A1), obtained by straight pressing (particle size of pulp was 3 x 3 x 10 mm).

The contributions of the analyzed compounds to the measured antioxidant activity of the enriched juices were 52, 59, and 38% in enriched juices from Elstar, Golden Delicious, and Jonagold apples, respectively. The proposed novel process of pomace extraction increased the antioxidant activity of enriched apple juices produced from three apple cultivars by a factor of between 2 and 7; therefore, the method can be used for different apple cultivars.

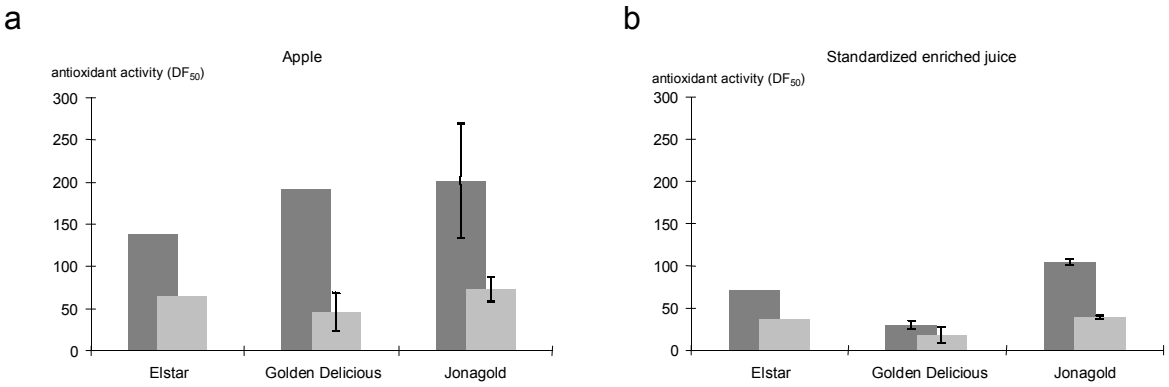


Figure 5. Antioxidant activity of apple and enriched juice prepared by ethanolic pomace extraction (A1); comparison of three cultivars used as starting material. Dark gray bars represent measured antioxidant activity. Light gray bars represent antioxidant activity calculated from composition. Values are mean ± SD. DF, dilution factor.

5.3.3. Sensory Properties of Raw and Enriched Juice.

Tto determine whether differences in taste existed between conventional and enriched Jonagold apple juices, they were evaluated using the triangle difference test. The untrained panelists identified the odd sample 18 times in a preliminary session, and after one training session 22 times; therefore, the tastes of conventional and enriched juices were significantly different at the 0.1% level. Panelists were not asked questions about preference, degree of difference, acceptance, or type of difference after identification of the odd sample.

The color of raw and enriched Jonagold apple juice can be described by the following *L**, *a**, and *b** values. For raw apple juice they are 46.3, 20.4, and 53.5, respectively, and for enriched juice, 54.2, 14.2, and 50.2. This indicates that both juices have an orange-brownish color. However, visually the enriched juice would be described as more reddish than the raw juice.

The concentrated extracts obtained from Jonagold and Elstar apples looked very red, but the concentrated extract obtained from Golden Delicious apples was green. This difference in color most probably can not be explained only by the lower cyanidin galactoside concentration present in Golden Delicious concentrated extract.

5.3.4. Technological Implications.

We showed that it is possible to enhance the activity and content of polyphenolic antioxidants in apple juice by performing an alcoholic extraction on the pomace or

pulp, followed by removal of the solvent from the obtained extract. This might be performed by multistage evaporation. Juice yield was improved considerably as well. The antioxidant activity of the final juice was 5 times increased and the concentration of polyphenolic antioxidants up to 9 times increased compared to conventional processing. Sensory evaluation showed that the enriched juice tasted different, but preferences were not determined. It is expected to be more adstringent than conventional juice, due to the presence of the extracted polyphenols (*27*). The color of the enriched juice was slightly different (more reddish) from that of conventional juice, which may be beneficial in marketing an enriched juice versus a conventional one.

Advantages of pomace extraction over pulp extraction are that less alcohol is needed and that apart from facilities for the pomace extraction only minor adjustments in the usual production facilities are needed. In both cases the production process will take more time than in conventional apple juice production, because of the extra time needed for extraction.

A hot water extraction of the pomace may be investigated as an alternative to the use of alcoholic pomace extraction. Extraction of the pomace with water at an elevated temperature might also produce a flavonoid-rich solution that can be added to the raw juice immediately or after concentration. As mentioned before, Spanos and co-workers *(12)* showed that diffusion extraction performed on apple pulp at elevated temperatures indeed increased flavonoid content of the juice 3–5-fold.

When using alcohols as extractant in food production, legislation and good manufacturing practices should be considered (*23*). Furthermore, the solvent should be recovered from the residue *(28)*, to meet environmental legislation.

Pomace extraction most probably has less influence on the taste of the obtained juice than pulp extraction, because the largest amount of the juice will be produced in the conventional and therefore known manner. During the evaporation process necessary to remove the alcohol from the extract, some of the aroma compounds may be lost, which will affect the taste of the final enriched juice. The use of aroma trapping may prevent the loss of aroma compounds and provide a tasty enriched apple juice.

The proposed novel production process can be a valuable approach to the design of new types of apple juice with an enhanced health-protecting capacity.

5.4. ABBREVIATIONS USED

Q-3-Ga, quercetin galactoside or hyperin; Q-3-Ru, quercetin rutinoside or rutin; Q-3-Gl, quercetin glucoside or isoquercitrin; Q-3-Xy, quercetin xyloside or reynoutrin; Q-3-Ar, quercetin arabinoside or avicularin; Q-3-Rh, quercetin rhamnoside or quercitrin; Cy-Ga, cyanidin galactoside or ideain.

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5.6. LITERATURE CITED

1. Middleton, E., Jr.; Kandaswami, C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In *The Flavonoids, Advances in Research since 1986*. Harborne, J. B., Ed.; Chapman and Hall: London, U.K.; **1994**; pp 619–652.
2. Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet*, **1993**, *342*, 1007–1011.
3. Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985–1012.
4. Bitsch, I.; Netzel, M.; Strass, G.; Janssen, M.; Kesenheimer, B.; Herbst, M.; Carle, E.; Bohm, V.; Harwat, M.; Rechner, A.; Dietrich, H.; Bitsch, R. High-quality fruit juices from special apple varieties – their contribution to a healthy diet according to the 'five-a-day' campaign. *Ernaehrungs Umschau* **2000**, *47*, 428–431.
5. Lister, C. E.; Lancaster, J. E.; Sutton, K. H. Developmental changes in the concentration and composition of flavonoids in skin of a red and green apple cultivar. *J. Sci. Food Agric.* **1994**, *64*, 155–161.
6. Macheix, J. J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*; CRC Press: Boca Raton, FL, **1990**; pp 68–71, 113.
7. Dekker, M.; Verkerk. R.; Jongen, W. M. F. Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. *Trends Food Sci. Technol.* **2000**, *11*, 174–181.

8. Van der Sluis, A. A.; Dekker, M.; Skrede, G.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *J. Agric. Food Chem.* **2002**, *50*, 7211–7219.

9. Mullineaux, P. M.; Creissen, G. P. Opportunities for the genetic manipulation of antioxidants in plant foods. *Biochem. Soc. Trans.* **1996**, *24*, 829–835.

10. Muir, S. R.; Collins, G. J.; Robinson, S.; Hughes, S.; Bovy, A.; de Vos, C. H. R.; van Tunen, A. J.; Verhoeyen, M. E. Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. *Nat. Biotechnol.* **2001**, *19*, 470-474.

11. Awad, M. A.; de Jager, A. Relationships between fruit nutrients and concentrations of flavonoids and chlorogenic acid in 'Elstar' apple skin. *Sci. Hortic.* (*Amsterdam*) **2002**, *92*, 265–276.

12. Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of processing and storage on the phenolic composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572–1579.

13. Von Haug, M.; Gierschner, K. Auswirkung unterschiedlicher industrieller Herstellungsverfahren (Auspressen, Extraktion) auf die phenolischen Verbindungen in Apfelsäften. 1. Mitteilung: Abtrennung und Dünnschichtchromatographie der Pflanzenphenole. *Dtsch. Lebensm. Rundsch.* **1979**, *75*, 248–253.

14. Schols, H. A.; In 't Veld, P. H.; van Deelen, W.; Voragen, A. G. J. The effect of the manufacturing method on the characteristics of apple juice. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 142–148.

15. Van der Sluis, A. A.; Dekker, M.; Jongen, W. M. F. Flavonoids as bioactive components in apple products. *Cancer Lett.* **1997**, *114*, 107–108.

16. Waterman, P. G.; Mole, S. *Analysis of Phenolic Plant Metabolites*. Blackwell Scientific Publications: Oxford, U.K., **1994**.

17. Van der Sluis, A. A.; Dekker, M.; de Jager, A.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple; effect of cultivar; harvest year and storage conditions. *J. Agric. Food Chem.* **2001**, *49*, 3606–3613.

18. Van der Sluis, A. A.; Dekker, M.; Verkerk, R.; Jongen, W. M. F. An improved, rapid *in vitro* method to measure antioxidant activity; application on selected flavonoids and apple juice. *J. Agric. Food Chem.* **2000**, *48*, 4116–4122.

19. Meilgaard, M. C.; Civille, G. V.; Carr, B. T.; *Sensory Evaluation Techniques, 2nd ed*.; CRC: Boca Raton, FL, **1991**; pp 60–98.

20. Robards, K.; Antolovich, M. Analytical chemistry of fruit bioflavonoids. *Analyst* **1997,** *122*, R11–R34.

21. Tura, D.; Robards, K. Sample handling strategies for the determination of biophenols in food and plants. *J. Chromatogr. A*, **2002**, *975,* 71–93.

22. Francken, J. M. *Warenwet* (Dutch Food Law), Band 2; Koninklijke Vermande BV: Lelystad, The Netherlands, **1990**.

23. EU Council Directive 88/344/EEC of 13 June 1988 on the approximation of the laws of the Member States on extraction solvents used in the production of foodstuffs and food ingredients. *Off. J. Eur. Communities* **1988**, *L157*, 0028-0033.

24. Lu, Y.; Foo, Y. L. Identification and quantification of major polyphenols in apple pomace. *Food Chem.* **1997**, *59*, 187–194.

25. Wolfe, K. L.; Liu, R. H. Apple peels as a value-added food ingredient. *J. Agric. Food Chem.* **2003**, *51*, 1676–1683.

26. Foo, Y. L.; Lu, Y. Isolation and identification of procyanidins in apple pomace. *Food Chem.* **1999**, *64*, 511–518.

27. Drewnowski, A.; Gomez Carneros, C. Bitter taste, phytonutrients, and the consumer: a review. *Am. J. Clin. Nutr*. **2000**, *6*, 1424–1435.

28. Starmans, D. A. J.; Nijhuis, H. H. Extraction of secondary metabolites from plant material: a review. *Trends Food Sci. Technol.* **1996**, *7*, 191–197.

CHAPTER 6

Activity and Concentration of Polyphenolic Antioxidants in Apple Juice. 3. Stability during Storage

ABSTRACT

Kinetic data are reported describing the stability of various classes of polyphenolic antioxidants in an apple juice enriched in these compounds as a function of storage temperature and oxygen concentration. The most thermally sensitive compounds were the various quercetin glycosides and epicatechin, whereas phloridzin and chlorogenic acid were more stable. The quercetin glycosides showed differences in their stability: quercetin galactoside ≈ quercetin rhamnoside > quercetin glucoside/rutinoside > quercetin arabinoside. The effect of the presence of oxygen on the degradation rates was clear for only quercetin and to a lesser extent for epicatechin. Accelerated shelf-life testing of enriched apple juice during 4 days at 80 °C showed decreases in the antioxidant activity of 20–40%. The parameters obtained were used to predict the stability at different storage conditions. Calculations showed that polyphenolic antioxidants and antioxidant activity of enriched apple juice will be quite stable at ambient or refrigerated storage conditions up to half a year.

Key words

Antioxidant activity; quercetin glycosides; catechins; phloridzin; anthocyanins; chlorogenic acid; storage; apple juice; kinetics; shelf-life

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6.1. INTRODUCTION

Six classes of polyphenols are present in apples: flavonol glycosides; catechins; anthocyanins; dihydrochalcones (phloretin glucoside and xyloglucoside); phenolic acids (chlorogenic acid and *p*-coumaroylquinic acid); and procyanidins (*1*).

From epidemiological research, the intake of fruits and vegetables has been widely acknowledged to be inversely related to cancer incidence and cardiovascular diseases (*2*, *3*). Antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties and capacity to modulate some key cellular enzyme functions are ascribed to many phenolic compounds (*4*). Therefore, these compounds might play a role in relation to human health (*5*).

Polyphenolic intake (phenolic acids, flavonols, catechin monomers, proanthocyanidins, flavanones, anthocyanins) is estimated at 1 g/day, but the structural diversity of polyphenols makes the estimation of their content in food difficult and, therefore, the estimation of daily intake as well (*6*). From this 1 g/day, phenolic acids provided ~30% and flavonoids accounted for the remainder. In a Western diet catechins from apples contribute 5–12% to their daily intake (*7*) and flavonols, 10% (*8*).

Apples can be further processed to, for example, apple juice, apple cider, or applesauce. In conventional apple juice production, juice poor in flavonoids and with only 3–10% of the antioxidant activity of the originating fruit is obtained (*9*). Applying an alcoholic extraction either on the pulp or on the pomace makes it possible to improve flavonoid content in apple juice as well as its antioxidant activity (*10*).

During the storage of apple juice or apple juice concentrates various changes may occur, for example, as a result of browning and degradation reactions. Comparison of apple juices prepared from concentrates that were not stored with concentrates that were stored at 25 °C for 9 months showed that quercetin glycoside and phloridzin concentrations decreased 54 and 32%, respectively. In the juice from the not-stored concentrate 2.9 mg/L of catechin and 6.1 mg/L of epicatechin was present, but in the juice from the stored concentrate these compounds were not detected at all (*11*). A decrease of flavonol glycosides in juice concentrates upon 90 days of storage at 30 °C was reported by van Buren et al. (*12*). Furthermore, carton-laminated packed commercial apple juices showed declines in phenolic acids and flavonoid content as well as in TEAC value after 11 months of storage at room temperature (*13*). This

indicates that storage of apple juice or juice concentrates affects flavonoid concentration and antioxidant activity in apple juice.

Using apple juice with increased flavonoid content gives the opportunity to study the behavior of polyphenolic antioxidants in apple juice during storage at normal and elevated temperatures. Therefore, the aim of the present study was to evaluate the effects of storage at 4 °C, at ambient temperature, and under conditions for accelerated shelf-life testing on the concentration and antioxidant activity of various polyphenolic compounds found in enriched apple juice. To distinguish between oxidative and nonoxidative breakdown reactions, storage was done with varying oxygen concentrations. A kinetic modeling approach was used to study the reaction pathways of breakdown and conversions of polyphenolic antioxidants and to be able to translate the results of the accelerated shelf-life testing into the stability of compounds under normal conditions.

6.2. MATERIALS AND METHODS

6.2.1. Materials.

Chemicals were the same as described earlier (*9*). Jonagold apples were used to produce apple juice and were harvested from commercial orchards in the years 1996 and 1998. Apples were harvested as described before (*9*) and stored until use (1–6 months) at controlled atmosphere (CA) conditions (1.5 °C, 1.2% O_2, and 2.5% CO_2), which is optimal for long-term storage.

6.2.2. Methods.

6.2.2.1. Apple Juice.

Apple juice used in cold storage and storage at room temperature was prepared by pomace extraction as described earlier (*10*). About 1.5 kg of Jonagold apples (harvest year 1998) was cleaned, and pulp was prepared using a domestic food processor. Apple pulp was pressed in a hydraulic manual press using a cheesecloth (two times pressing to 100 bar). Raw juice was obtained, and pomaces were extracted with ethanol (4 h at room temperature) in 1:1 proportion (on weight basis), under continuous stirring. Extracted pomaces were pressed in a hydraulic manual press. Juice containing ethanol was obtained, and the ethanol was removed in a rotary evaporator by concentration to 50–60 °Brix. The obtained concentrate was

diluted with water to 11 °Brix, and this diluted extract was added to the earlier produced raw juice. The storage experiments started immediately after apple juice production.

Apple juice used in the accelerated storage experiments was prepared according to the pulp extraction method described earlier (*10*). About 1.5 kg of Jonagold apples (harvest year 1996) was cleaned by washing, stems were removed, and the fruit was cut in four pieces. Apple pulp was prepared by quick slicing in a domestic food processor (Braun). Apple pulp was aerated for 60 min. Methanol was added in a 1:1 proportion (on weight basis) to the pulp, which was then extracted for 45 or 90 min in a 30 °C shaking water bath. No difference was observed between the extraction times of 45 and 90 min (data not shown). Extracted apple pulp was pressed in a hydraulic manual press. The obtained juice was concentrated to 65–75 °Brix using a rotary evaporator and diluted to 11 °Brix with water. Apple juice was used immediately or stored at –20 °C for a maximum of 2 weeks until use in the storage experiments.

6.2.2.2. Storage Experiments.

a) Cold Storage and Storage at Room Temperature.

Sterilized glass bottles were filled with 100 mL of apple juice (raw or enriched juice, noncentrifuged, pasteurized for 30 s at 90 °C) and sealed. Bottles were stored in a cold room (4 °C) or at room temperature (20 °C). For sampling at different times, different bottles were used.

b) Accelerated Storage.

Three flasks filled with 170–350 mL of apple juice each were placed in a water bath of 70, 80, or 90 °C or in a glycerol/water (1:1) bath of 100 °C. Each of the flasks was continuously flushed with nitrogen, air, or oxygen, which was bubbled straight into the juice, with a flow rate of ~100 mL/min. The flow rate was measured by an electrical flow meter. The flasks were connected to condensers in order to minimize possible losses of water and volatiles from the apple juice by evaporation.

6.2.2.3. Sampling and Analysis.

In cold storage and storage at room temperature an aliquot of apple juice was taken from a bottle and stored immediately at –20 °C until analysis. Apple juice (5 mL) was

extracted with 5 mL of methanol and sonicated for 30 min followed by 10 min of centrifugation at 2500 rpm. The supernatant was filtered through a 0.45-µm CA filter (Schleicher and Schuell). Extractions were performed in duplicate. The same extract was used for both HPLC analysis and antioxidant activity determination.

During the accelerated storage experiments apple juice samples (20–25 mL) were taken regularly and stored immediately at –20 °C until lyophilization. Dry weight was determined from the sample weight before and after lyophilization. Lyophilized apple samples were stored at –20 °C until analyzed. Apple juice samples were extracted in duplicate before HPLC analysis. Lyophilized sample (0.5 g) was extracted with 10 mL of 15% acetic acid in methanol and sonicated for 30 min followed by filtration through a 0.45-µm CA filter (Schleicher and Schuell).

Quantification of flavonoids by HPLC and HPLC equipment was as described earlier (*14*). Flavonoid and chlorogenic acid standards were dissolved in methanol.

The antioxidant concentration at which 50% inhibition of lipid peroxidation occurs (IC_{50}) was calculated from triplicate determination of six different antioxidant concentrations ranging from no to full inhibition of lipid peroxidation, which was assessed by measuring thiobarbituric acid reactive species (TBARS) after heating. Absorption was read at 540 nm (color) versus 620 nm (turbidity correction) by an ELISA reader (*15*). Antioxidant activity was expressed as DF_{50}, which corresponds to $1000/IC_{50}$ (*9*). The higher the DF_{50} value, the higher the antioxidant activity of a sample is.

6.2.3. Modeling and Statistical Analysis.

Analyses were performed at least in duplicate, and results were expressed on a fresh weight basis. The analyzed polyphenols (chlorogenic acid, phloridzin, epicatechin, quercetin, Q-3-Ga, Q-3-Gl/Ru, Q-3-Ar, Q-3-Rh) were all expressed as millimolar in the juice. Oxygen concentrations were calculated from the temperature-dependent Henry coefficients [derived from Rooney and Daniels (*16*)], based on the gas composition that was led through the juice (0, 21, and 100% oxygen) and were also expressed as millimolar.

Reaction kinetics was studied by multiresponse modeling using the determinant criterion (*17*). Multiresponse modeling implies that more than one reactant or product is taken into account. The determinant criterion is then more suitable than the familiar least-squares criterion. The software package Athena Visual Workbench

(www.athenavisual.com) was used for numerical integration of differential equations as well as parameter estimation of the rate constants in the differential equations following minimization of the determinant in order to obtain the reaction kinetic parameters (rate constant *k* and activation energy E_a).

6.3. RESULTS AND DISCUSSION

6.3.1. Cold Storage and Storage at Room Temperature.

Figure 1 shows the effect of storage of raw apple juice and enriched apple juice at 4 °C or at 20 °C for 1 month. During storage the °Brix values of the apple juices remained unchanged (11.3 ± 0.1 for raw apple juice and 11.2 ± 0.1 for enriched apple juice) at both storage conditions. Also , the pH did not change (values between 3.6 and 3.7). During storage at 4 °C and at 20 °C no significant changes were observed at a 5% significance level in concentrations of total quercetin glycosides, total catechins, chlorogenic acid, phloridzin, and cyanidin galactoside. During the storage time no quercetin aglycon was formed. The above indicates that a 1 month storage of apple juice in a refrigerator or even at room temperature will not lower the concentration of the present polyphenolic antioxidants.

6.3.2. Stability of the Polyphenolic Antioxidants at Elevated Temperatures.

In accelerated storage experiments the stability of eight polyphenolic antioxidants was studied in enriched apple juice, and the results are shown for three oxygen pressures (0, 21, and 100%) at 80 °C in Figure 2a–h. Breakdown of quercetin glycosides showed no dependency on oxygen pressure. A clear difference in degradation rate between the various glycosides was observed (Fig 2a–d). The stability decreased in the order Q-Ga ≈ Q-Rh > Q-Gl/Ru > Q-Ar. During the incubations an increase in the amount of quercetin aglycon (Q) was observed (Fig. 2e). The aglycon was present at only very low amounts initially (<3 μM) but was formed during the incubation, presumably by the hydrolysis of the various quercetin glycosides. Acid hydrolysis of quercetin glycosides is a well-known phenomenon, but, to our knowledge, has not been described in apple juice before. In Figure 2e it is seen that the formed quercetin aglycon was not stable but was further degraded. This degradation of quercetin showed a clear dependency on the amount of oxygen, indicating an oxidative pathway. Phloridzin and chlorogenic acid (Fig. 2f–g) showed minor degradation rates. Both compounds showed some 20-30% decrease in levels

over 100 h of incubation at 80 °C. The presence of oxygen did not have a big effect, if any, on the stability of these compounds. Epicatechin degradation profiles showed a decrease of 80% of the initial values after 50–100 h (depending on the oxygen pressure). The concentration remained stable at this 20% level. For epicatechin in

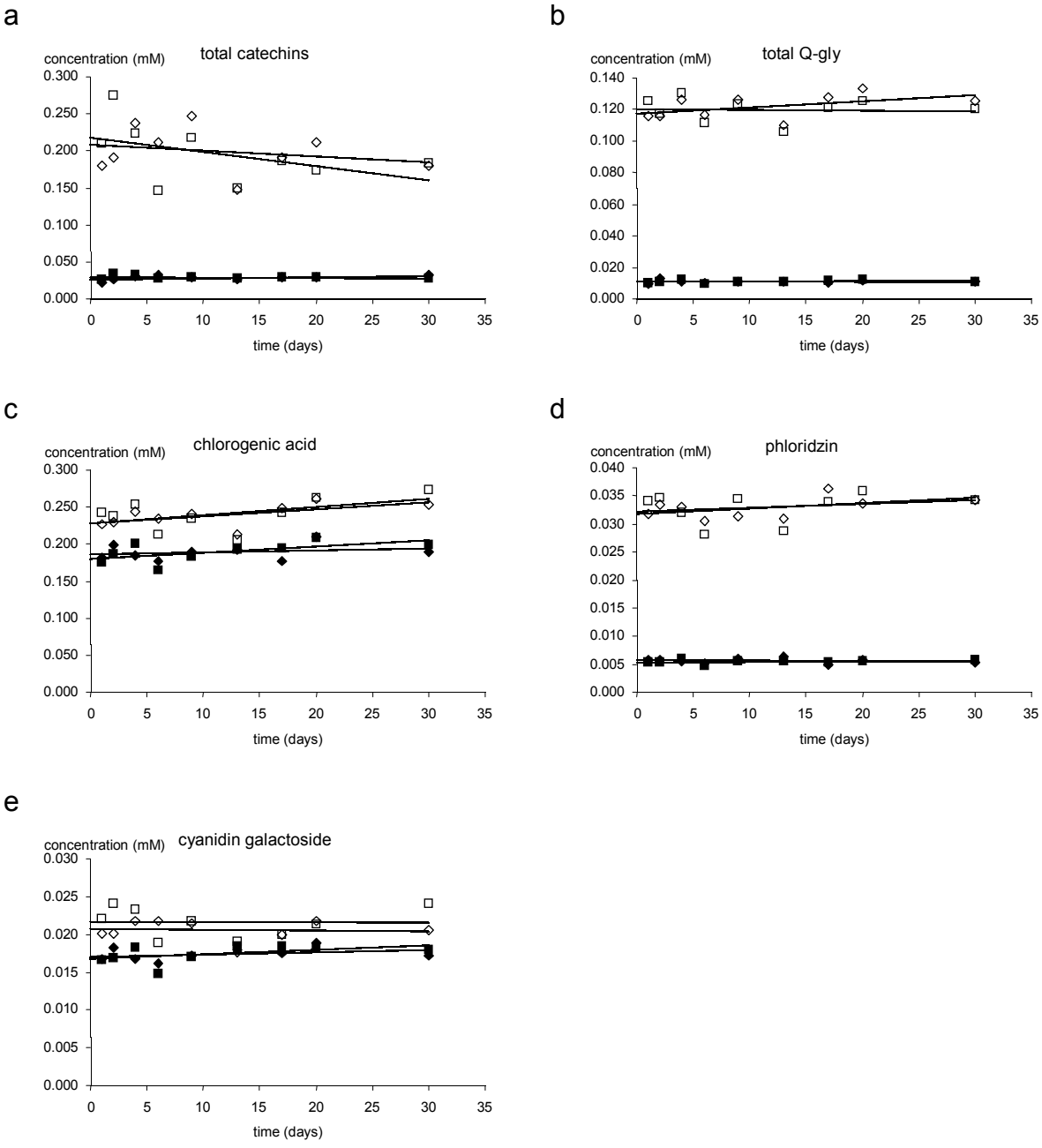


Figure 1. Effect of storage conditions on raw apple juice (solid symbols) and enriched apple juice (open symbols). Juices were stored at 4 °C (◊) or at 20 °C (□) for 1 month. Enriched apple juice was prepared by ethanolic pomace extraction. SD = 1–20%. Lines are linear trend lines. The group 'total Q glycosides' consists of the compounds Q-3-Ga, Q-3-Ru/Gl, Q-3-Ar, and Q-3-Rh. The group 'total catechins' consists of the compounds catechin and epicatechin.

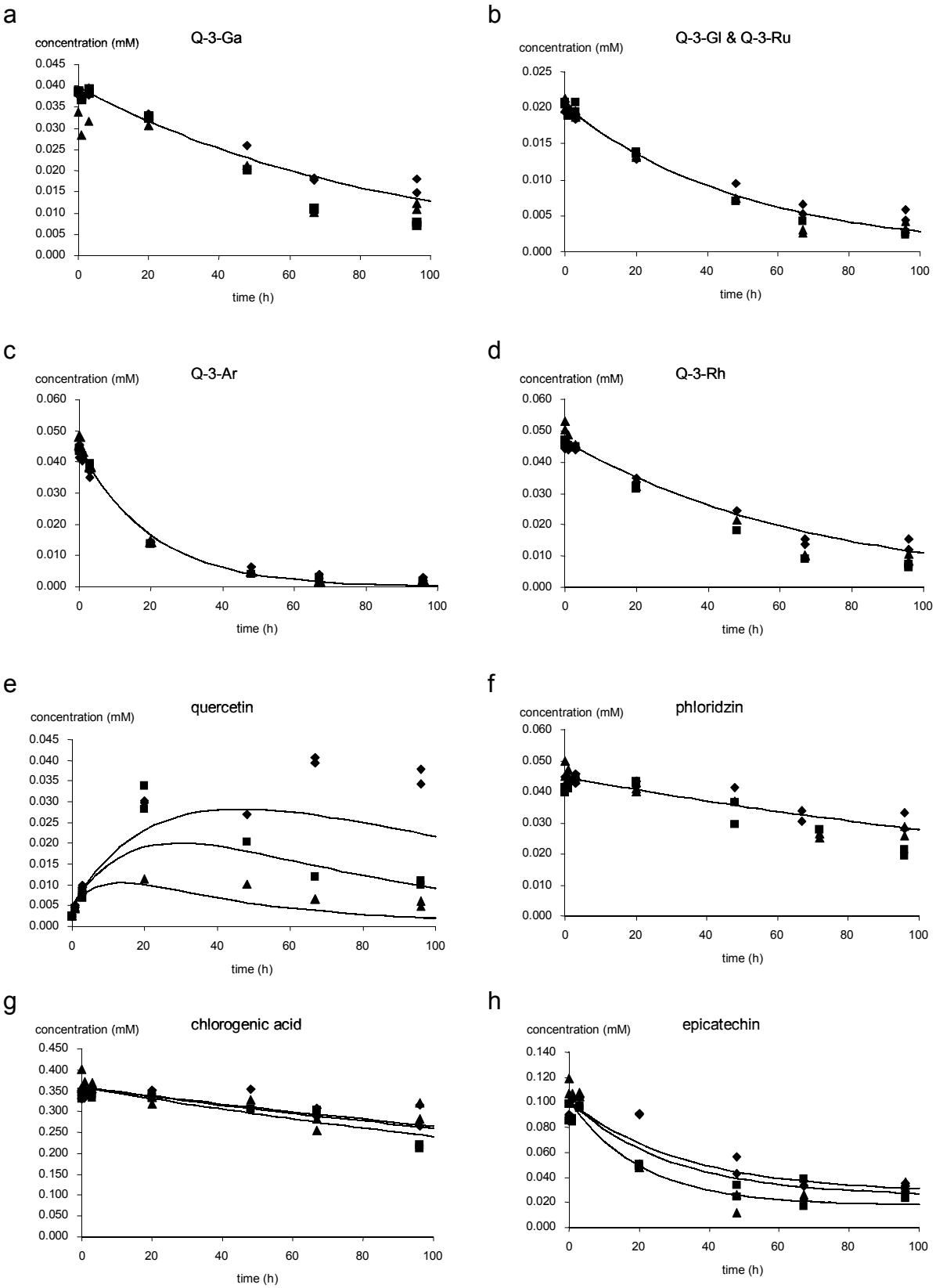


Figure 2. Effect of storage conditions on enriched apple juice. Juices were stored at 80 °C while bubbled with 0% O_2 (♦), 21% O_2 (■), or 100% O_2 (▲), for 4 days. Enriched apple juice was prepared by methanolic pulp extraction. Values are from duplicate extractions. Lines are fitted lines using eqs 5–8. Estimated parameters are given in Table 1–3.

chocolate it has been observed that epicatechin can be formed from the polymerized forms (procyanidins), which are also present in the product, at elevated temperatures (*18*). Because procyanidins are present in considerable concentrations in apple [0.5–1 g/kg, (*19*)], they are expected to be present in high levels in enriched apple juice as well; therefore, it can be assumed that the seemingly stable remaining levels are in fact a quasi steady state as a consequence of a formation and a degradation reaction.

Incubation of the apple juices at various temperatures (70, 80, 90, and 100 °C) for different oxygen pressures (0, 21, and 100%) showed a clear effect of temperature on the stability of all polyphenolic antioxidants studied. In Figure 3 results of Q-Rh at 0% oxygen are shown as an example. At 70 °C ~50% breakdown was observed after 100 h of incubation, whereas at 100 °C >95% breakdown was observed after 50 h.

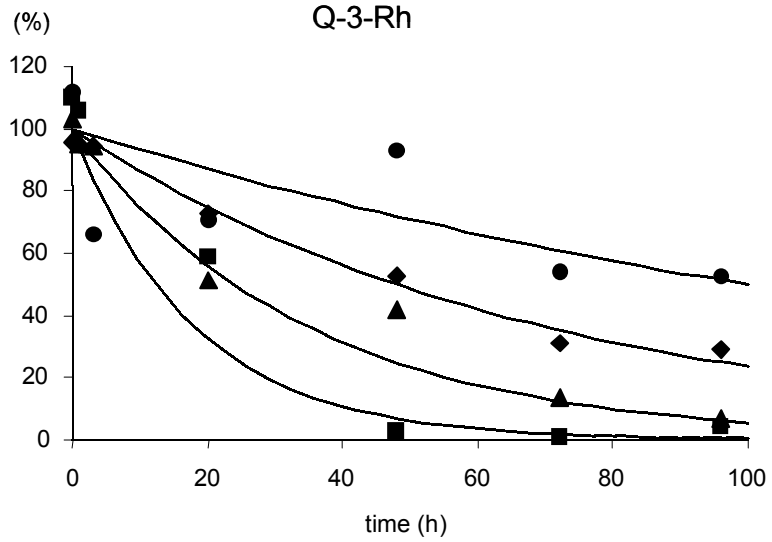


Figure 3. Effect of temperature on the stability of Q-Rh in enriched apple juice stored at 0% oxygen. Juices were incubated at 70 °C (●), 80 °C (♦), 90 °C (▲), or at 100 °C (■). Enriched apple juice was prepared by methanolic pulp extraction. Lines are fitted lines.

6.3.3. Antioxidant Activity.

The effect of incubation at 80 °C at various oxygen pressures on the antioxidant activity of the apple juice is shown in Figure 4. The activity is expressed as the dilution factor (DF_{50}) to reach 50% inhibition of the lipid oxidation in the microsomal assay. A higher DF_{50} value therefore indicates a higher antioxidant activity of the

juice. It can be seen that the antioxidant activity was reduced after incubation of 100 h at 80 °C with 20% (0 and 21% oxygen) until 40% (100% oxygen). Except for the 0% oxygen incubation an initial increase in the antioxidant activity was observed in the first hours of incubation. The reason for this is not clear. It is known that the formation of quercetin aglycon by hydrolysis of quercetin glycosides results in an increase in activity, because the aglycon has around twice the antioxidant activity compared to the average quercetin glycoside activity (*15*). The time frame of this reaction, however, is much longer than that of the observed increase. Because also in some of the analyses of the individual quercetin glycosides a slight increase was observed in the first few hours of incubation, an explanation of the increase could be an increase in extractability of the freeze-dried samples after heating. This phenomenon could be due to protein denaturation leading to reduced complex formation between protein and flavonols.

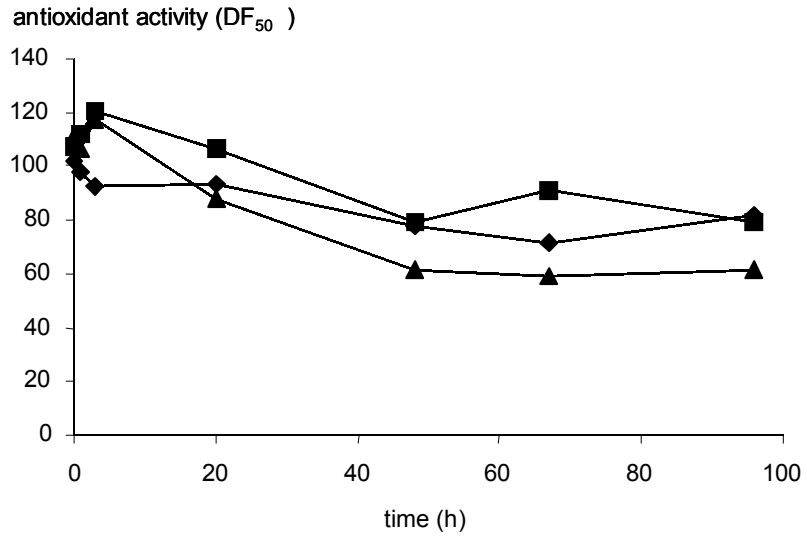


Figure 4. The effect of storage on the antioxidant activity of enriched apple juice. Juices were stored at 80 °C while bubbled with 0% O_2 (♦), 21% O_2 (■), or 100% O_2 (▲), for 4 days. Enriched apple juice was prepared by methanolic pulp extraction.

6.3.4. Modeling the Degradation Reactions of the Polyphenolic Antioxidants.

The complete data set of all the analyzed concentrations after incubation of the apple juice for the different conditions (time, temperature, and oxygen pressure) was used to estimate the Arrhenius parameters for all of the reactions as described in

equations 1–10 below. This was a total of 206 time/temperature/oxygen combinations with each seven (or eight at 80 °C) different polyphenolics analyzed. The results of this analysis are shown in Tables 1 and 2, which show the estimates of the reference rate constants and their activation energies together with their 95% confidence intervals as determined by the multiresponse fitting procedure described in the modeling section under Materials and Methods. The modeling results will be discussed for each group of components separately.

The non enzymatic degradation of polyphenols can be divided in oxidative degradation and nonoxidative degradation. The proposed pathways of the two types of degradation are shown in the general reaction scheme:

$$\mathrm{PP} \xrightarrow{k_\mathrm{d}} \mathrm{X}_1 \tag{1}$$

$$\mathrm{PP} + \mathrm{O}_2 \xrightarrow{k_\mathrm{o}} \mathrm{X}_2 \tag{2}$$

where PP indicates polyphenol, X_n indicates breakdown products, k_d is the nonoxidative degradation rate constant, and k_o is the oxidative degradation rate constant.

In the case of quercetin glycosides also a hydrolysis reaction may be involved in the degradation pathway:

$$\mathrm{QG} \xrightarrow{k_\mathrm{h}} \mathrm{Q} + \mathrm{G} \tag{3}$$

QG is quercetin glycoside, Q is quercetin, G is sugar residue, and k_h is the hydrolysis rate constant.

Both the quercetin glycoside and the aglycon can subsequently be degraded as described by the reactions 1 and 2.

In the case of epicatechin a steady state was observed after an initial breakdown. This can be modeled by taking into account the possibility of the formation of epicatechin from polymeric forms (procyanidins):

$$\mathrm{PC} \xrightarrow{k_\mathrm{f}} \mathrm{EC} \tag{4}$$

PC indicates procyanidins, EC indicates epicatechin, and k_f is the formation rate constant.

The reaction schemes were translated into the following differential equations:

$$\frac{\mathrm{d[PP]}}{\mathrm{d}t} = -k_{\mathrm{d,PP}}[\mathrm{PP}] - k_{\mathrm{o,PP}}[\mathrm{PP}][\mathrm{O}_2] \tag{5}$$

$$\frac{\mathrm{d[QG_n]}}{\mathrm{d}t} = -k_{\mathrm{h,QG_n}}[\mathrm{QG_n}] - k_{\mathrm{d,QG_n}}[\mathrm{QG_n}] - k_{\mathrm{o,QG_n}}[\mathrm{QG_n}][\mathrm{O}_2] \tag{6}$$

$$\frac{\mathrm{d[Q]}}{\mathrm{d}t} = +\sum_{1}^{4} k_{\mathrm{h,QG_n}}[\mathrm{QG_n}] - k_{\mathrm{d,Q}}[\mathrm{Q}] - k_{\mathrm{o,Q}}[\mathrm{Q}][\mathrm{O}_2] \tag{7}$$

$$\frac{d[EC]}{dt} = +k_f[PC] - k_{d,EC}[EC] - k_{o,EC}[EC][O_2] \quad (8)$$

The PP are chlorogenic acid, phloridzin and epicatechin. k_d is the nonoxidative degradation rate constant, and k_o is the oxidative degradation rate constant.

The temperature dependence of the reaction rate constants was described by the Arrhenius equation:

$$k = k_0 \exp(-\frac{E_a}{RT}) \quad (9)$$

which was rearranged to:

$$k = k_{ref} \exp((\frac{E_a}{R})(\frac{1}{T_{ref}} - \frac{1}{T})) \quad (10)$$

The temperature of 70 °C was used as the reference temperature (T_{ref} = 343 K). Estimation of the k_{ref} and E_a of all reactions was done by fitting the equations simultaneously for the experimental data of all four temperatures investigated. The initial concentrations were estimated by the fitting procedure as well, to allow for uncertainty in the experimental observation at t = 0.

Table 1. Estimated Reference Rate Constants for the Hydrolysis of Quercetin Glycosides and Their Activation Energies

compound	$k_{h,70}$ (h^{-1})	$E_{a,h}$ (kJ/mol)
quercetin galactoside	2.2×10^{-3} (0.2×10^{-3}) [a]	78 (5)
quercetin glucoside/rutinoside	4.3×10^{-3} (0.3×10^{-3})	66 (3)
quercetin arabinoside	11.8×10^{-3} (0.7×10^{-3})	57 (3)
quercetin rhamnoside	2.9×10^{-3} (0.2×10^{-2})	74 (4)

[a] Values in parentheses indicate the 95% confidence interval.

6.3.4.1. Quercetin Glycosides and Quercetin Aglycon.

The stability of the four different quercetin glycosides and quercetin aglycon was modeled simultaneously because the formation of quercetin aglycon in the juice was a direct result from the hydrolysis rate of the quercetin glycosides. It was not possible to estimate the individual nonoxidative and oxidative reaction rate constants and hydrolysis rate constants for all different glycosides separately, because of the high correlation between them. Therefore, it was assumed that the individual quercetin

glycosides had a degradation rate which is the summation of the nonoxidative degradation reaction rate and the hydrolysis rate of quercetin. The oxidative degradation reaction was neglected because no effect of oxygen pressure on the stability of the quercetin glycosides was observed at all temperatures studied (Figure 2). The ratio between breakdown and hydrolysis of the glycosides was assumed to be constant for all quercetin glycosides and was estimated by the modeling procedure. This resulted in the estimation that 43.5 ± 2.0% of the observed breakdown of quercetin glycosides occurred through hydrolysis, resulting in the formation of quercetin aglycon. This percentage showed no temperature dependence in the range of 70–100 °C. The hydrolysis rate constants (Table 1) of the individual glycosides varied with a factor of 5. The stability decreased in the order quercetin galactoside ≈ quercetin rhamnoside > quercetin glucoside/rutinoside > quercetin arabinoside. The quercetin aglycon was formed during the incubations and was subsequently degraded both oxidatively and nonoxidatively, with the oxidative reaction being dominant at high oxygen pressures (see also Figure 2). The fit of the data of quercetin, as shown in Figure 2e, showed an underestimation for the data of the anaerobic incubations at 80 °C. The deviations at the other temperatures were less marked and showed a small underestimation at 70 and 80 °C and a small overestimation at 100 °C. These deviations could indicate that an additional reaction pathway is involved for the nonoxidative degradation of quercetin.

Table 2. Estimated Reference Rate Constants for the Nonoxidative and Oxidative Degradation of Chlorogenic Acid, Phloridzin and Quercetin Aglycon and Their Activation Energies

compound	$k_{d,70}$ (h^{-1})	$E_{a,d}$ (kJ/mol)	$k_{o,70}$ (h^{-1})	$E_{a,o}$ (kJ/mol)
chlorogenic acid	1.8×10^{-3} (0.3×10^{-3}) [a]	52 (8)	1.0×10^{-4} (0.6×10^{-4})	187 (25)
phloridzin	2.3×10^{-3} (0.5×10^{-3})	73 (10)	0	-
quercetin aglycon	1.1×10^{-2} (0.1×10^{-2})	31 (5)	3.8×10^{-2} (0.6×10^{-2})	30 (8)

[a] Values in parentheses indicate the 95% confidence interval.

6.3.4.2. Phloridzin.

The main degradation route for phloridzin was the nonoxidative pathway. No significant effect of different oxygen pressures was observed at any temperatures studied (Table 2).

6.3.4.3. Chlorogenic Acid.

The main degradation route at temperatures lower than 90 °C for chlorogenic acid was the nonoxidative pathway. The oxidative degradation was observed at only 90 and 100 °C. This resulted in a quite large confidence interval for the oxidative reaction rate constant and its activation energy. A high correlation coefficient was also observed between these two parameters (–0.978). At normal storage conditions the nonoxidative reaction will be dominant.

The above does not include enzymatic degradation. At ambient conditions oxidative degradation of chlorogenic acid and catechin by the enzyme polyphenol oxidase is also possible, as chlorogenic acid and epicatechin are good substrates for this enzyme (*12*); however, at elevated temperatures, the enzyme will be inactivated.

Table 2 shows that the degradation rate constants for phloridzin and chlorogenic acid are a factor 10 lower than that of quercetin aglycon, which indicates that phloridzin and chlorogenic acid are more stable than quercetin aglycon upon heating.

6.3.4.4. Epicatechin.

Epicatechin degradation was investigated at only 80 °C; therefore, no effect of temperature on the reaction rate could be analyzed. As mentioned in the discussion of the general observations of the trends, a stable level of ~20% of the compound remained in the juice after the initial breakdown. This behavior could be modeled by taking into account the formation of epicatechin from the hydrolysis of its polymeric form (procyanidins). Procyanidins were not analyzed in the present study, but it is expected that enriched apple juice contains high levels of these compounds. Because this is most probably higher than the concentration of epicatechin, it was assumed that the hydrolysis rate remained constant during the incubations. From the parameter estimation it can be concluded that the nonoxidative degradation is slightly higher than the oxidative degradation at 100% oxygen (Table 3).

Table 3. Estimated Rate Constants for the Nonoxidative and Oxidative Degradation of Epicatechin and the Formation Rate Constant at 80 °C

compound	$k_{d,80}$ (h^{-1})	$k_{o,80}$ (h^{-1})	$k_{f,80}$·[PC] (mM/h)
epicatechin	31.2×10^{-3} (5.2×10^{-3}) [a]	11.3×10^{-3} (8.6×10^{-3})	8.7×10^{-4} (2.0×10^{-4})

[a] Values in parentheses indicate the 95% confidence interval.

6.3.5. Prediction of the Effect of Ambient Storage on the Levels of Polyphenolic Antioxidants in Apple Juice.

With the estimated rate constants and their activation energies it was possible to predict the effect of practical storage conditions on the level of polyphenolic antioxidants in apple juice. This is shown in Figure 5, where the effect of 200 days of storage at 20 °C in the absence of oxygen is shown. According to this simulation both chlorogenic acid and quercetin glycosides declined roughly by 40%, a small amount of quercetin aglycon was formed, and phloridzin remained quite stable. In carton-laminated packed commercial apple juices a decline of phenolic acids (5–21%) and in flavonoid content (14%–18%; quercetin glycosides and phloridzin together) is reported after 11 months of storage at room temperature (*13*), which is roughly half of our prediction for enriched apple juice.

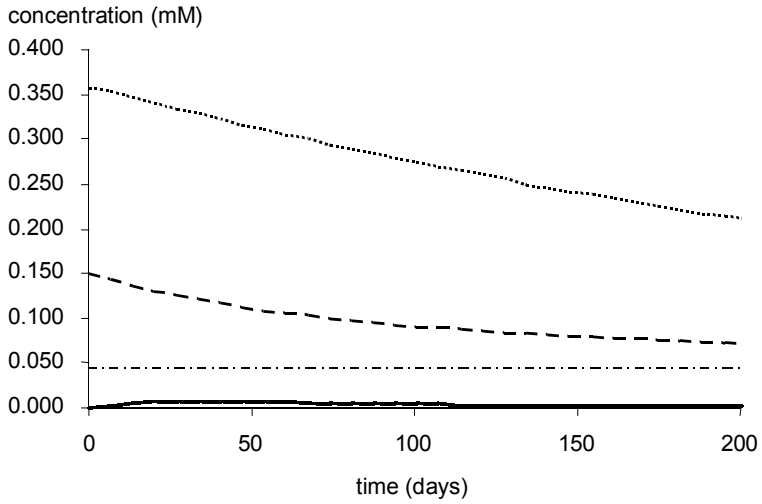


Figure 5. Prediction of the effect of practical storage conditions (200 days at 20 °C in the absence of oxygen) on the level of chlorogenic acid (·····), total quercetin glycosides (- - -), phloridzin (-·-·-), or quercetin aglycon (—) in enriched apple juice. Estimated rate constants and their activation energies were used as input.

To check if it was possible to use the estimated rate constants and their activation energies for temperatures that lie outside the range of the measured temperatures (70–100 °C) predicted changes in polyphenolic antioxidant concentrations were compared with measured ones (as shown in Figure 1). The predicted decreases in quercetin glycoside and chlorogenic acid concentration after 30 days of storage at room temperature were 17 and 7%, respectively, where in the experiment a stability of these compounds at 20 °C was observed. Phloridzin remained stable in both the model predictions and the measurements. The storage experiment showed no formation of quercetin aglycon at 20 °C, where the model predicted the formation of a few micromoles. This indicates that for quercetin glycosides the prediction of breakdown rates slightly overestimates reality during extrapolation to lower temperatures.

6.3.6. Prediction of the Effect of Heat Treatments on the Level of Quercetin in Apple Juice.

With the estimated rate constants and their activation energies it was also possible to predict the effect of various heat treatments such as pasteurization and sterilization on the level of antioxidants in apple juice. Data were extrapolated to heat treatments of maximal 1h at 140 °C, and as an example Figure 6a shows the effect of heating time and temperature on the decrease in quercetin content. Whereas quercetin glycoside concentration decreased, quercetin aglycon concentration increased (as mentioned earlier), those two effects were added in Figure 6a. This figure shows that pasteurization [bottles, 10–20 min in water of 80–90 °C, (*20*)] will not affect quercetin concentration in apple juice, nor will sterilization (90 s at 120 °C). After 1 h at 120 °C a decrease in quercetin glycoside concentration of 12% will be observed, which is a treatment one will not find in practice.

In Figure 6b the effect of heat treatments on the calculated antioxidant activity of quercetin glycosides and quercetin aglycon is shown, using the concentrations given in Figure 6a and the antioxidant activity of the individual compounds reported earlier (*15*).

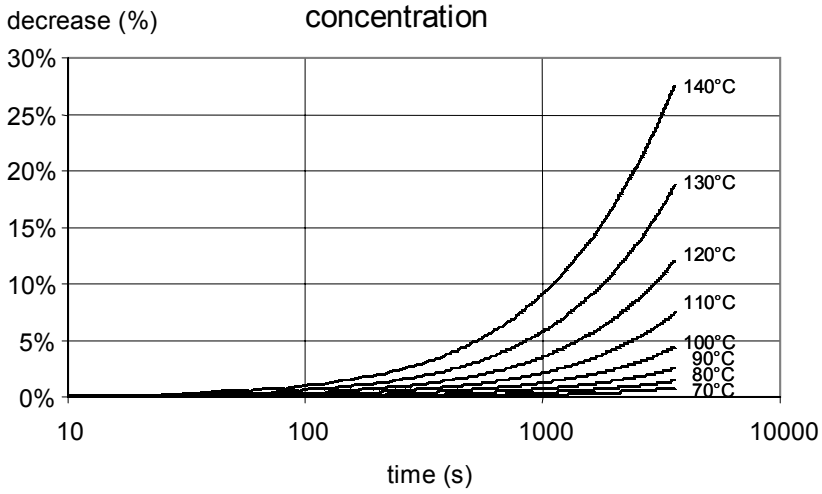
The calculated antioxidant activity is derived as follows

$$\sum_{i=1}^{n} \frac{C_i}{IC_{50,i}} \qquad (11)$$

where C_i is the concentration of component i (mM) and $IC_{50,i}$ is the IC_{50} value of component i (mM).

Figure 6 b shows that the decrease in calculated antioxidant activity is lower than the decrease in concentrations predicted as a result of heat treatments, due to the formation of quercetin aglycon, which has an almost 2-fold higher antioxidant activity than its corresponding glycosides (*15*). After 1 h at 120 °C a decrease of only 5% in calculated antioxidant activity will be observed. In the mentioned calculated antioxidant activity only the contribution of quercetin glycosides and quercetin aglycon is taken into account; in this case the contribution of other antioxidants and their possible interaction are not included.

a



b

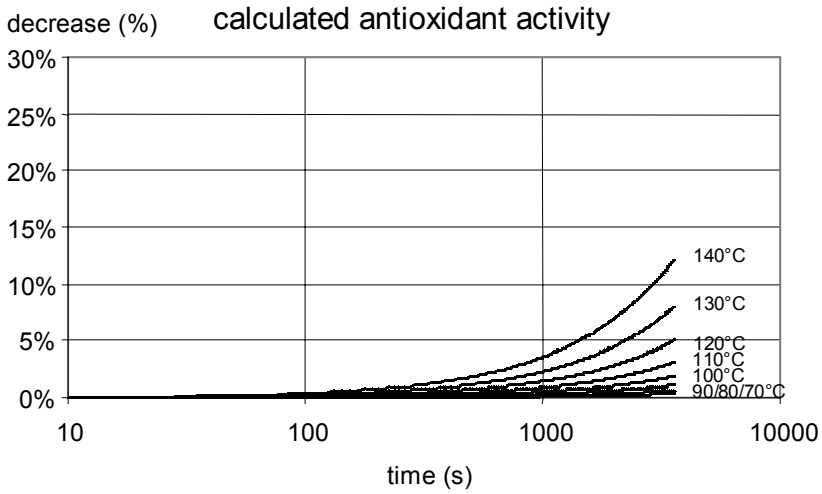


Figure 6. Prediction of the effect of heat treatments (such as pasteurization) in the presence of 21% oxygen on the decrease in concentration and calculated antioxidant activity of total quercetin glycosides including quercetin aglycon in apple juice. Estimated rate constants and their activation energies were used as input.

6.3.7. Practical Implications

It has been possible to obtain information about the stability of polyphenolic antioxidants from accelerated storage experiments. Using kinetic modeling, predictions about the stability of quercetin glycosides, chlorogenic acid, and phloridzin in apple juice during storage and various heat treatments could be made. It showed that the results of the predictions are in accordance with our own results, and the compounds are stable during normal storage temperatures (4 and 20 °C). Furthermore, it was predicted that quercetin glycosides can withstand 1.5 min at 140 °C or 1 h at 70 °C without major degradation.

Upon heating, the various quercetin glycosides and epicatechin were the most sensitive compounds, whereas phloridzin and chlorogenic acid were more stable. Quercetin glycosides showed differences in their stability. The decrease in concentration as a consequence of heat treatments is higher than the decrease in calculated antioxidant activity.

The prediction does not include enzymatic degradation of chlorogenic acid at lower temperatures; however, at elevated temperatures, the enzyme polyphenol oxidase will be inactivated. Quercetin glycosides are not substrates for polyphenol oxidase.

Substantial differences have been observed in terms of activation energies for the various polyphenolic antioxidants studied. This can be used for optimizing heat treatments, for example, regarding a specific compound of interest.

The effect of the presence of oxygen on the degradation rates was clear for only quercetin aglycon and to a lesser extent for epicatechin, which indicates that there was not much of an effect of oxygen concentration observed and that with respect to packaging and heat treatments no special precautions regarding oxygen have to be taken.

6.4. ABBREVIATIONS USED

Cy-Ga, cyanidin galactoside or ideain; EC, epicatechin; k_d, nonoxidative degradation rate constant; k_o, oxidative degradation rate constant; k_f, formation rate constant; PC, procyanidins; PP, polyphenol; Q, quercetin; QG, quercetin glycoside; Q-3-Ga, quercetin galactoside or hyperin; Q-3-Ru, quercetin rutinoside or rutin; Q-3-Gl, quercetin glucoside or isoquercitrin; Q-3-Ar, quercetin arabinoside or avicularin; Q-3-Rh, quercetin rhamnoside or quercitrin; X_n, breakdown products.

6.5. ACKNOWLEDGMENT

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6.6. LITERATURE CITED

1. Miller, N. J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem*. **1995**, *43*, 1794–1801.
2. Block, G.; Patterson, B.; Subar, A. 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18*, 1–29.
3. Steinmetz, K. A.; Potter, J. D. Vegetables, fruit, and cancer prevention: a review. *J. Am. Diet. Assoc*. **1996**, *96*, 1027–39.
4. Tanaka, T. Cancer chemoprevention by natural products (review). *Oncol. Rep.* **1994**, *1*, 1139–1155.
5. Kühnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet*. **1976**, *24*, 117–191.
6. Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.*, **2000**, *130*, 2073S–2085S.
7. Arts, I.; Hollman, P. C. H.; Feskens, E. J. M.; Bueno de Mesquita, H. B.; Kromhout, D. Catechin intake and associated dietary lifestyle factors in a representative sample of Dutch men and women. *Eur. J. Clin. Nutr.* **2001**, 55, 76–81.
8. Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet*, **1993**, *342*, 1007–1011.
9. Van der Sluis, A. A.; Dekker, M.; Skrede, G.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *J. Agric. Food Chem.* **2002**, *50*, 7211–7219.
10. Van der Sluis, A. A.; Dekker, M.; Skrede, G.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple juice. 2. Effect of novel production methods. *J. Agric. Food Chem.* **2004**, *52*, 2840–2848.
11. Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of processing and storage on the phenolic composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572–1579.
12. Van Buren, J.; de Vos, L.; Pilnik, W. Polyphenols in Golden Delicious apple juice in relation to method of preparation. *J. Agric. Food Chem.* **1976**, 24, 448–451.
13. Gliszczynska Swiglo, A.; Tyrakowska, B. Quality of commercial apple juices evaluated on the basis of the polyphenol content and the TEAC antioxidant activity. *J. Food Sci.* **2003**, *68*, 1844–1849.

14. Van der Sluis, A. A.; Dekker, M.; de Jager, A.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple; effect of cultivar; harvest year and storage conditions. *J. Agric. Food Chem.* **2001**, *49*, 3606–3613.

15. Van der Sluis, A. A.; Dekker, M.; Verkerk, R.; Jongen W. M. F. An improved, rapid in vitro method to measure antioxidant activity; application on selected flavonoids and apple juice. *J. Agric. Food Chem*. **2000**, *48*, 4116–4122.

16. Rooney, P. C.; Daniels, D. D..Oxygen solubility in various alkanolamine/water mixtures. *Petroleum Technol. Q.* **1998**, Spring, 97–101.

17. Stewart, W. E.; Caracotsios, M.; Sørensen, J. P. Parameter estimation from multiresponse data. *AIChE J.* **1992**, *38*, 641–650; erratum, **1992**, *38*, 1302.

18. Zhu, Q. Y.; Holt, R. R.; Lazarus, S. A.; Ensunsa, J. L.; Hammerstone, J. F.; Schmitz, H. H.; Keen, C. L. Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. *J. Agric. Food Chem.* **2002**, *50*, 1700–1705.

19. Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H. Procyanidin content and variation in some commonly consumed foods. *J. Nutr.* **2000**, *130*, 2086S–2092S.

20. Fellows, P. *Guidelines for Small-Scale Fruit and Vegetable Processors*; FAO Agricultural Services Bulletin 127; retrieved from the Web April 21, 2004; http://www.fao.org/docrep/W6864E/w6864e08.htm#pasteurisation

CHAPTER 7

General Discussion

7.1. INTRODUCTION

In this thesis the apple production chain (harvest, storage, processing and further storage of the product) was investigated, with a focus on the effects on the level of polyphenolic compounds and antioxidant activity. This knowledge was used to design a process to obtain an enriched product.

To be able to determine antioxidant activity of pure compounds as well as of extracts of apple products, a method was optimized enabling analysis of large amounts of samples.

The effect of storage and processing on polyphenolic compound concentrations and antioxidant activity of apple and its juice was determined. During apple storage polyphenolic compound concentrations remained quite stable, only slight differences between individual compounds were observed. Antioxidant activity remained stable.

During processing of apples to apple juice in a conventional way the largest part of the polyphenolic compounds remained in the pomace and only 3–10% of the antioxidant activity present in fresh apples was found in the juice. A novel production method was developed in order to produce an apple juice with an enhanced concentration of these compounds. This was achieved by applying an alcoholic extraction on the pulp or on the press cake. In such an enriched apple juice more than 50% of the antioxidant activity present in fresh apples was found.

Finally the effect of storage conditions (temperature and oxygen concentration) on polyphenolic antioxidants present in enriched apple juice was determined. The most (thermally) sensitive compounds were the various quercetin glycosides and epicatechin, whereas phloridzin and chlorogenic acid were more stable. Accelerated shelf life testing of enriched apple juice during 4 days at 80 °C showed decreases in the antioxidant activity of 20–40%. Model predictions showed that polyphenolic antioxidant concentrations and antioxidant activity of enriched apple juice should be quite stable at ambient or refrigerated storage conditions up to half a year.

Summarizing it can be concluded that the research objectives were reached: the main effect of the production chain on polyphenolic antioxidants occurs during conventional processing into juice, where more than 90% of the antioxidant activity is lost, and a process yielding a product with a higher polyphenolic content as well as a higher antioxidant activity was developed. In section 7.2 this new processing method is critically evaluated and in section 7.3 the implications of our findings for other apple

products are indicated. In section 7.4 the method that was used to determine antioxidant activity is evaluated, followed by some considerations regarding the bioavailability of quercetin glycosides present in apple in section 7.5. As the implications of enriched apple products for human health remain to be studied, in section 7.6 some predictions of the possible impact on intake of polyphenolic compounds are presented. The discussion is finished with recommendations for further research in section 7.7.

7.2. DISCUSSION OF THE NEWLY DESIGNED METHOD

7.2.1. Conventional versus Enriched Apple Juice Production.

In both conventional and enriched apple juice production the choice of the apple cultivar to use is important as was shown in Chapter 3. Apple storage time is less important. Chapters 4 and 5 have shown the importance of the choice of processing method and conditions on the final levels and antioxidant activity of the raw and enriched juices. Figure 1 gives some options for further processing of raw (or enriched) apple juice. It indicates that in further processing of juice, clarification treatments, concentration practices (to lower the volume of product to store and transport), pasteurization and packaging can potentially affect the product. These treatments may affect polyphenol content and antioxidant activity of the final product, as well as other quality parameters (such as pH of juice, °Brix, color, taste, and aroma).

The newly designed method of alcoholic pomace extraction consists of adjustments in the processing method. By doing so, the effect of changes in production and processing methods on other quality factors (such as taste, color, keepability), production efficiency, economic feasibility and consumer acceptance should be taken into account. Restrictions of food laws regarding the final product should be obeyed as well.

Not all these aspects were included in the research described in this thesis. The effect of clarification methods on polyphenolic content in apple juice and antioxidant activity was out of the scope of this research, but clarification methods are intended to reduce turbidity and to prevent undesired browning of the juice. A decrease in polyphenol content of juices can be expected as polyphenols can bind to proteins causing turbidity. Production efficiency and economic feasibility have not been

investigated, but alcoholic extraction procedures will increase production time compared to conventional juice processing, and an extraction vessel with stirrer will be needed. If in conventional apple juice production a concentrate is prepared to facilitate storage and transport, these facilities can be used as well for the evaporation of ethanol from the extract. The ethanol needed for alcoholic pomace extraction can be reused.

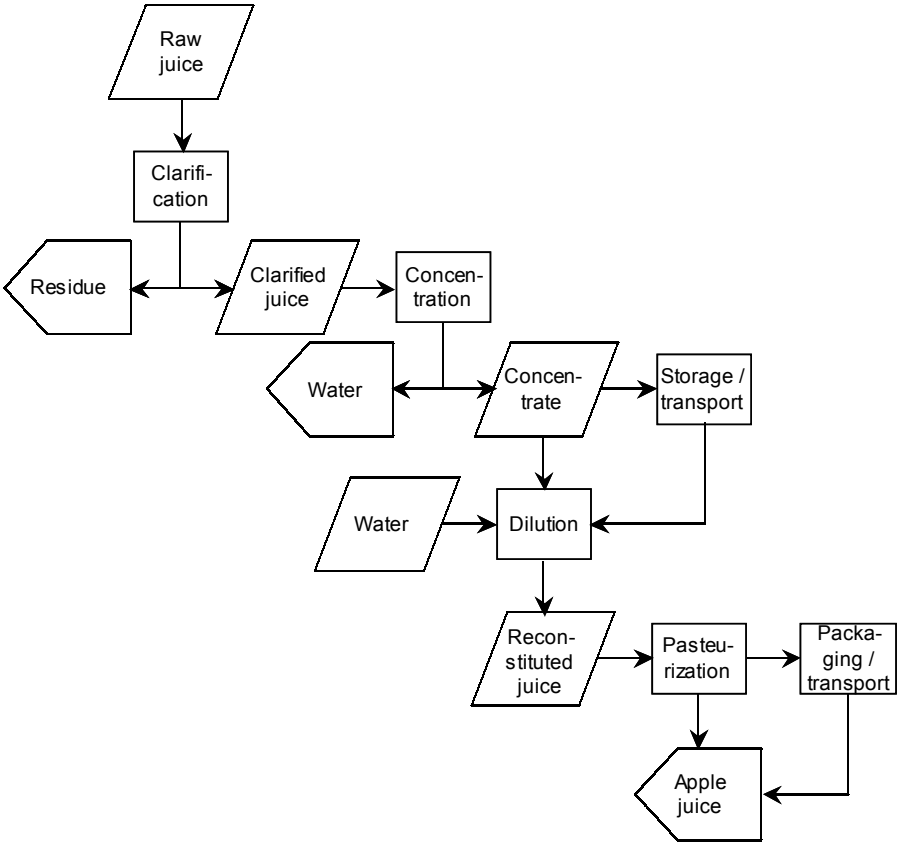

Figure 1. Options for further processing of raw apple juice.

The relative effect of pasteurization (described in Chapter 6) will be the same for conventional and enriched apple juice production. The effect of packaging was not studied in this thesis, but from Chapter 6 it can be concluded that packaging with the exclusion of oxygen will provide minor additional stability for the polyphenolic antioxidants.

Not included in the research described in this thesis was the polyphenolic group of the procyanidins. Procyanidins are polymerized epicatechin and catechin compounds with a degree of polymerization between 2 and 7 in apples. These compounds can

be quantified by HPLC after a thiolysis reaction (*1*). It appeared that procyanidin concentration in apple flesh can be 6–10 times higher than the concentration of epicatechin and catechin monomers together. In apple skin their concentration is even higher. It is expected that most procyanidins behave quite similar as their monomers catechin and epicatechin with respect to solubility in ethanol or juice, but with increasing degree of polymerization their solubility lowers. Therefore it is expected that procyanidins will be present in enriched apple juices in higher quantities than their monomers. As hypothesized in Chapters 4 and 5, the unexplained part of apple (juice) antioxidant activity is likely to be caused by the presence of procyanidins.

7.2.2. Possible Coextraction of Other Compounds.

A possible disadvantage of the novel processing method might be that a coextraction of undesired compounds from apple in the alcoholic pomace extraction occurs. This could be the case for the toxin amygdalin from seeds and for ethanol soluble pesticides residues, although pesticides should not be present at toxic levels according to food safety legislation.

Amygdalin is a seed constituent that belongs to the cyanoglycosides, it contains sugar and produces hydrogen cyanide (a toxic compound) as a result of coming into contact with beta-glucosidase, a hydrolytic enzyme which is released when the cell structure is disrupted. Amygdalin concentration in apple seed has been reported to be 790 mg/kg (*2*). The presence of amygdalin in alcohol extracted apple juice was not investigated in this thesis, but a worst-case safety evaluation can be made. The ADI (acceptable daily intake) described for cyanide has been reported at 0.05 mg/kg (*3*). If all amygdalin would be converted into cyanide this would corresponds to an ADI for amygdalin of 0.85 mg/kg. For a person of 75 kg this equals an acceptable intake of amygdalin of 63 mg daily. Assuming that all amygdalin from apple seeds is extracted from the seeds (which will be an overestimation), this equals the consumption of 80 g of apple seeds. Assuming furthermore that apple seeds constitute 0.2% of the fresh apple, this means that when all amygdalin would be extracted from the seeds into apple juice, a juice will be obtained containing 1.6 mg/L amygdalin. A person of 75 kg would need to consume 40 L of enriched apple juice daily to meet that risk, which is unlikely to occur. Even for small children (10 kg)

problems with amygdalin are unlikely, since they would need to consume over 5 L juice daily before exceeding the ADI value.

7.2.3. Alternatives for Alcoholic Pomace Extraction.

An alternative to the use of an alcoholic extraction procedure to obtain apple juice with a higher content of polyphenolic compounds, might be to heat the apple pulp before pressing using microwave. An illustration of this procedure is given by Gerard and Roberts (*4*). Fuji and McIntosh apple pulp were heated to bulk temperatures of 40 °C, 50 °C, 60 °C and 70 °C in a 2450 MHz microwave oven at 1500 W and compared to juice produced from unheated apple pulp at 21 °C. The desired temperatures were reached in 4, 7, 11 and 16 min, respectively (using 3 kg of apple pulp). Microwave heating has the advantage over conventional heating that enzymes are inactivated more quickly and that browning is minimized. Juice yield increased when pulp was microwave heated before pressing, and juice produced from the heated pulp had comparable pH, titratable acidity, and sensory characteristics to juice produced from room temperature pulps. Total phenolic and flavonoid content (determined spectrophotometrically) of the juice increased with increasing pulp temperature. Microwave heating to 60 °C was considered to be optimal, resulting in a juice with a total polyphenolic content increase of 20–40% and a flavonoid increase of ~20%.

Diffusion extraction at a higher temperature is another possibility to increase polyphenol content in apple juice. As mentioned before in Chapter 1, Spanos and co-workers (*5*) showed that diffusion extraction performed on apple pulp at elevated temperatures indeed increased flavonoid content of the juice 3–5-fold. However, the concentration of polyphenolic compounds extracted into the juice using these methods is lower compared to juice obtained by alcoholic pulp or pomace extraction procedures.

7.3. IMPLICATIONS OF THE RESULTS FOR OTHER APPLE PRODUCTS.

In the following sections possible implications of our findings for other apple products are indicated, cider and applesauce processing were taken as examples.

7.3.1. Cider Production.

Cider is a fermented apple juice, called 'hard cider' in the USA, while freshly expressed apple juice is confusingly called 'cider' (*6*). There are various types of cider, with an alcohol percentage varying between 1% (sparkling sweet cider) to 6-7% (dry cider). Cider production is interesting in relation to polyphenol contents because of two facts: apples used for cider production (cider apples) contain high concentrations of these compounds, sometimes 10-fold higher than dessert apples (*7*). Furthermore, there is alcohol present in the final juice, which favors solubility of polyphenolic compounds. Cider apples also contain a higher sugar content than dessert apples, and are lower in acid. Dessert apples are not used for cider making because they loose flavor and body during fermentation. Methods for milling and pressing apples for cider production are the same as those used in the production of juice. Fermentation of the juice can occur naturally or after application of sulfur dioxide and inoculation with a desired yeast strain. The best flavored cider is generally produced by a slow fermentation at low temperature (16 °C). Clarification methods for cider are similar to those used for apple juice. The cider can be pasteurized (*6*). The cider processing method indicates two things: during cider production apple juice is used and fermented. This juice will contain a low concentration of polyphenolic compounds, as the largest part remains bound in the pomace. Furthermore a pomace is produced, which can be used for alcoholic pomace extraction to create an extract that can be added to the cider, like described for apple juice in Chapter 5. For cider this is more practical, because there is no need to remove all the ethanol. At what stage the extract should be added to the cider needs to be established, it might be added before fermentation or afterwards. When added before, the effect of fermentation on the polyphenolic components in the extract needs to be investigated.

Comparison of Basque and French cider shows that total polyphenol content of Basque and French ciders differs notably, and is almost 8-fold higher in French ciders (*8*). This is ascribed to the cider production methods, which differ from each other. In Basque cider production a higher polyphenol oxidation of the must during maceration and long pressing steps leads to ciders with lower amounts of phenolic compounds, and it is followed by a speedy fermentation to dryness. In French cidermaking must polyphenol oxidation is minimized, and fermentation is slowed down by removing most of the yeast microflora (*8*). This indicates that in cider

production (as well as in apple juice production), processing methods play a very important role in determining the content of polyphenolic compounds in the final product.

In the aforementioned research, ciders were obtained from different cidermakers, and the apple varieties that were used were not mentioned, so it must be assumed that the mixture of apples that is used for preparation of Basque and French ciders are comparable (different varieties are used, and the variation between cider apple varieties is large (*7*, *9*)).

7.3.2. Applesauce Production.

In industrial applesauce production, apples are not peeled. Apples are chopped, and at the same time blanched to prevent discoloration (browning as a result of polyphenol oxidase). These apple pieces (included core and peel) are heated until done. Then they are passed through a sieve with holes sized ~0.8 mm. Peels and core parts don't pass the sieve (*10*, *11*). The production process for apple compote is slightly different: the apples are peeled in advance (*10*). Therefore it is expected that applesauce will contain a higher flavonol content than apple compote. In Chapter 1 it was already described that applesauce contained 50% of the quercetin (analyzed as aglycon) present in fresh apple, which is considerably higher than the concentration found in apple juice. Most probably this is due to the longer contact time with the peel (favoring solubilization from the peel), which occurs at elevated temperatures in apple sauce processing. It is expected that chlorogenic acid and total catechin content of applesauce is higher than that of apple juice as well, due to the inactivation of polyphenol oxidase upon heating.

7.4. ANTIOXIDANT ACTIVITY DETERMINATION.

In Table 1 antioxidant activity of various compounds that are present in apple and apple juice is given, measured by different methods. Quercetin, as aglycon, has the strongest antioxidant activity in the microsome assay and in the TEAC assay. When a sugar residue is attached to the aglycon, the antioxidant activity is 2–2.5 fold lower in all three assays. Cyanidin is almost 2.5 times less active than quercetin in the microsome assay, but in the TEAC test and LDL oxidation model both compounds are almost equally strong. In the microsome assay and TEAC test flavanols have a lower antioxidant activity than quercetin, but in the LDL oxidation model epicatechin

is the strongest antioxidant. Trolox and vitamin C are equally strong in the TEAC assay, while vitamin C shows almost no activity in the microsome test. Chlorogenic acid and phloridzin are powerful antioxidants according to the TEAC test (although less powerful than quercetin), but in the microsome assay both compounds show low activity. This indicates that antioxidant activity of compounds analyzed in different test systems differs considerably, and comparison of studies using different test systems should be done with great care.

Table 1. Antioxidant Activity of Some Components Present in Apple and Apple Juice

	LPO microsome assay IC_{50} (μM) (ref *12*)	in vitro oxidation model of low-density lipoproteins IC_{50} (μM) (ref *14*)	Trolox Equivalent Antioxidant Capacity TEAC (ref *15*)
flavonols:			
Q (aglycone)	9.7	0.22	4.72
Q-3-Ru	22.3	0.512	2.42
flavanols:			
catechin	15.6	n.d.	2.40
epicatechin	12.2	0.187	2.50
anthocyanidins:			
cyanidin galactoside	25.5	0.212	4.42
chlorogenic acid	123.4	0.296	1.24
phloridzin	1925.5	n.d.	2.38
vitamin C	952 (ref *13*)	n.d.	0.99
Trolox	18.0 (ref *13*)	n.d.	1.00

Increasing antioxidant activity corresponds with decreasing IC_{50} values and increasing TEAC values. n.d.: not determined.

Advantages and Disadvantages of The Microsome Assay.

To measure antioxidant activity in this project, rat liver microsomes were chosen as an oxidative system, because it is close to the *in vivo* situation where both an aqueous phase and a lipid phase are present. Microsomes are a complex and not well defined substrate (consisting of membranes with cytostolic enzymes included), and are subject to biological variability. This system with a combination of aqueous

and lipid phases provides good possibilities to serve as a very general system to measure antioxidant activity, especially of flavonoids, which mostly are not completely water-soluble antioxidants. However, other antioxidants can be measured using this system as well.

The TBARS procedure, which is used in the microsome assay to measure reaction products, is widely used, even though the reaction is not very specific. A disadvantage of the TBARS procedure is that reaction conditions have a large effect on color development (*16*). Selectivity of the TBARS procedure is improved by the use of HPLC with which reaction products can be monitored more selectively, but HPLC is more time consuming than a spectrophotometric color measurement, which increases the time needed for analyis.

Although many different methods to assess antioxidant activity exist, it seems justified to stick to one method to compare the relative effects of technological actions over the chain. It is advisable to use more assays, especially when the product of investigation contains different types of antioxidants that have distinct activities in different test systems.

7.5. BIOAVAILABILITY.

The most abundant quercetin glycosides present in apple are Q-3-Rh, Q-3-Ar, and Q-3-Ga. It appears that in an *in situ* rat intestinal perfusion model these compounds (dissolved in a potassium phosphate buffer) were hardly absorbed (*17*). Quercetin aglycon and its methylated conjugates did not appear in plasma and bile. Q-3-Gl was better absorbed, but its concentration in apple is 5–10 times lower than the other quercetin glycosides (depending on cultivar and compound). This indicates that the bioavailability of apple quercetin glycosides may be very poor. However, in this intestinal perfusion model the quercetin glycosides were not exposed to digestive juices such as saliva and pancreatic juice (*17*). The effect of these digestive juices on the absorption of the most important apple quercetin glycosides still needs to be established. Even if absorption of quercetin glycosides is very low, they may exert their protective action in the gut unabsorbed and act as a local antioxidant.

7.6. CONSEQUENCES OF CONSUMPTION OF ENRICHED APPLE JUICE.

It would be interesting to study the changes in flavonoid consumption by consumers/consumer groups as a result of putting an enriched apple juice on the

market. What happens with the total flavonoid and chlorogenic acid intake if the consumer replaces current apple juice consumption by drinking the same amount of enriched apple juice? To answer that question consumption data are needed as well as the polyphenol concentrations of the consumed products. It was decided to focus on intake from apple and apple products only, as total catechin and quercetin intake from the diet are already known for the Dutch population, as shown in the Introduction. Furthermore, in the United States, 22% of the phenolic compounds (measured by Folin-Ciocalteu reagent) consumed from fruits are from apples (*18*).

Consumption data for apple and apple products were retrieved from the Dutch National Food Consumption Survey 1998, a dietary survey carried out in 1997–1998 among a representative sample of 6200 men and women aged 1–97 year, in which consumption data were collected using a 2 day dietary record method. Results are shown in Table 2, where consumption data are given for the total population, for young children aged 1–4 years and for the group aged 5–97 years.

Table 2. Consumption Data of Apple and Apple Products from the Dutch Food Consumption Survey (in Gram per Person per Day)

product	population	mean	SD	min	max	no. of persons
Apple + peel	total	68	18	8	203	4708
	1–4 year	52	20	8	101	290
	5–97 year	69	17	13	203	4418
Apple – peel	total	48	25	1	203	1442
	1–4 year	38	19	1	80	125
	5–97 year	49	25	1	203	1317
Applesauce	total	62	35	6	284	575
can/glass	1–4 year	40	23	6	103	42
	5–97 year	63	36	10	284	533
Apple juice	total	102	52	8	450	2723
	1–4 year	86	37	24	300	177
	5–97 year	105	54	8	450	2546

The data are for apple and apple products only, in the calculations zero users (people who did not use apple products in the 2 days survey) are excluded. No distinction to gender was made in these calculations.

To describe the level and variation of flavonoid and chlorogenic acid content in apple and apple products, the following data are needed:

- variation between various apple cultivars
- variation between storage methods
- variation as a result of storage time
- variation between juice production methods

The variation in flavonoid and chlorogenic acid concentration between various apple cultivars can be derived from Table 4 presented in Chapter 3, in which data for apples with peel were presented. Data needed for apples without peel were derived from those using Table 2 from Chapter 3 in which the concentration of quercetin glycosides, phloridzin, and chlorogenic acid in Jonagold apple peel and flesh were presented. Cyanidin galactoside was assumed to be absent in apple flesh, and total catechin content in apple without peel was calculated to be 72% of total catechin content present in apple with peel, using data from Arts and coworkers (*19*).

The concentration of flavonoids and chlorogenic acid in applesauce was estimated at 50% of the concentration analyzed in apples with peel, based on data for quercetin (*20*).

Variation between storage methods and storage time is described in Chapter 3 as well, and for these calculations they were assumed to be zero. The variation between juice production methods can be derived from Chapter 4 and 5. In Table 3 the concentrations that were used in the estimation of consumption are given.

Monte Carlo simulations were used to assess dietary intake of flavonoids and chlorogenic acid from apple and apple products. Consumption data and flavonoid/chlorogenic acid concentrations of apple and apple products were assumed to be normally distributed (distributions were truncated, to omit negative values) and mean values and standard deviations as described in Table 2 and 3 were used as input for Monte Carlo simulations (1000 iterations). In these simulations the variation in consumption figures and variation in product composition can be taken into account by randomly picking consumption figures and levels for a specific product from the distributions described in Table 2 and 3. The component intake was then calculated by multiplying these randomly picked consumption figures with the randomly picked concentration of the component present in the apple product (*21*). Simulations were done using @risk software (Palisade), an add-in in Microsoft's spreadsheet Excel.

Table 3. Variation in Concentration (Milligrams per Kilogram of Fresh Weight) of Flavonoids and Chlorogenic Acid in Apple and Apple Products

	apple + peel [a]		apple – peel [b]		apple sauce [c]	
compound	mean	SD	mean	SD	mean	SD
total Q-glycosides	70	21	9	3	35	10
total catechins	169	32	122	23	84	16
phloridzin	26	14	14	8	13	7
chlorogenic acid	128	63	170	84	64	32
Cy-Ga	4	3	0	0	2	1
	conventional apple juice [d]		enriched apple juice [e]			
compound	mean	SD	mean	SD		
total Q-glycosides	9	3	92	24		
total catechins	18	14	59	28		
phloridzin	4	1	29	7		
chlorogenic acid	66	60	102	67		
Cy-Ga	3	1	6	2		

[a] Average from 4 cultivars, data from Chapter 3, Table 4. [b] Average from 4 cultivars, data derived from apple+peel and Chapter 3, Table 2. [c] Estimated to be 50% of the values analyzed in apples with peel (*20*). [d] Average from 3 cultivars, juices obtained by straight pressing, data from Chapter 4, Table 2. [e] Average from 3 cultivars, data from Chapter 5, Table 2.

Two scenarios were compared: scenario 1 in which conventional apple juice is consumed and scenario 2 in which the same amount of apple juice is replaced by enriched apple juice. In both cases intake of flavonoids and chlorogenic acid from apple and apple sauce consumption were included in the estimations. The results for mean intakes are presented in Figure 2.

Figure 2a clearly shows that replacing conventional apple juice consumption by the consumption of the enriched juice has the largest effect on quercetin glycoside intake. A twofold increase is expected to result from it. For the other components smaller increases in daily intake figures are estimated. The same was observed for all three age groups (Figure 2b and 2c).

The minor increase in total catechin and chlorogenic acid intake was expected, as these compounds are present in the apple flesh, which gives a higher intake of these compounds from peeled apples.

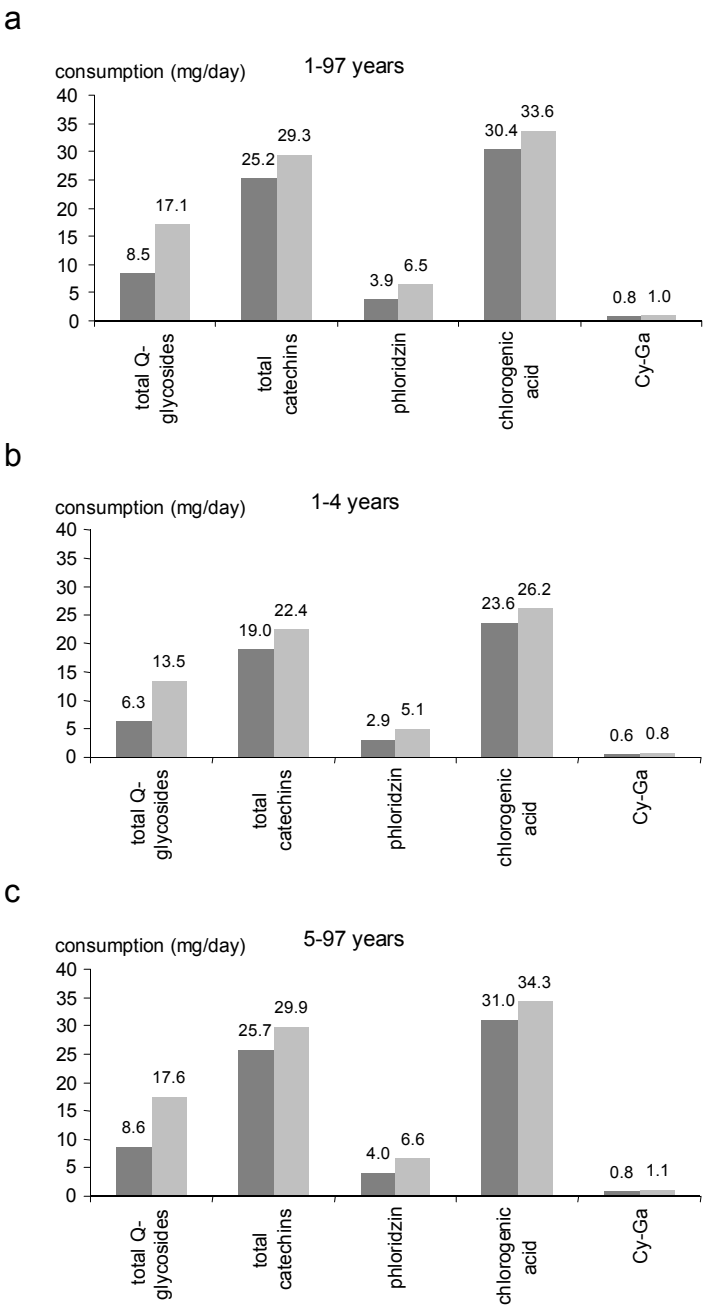

Figure 2. Daily consumption of flavonoids and chlorogenic acid (in mg) from apple (with or without peel), applesauce, and apple juice for 3 different age groups (a=1–97 years, b=1–4 years, c=5–97 years). Dark gray: scenario 1: conventional apple juice is consumed. Gray: scenario 2: conventional apple juice is replaced by consumption of the same amount of enriched apple juice.

With the Monte Carlo simulations it is also possible to determine which part of a population is at "risk" to consume less than a certain amount of a specific component. Presently, no optimal or minimum intake levels are known for polyphenolic compounds, so a boundary was arbitrarily chosen as 25% of the daily intake already described in literature. Daily intakes are ~25 mg/day for flavonols and flavons (*22*)

and 50 mg/day for total catechins (*23*). The flavonol and flavon intake estimate is based on 4112 Dutch adults, aged 19–74 (*22*). The catechin intake estimate is based on the same study population as used in our calculations (6200 men and women aged 1–97 year) (*23*). Calculations were performed only for total quercetin glycosides and total catechins, as total and reference intake data for chlorogenic acid, phloridzin and cyanidin galactoside are inconclusive or absent. In Table 4 the "risk" assessment for the three consumer groups in the two scenarios are presented.

Table 4. Percentage of Consumers that Fall Below a Given Boundary (Intake of Compound from Apples, Apple Sauce, and Apple Juice in Milligrams per Day)

	consumer group	scenario 1 [a]	scenario 2 [b]
total Q-glycosides; boundary = 6.25 mg/day	total (1–97 years old)	20.1%	1.0%
	1–4 years old	51.4%	4.1%
	5–97 years old	16.7%	0.7%
total catechins; boundary = 12.5 mg/day	total (1–97 years old)	1.1%	0.4%
	1–4 years old	10.1%	3.2%
	5–97 years old	0.6%	0.4%

[a] conventional apple juice is consumed. [b] conventional apple juice is replaced by consumption of the same amount of enriched apple juice.

Table 4 also shows that replacing conventional apple juice by enriched juice would have the largest effect on quercetin glycoside intake. The percentage of consumers that fall below the chosen intake boundary is substantially lower in the case that enriched apple juice is consumed. This was observed for the total population as well as for small children. For total catechins the reduction of the percentage of consumers that fall below the chosen intake boundary is only small, as can be expected, because intake levels (Figure 2) did not increase much as a result of replacing conventional apple juice by enriched juice. A similar analysis can be made for potential 'overconsumption' of compounds.

The above indicates that it is technically possible to put an enriched apple juice on the market as a replacement for conventional apple juice. The major effect is on the intake of quercetin glycosides, daily consumption of these compounds from apple and apple products will be doubled, but as a result, the increase in quercetin

glycoside intake from the diet will be less than doubled. There are people who consume higher daily doses of quercetin glycosides from supplements, without adverse effects. But as at present, no recommended daily intakes are available for polyphenolic compounds, and intake levels above which adverse effects might occur are unknown as well, care should be taken.

Although minimum/optimal intake levels are missing, the possibilities and restrictions of a procedure to obtain an enriched product and how to evaluate the effects on dietary intake from polyphenolic compounds present in such a product, is illustrated here.

7.7. RECOMMENDATIONS FOR FURTHER RESEARCH.

- As polyphenolic compounds are structurally very heterogeneous, and most probably not all polyphenolic compounds are identified yet, application of advanced metabolomic techniques offers the potential to increase knowledge in polyphenol research. The use of mass spectrometers coupled to GC or LC equipment makes it possible to measure and identify polyphenolic compounds and their metabolites with more selectivity and more sensitivity. When analytical methods are improved, better food composition tables can be produced, which are indispensable in epidemiological research. Also human studies regarding bioavailability will benefit from better analytical procedures. The above-mentioned can lead to an increased understanding of the relationships between dietary intake of polyphenolic compounds and human health.
- To understand the role of dietary polyphenols in human health it is important to identify and measure the physiological polyphenol conjugates. Once these are known it is possible to use in-vitro models for further investigation of physiologically relevant flavonoids and their conjugates at appropriate concentrations (*24*). This is needed because of the limitations imposed on sampling in humans. Also investigation of suitable animal models to determine the relationships between blood and tissue levels of antioxidants is needed. Furthermore, knowledge about the metabolism, and identification of metabolites, is vital to understanding mechanisms of bioactivity and ultimately identification of active compounds (*25*).

- More research is needed on how storage conditions, industrial food processing and preparation at home, influence the concentration, release and uptake of polyphenolic antioxidants from the food matrix.
- Because there are innumerable amounts of in vitro antioxidant activity assays, there is a need to make a selection of tests. This should be based on an understanding of the mechanisms involved, and the purpose of the research. Some of the assays are done at non-physiological pH values, in that case it is impossible to extrapolate the results to a physiological environment.

  The consensus of opinion is that a mix of these tools should be used in assessing the antioxidant activities in vitro. Measurements of lipid peroxidation using rat liver or cardiac microsomes, ox-brain phopholipid liposomes, arachidonic acid, and other lipid model systems (e.g. bulk oil and emulsified oil systems) should be the first line of tests to establish the potential antioxidant action of dietary antioxidant compounds (*26*).

All this information needs to be used to identify optimal levels of antioxidants, such as polyphenolic compounds, present in foods, based on an evaluation of benefits and potential risks (*25*). When optimal dose levels are available and recommended daily intakes can be given and also relations between human health and polyphenolic intake are clearer, the food industry can focus on the development of functional foods containing these compounds. This will require knowledge on how dietary sources can be modified to improve the availability and delivery of beneficial compounds. In this thesis an example was presented on how it is possible to increase polyphenolic content and antioxidant activity of apple juice.

7.8. ACKNOWLEDGMENT

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7.9. LITERATURE CITED

1. Guyot, S.; Le Bourvellec, C.; Marnet, N.; Drilleau, J. F. Procyanidins are the most abundant polyphenols in dessert apples at maturity. *Lebensm. –Wiss. u. –Technol*. **2002**, *35*, 289–291.
2. Haque, M. R.; Bradbury, J. H. Total cyanide determination of plants and foods using the picrate and acid hydrolysis methods. *Food Chem*. **2002**, *77*, 107–114.

3. Van Heijst, A. N. P. *Cyanide: Acceptable Daily Intake (ADI) and Other Guideline Levels*, **1997**. retrieved from the Web April 11, 2005; http://www.inchem.org/documents/pims/chemical/pimg003.htm

4. Gerard, K. A.; Roberts, J. S. Microwave heating of apple mash to improve Juice yield and quality. *Lebensm. –Wiss. u. –Technol*. **2004**, *37*, 551–557.

5. Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of processing and storage on the phenolic composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572–1579.

6. Downing, D. L. Apple cider. In: *Processed apple products*. Downing, D. L. (ed.). AVI (Van Nostrand Rheinhold), New York, **1989**, 169–188.

7. Sanoner, P.; Guyot, S.; Marnet, N.; Molle, D.; Drilleau, J. F. Polyphenol profiles of French cider apple varieties (*Malus domestica* sp.). *J. Agric. Food Chem*. **1999**, *47*, 4847–4853.

8. Alonso Salces, R. M.; Guyot, S.; Herrero, C.; Berrueta, L. A.; Drilleau, J. F.; Gallo, B.; Vicente, F. Chemometric characterisation of Basque and French ciders according to their polyphenolic profiles. *Anal. Bioanal. Chem.* **2004**, *379*, 464–475.

9. Alonso Salces, R. M.; Korta, E. .; Barranco, A.; Berrueta, L. A.; Gallo, B.; Vicente, F. Determination of polyphenolic profiles of basque cider apple varieties using accelerated solvent extraction. *J. Agric. Food Chem*. **2001**, *49*, 3761–3767.

10. Personal communication: Schijvens, E. Agrotechnology and Food Innovations BV, Wageningen University and Research Center, **2005**.

11. Wiley, R. C.; Binkley, C. R. Applesauce and other canned apple products. In: *Processed apple products*. Downing, D. L. (ed.). AVI (Van Nostrand Rheinhold), New York, **1989**, 215–238.

12. Van der Sluis, A. A.; Dekker, M.; Verkerk, R.; Jongen W. M. F. An improved, rapid in vitro method to measure antioxidant activity; application on selected flavonoids and apple juice. *J. Agric. Food Chem*. **2000**, *48*, 4116–4122.

13. Wilborts, M. *Evaluation of different antioxidant activity assays*. MSc Thesis, Wageningen University. **2003**, 39p.

14. Vinson, J. A.; Jang, J.; Dabbagh, Y. A.; Serry, M. M.; Cai, S. Plant polyphenols exhibit lipoprotein-bound antioxidant activity using an in vitro oxidation model for heart disease. *J. Agric. Food Chem*. **1995c**, *43*, 2798–2799

15. Miller, N. J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem*. **1995**, *43*, 1794–1801.

16. Antolovich, M.; Prenzler, P. D.;Patsalides, E.; McDonald, S.; Robards, K. Methods for testing antioxidant activity. *Analyst* **2002**, *127*, 183–198.

17. Arts, I. C. W.; Sesink, A. L. A.; Faassen Peters, M.; Hollman, P. C. H. The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent biliary excretion of dietary quercetin glycosides. *Brit. J Nutr.* **2004**, *91*, 841–847.

18. Vinson, J. A.; Su, X. H.; Zubik, L.; Bose, P. Phenol antioxidant quantity and quality in foods: Fruits. *J. Agric. Food Chem*. **2001**, 49, 5315–5321.

19. Arts, I. C. W.; van de Putte, B.; Hollman, P. C. H. Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, Vegetables, Staple foods, and processed foods. *J. Agric. Food Chem*. **2000**, *48*, 1746–1751.

20. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J. Agric. Food Chem*. **1992**, *40*, 2379-2383.

21. Dekker, M.; Verkerk. R. Dealing with variability in food production chains: a tool to enhance the sensitivity of epidemiological studies on phytochemicals. *Eur. J. Nutr.* **2003**, *42*, 67–72.

22. Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutr. Cancer* **1993b**, *20*, 21–29.

23. Arts, I. C. W.; Hollman, P. C. H.; Feskens, E. J. M.; Bueno de Mesquita, H. B.; Kromhout, D. Catechin intake and associated dietary lifestyle factors in a representative sample of Dutch men and women. *Eur. J. Clin. Nutr.* **2001**, *55*, 76–81.

24. Kroon, P. A.; Clifford, M. N.; Crozier, A.; Day, A. J.; Donovan, J. L.; Manach, C.; Williamson, G. How should we assess the effects of exposure to dietary polyphenols in vitro? *Am. J. Clin. Nutr*. **2004**, *80*, 15–21.

25. Stahl, W.; van den Berg, H.; Arthur, J.; Bast, A.; Dainty, J.; Faulks, R. M.; Gärtner, C.; Haenen, G.; Hollman, P.; Holst, B.; Kelly, F. J.; Polidori, M. C.; Rice-Evans, C.; Southon, S.; van Vliet, T.; Viña-Ribes, J.; Williamson, G.; Astley. S. B. Bioavailability and metabolism. *Mol. Aspects Med.* **2002**, *23*, 39–100.

26. Aruoma, O. I. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat. Res.-Fund. Mol. M.* **2003**, *Si*, 9–20.

SUMMARY

In the last years a growing interest exists for potential health-protecting compounds present in our food. Formerly these compounds have been regarded as non-nutrients, components that hardly contribute to the nutritive value of the food product. Especially secondary plant metabolites are of interest. From epidemiological research it appeared that these compounds might play a protective role in aging diseases, such as coronary heart diseases and cancers. The protective mechanism is not clear, but a possible explanation is through their antioxidant activity (quenching of harmful radicals in the human body) or the stimulation of naturally present protection mechanisms (e.g. detoxification enzymes or the immune system). For that reason consumers are becoming more interested in food products with high levels of health-protecting compounds. Knowledge about the influence of various steps occurring in the production chain (cultivation, breeding, industrial processing, distribution, storage and processing by the consumer) on such products is needed. This thesis describes the process of acquiring, processing and applying such knowledge with a product-oriented approach, using polyphenolic antioxidants from apples as an example. Polyphenolic compounds are present in apples, and contribute to color and taste. One of the properties of these compounds that are interesting for human health is their antioxidant function. Therefore, the aim of the research described in this thesis is to investigate the apple production chain (harvest, storage, processing and further storage of the product), with a focus on the effects on the level of polyphenolic compounds and antioxidant activity.

Introductory Chapter 1 provides an overview of polyphenolic compounds, their biochemical properties, their occurrence in apples, and aspects regarding cultivation, storage and processing into apple juice are described. Furthermore, the possible relation between polyphenolic compounds and health is dealt with. Polyphenolic intake from the diet is estimated on 1 g/day and in general, after the consumption of 10–100 mg of a phenolic compound, the maximum concentration observed in human plasma is about 1 μM. For polyphenolic compounds and flavonoids optimal plasma levels are still lacking and no data are available with respect to recommended daily intake.

A method to measure antioxidant activity *in vitro*, using rat liver microsomes, was optimized in order to be able to analyze large amounts of samples (Chapter 2). Antioxidant activity of pure compounds as well as of extracts of apple products was determined. The quercetin aglycon was a stronger antioxidant than its glycosides in the test used. Apple polyphenol antioxidant activity ranked in the following order: Q > Q-3-xyloside ≈ epicatechin > catechin ≈ Q-3-galactoside ≈ Q-3-glucoside ≈ Q-3-arabinoside ≈ Q-3-rhamnoside > Q-3-rutinoside > cyanidin galactoside >> chlorogenic acid >>> phloridzin. When testing apple juice to determine its antioxidant activity, no interference of the sugars present in the juice matrix was observed using this optimized assay.

Polyphenol concentrations differed greatly between apple varieties and apple tissues (Chapter 3). Four apple cultivars (Jonagold, Golden Delicious, Cox's Orange, and Elstar), which can be used as fresh apples or in processed apple products, were compared. Of these cultivars Jonagold possessed the highest flavonoid concentration and the highest antioxidant activity. With respect to antioxidant activity the other cultivars ranked Elstar > Cox's Orange > Golden Delicious. To study seasonal differences, apples from three different harvest years were analyzed, but in three out of four cultivars no effect on flavonoid concentration was observed, only in Cox's Orange significant differences were observed between the 5 and 10% levels. No seasonal effect on antioxidant activity was observed. Furthermore, the effect of long-term storage, both at refrigerator temperature and under controlled atmosphere conditions (CA) was assessed, and in both storage procedures polyphenolic compound concentrations remained quite stable, only slight differences between individual compounds were observed. For example, in Jonagold apples levels of total quercetin glycosides, phloridzin, and cyanidin galactoside were stable, but chlorogenic acid and total catechin concentrations decreases were significant. The observed decreases were not very high (18 and 40%, respectively), and after 52 weeks of storage, still substantial amounts were present. Antioxidant activity of the four cultivars remained stable during storage.

In conventional apple juice production most of the polyphenolic compounds appeared to be lost (Chapter 4). Jonagold pulp enzyming (2h at room temperature, with stirring) caused loss of phloridzin, chlorogenic acid, and total catechins, however no loss of cyanidin galactoside and total Q-glycosides was observed. Polyphenolic compounds predominantly remained in the pomace, and in the juice only 50% of the chlorogenic

acid and 3% of the catechins was detected. Chlorogenic acid was the predominant polyphenol of the ones analyzed that was found in apple juice, as it is the most water soluble. Antioxidant activity of conventional apple juice was only 3–10% of that of the apples it was originating from. Mass balances were used at various points in the juice making process to identify the most important processing steps that affect the concentrations of polyphenolic compounds contributing to the antioxidant activity of the product. It revealed that, besides the partitioning phenomenon that caused losses of the antioxidant compounds during the production, another process, most probably oxidation (especially of the catechins), resulted in considerable losses during the pressing step. Three apple cultivars (Jonagold, Golden Delicious, and Elstar) were compared as raw material used for the processing, and for all three varieties convential processing had similar effects. Antioxidant activity analysis showed that about 35% of the antioxidant activity of fresh apples, pulp, and pomace and 45% of the activity of the raw juice could be ascribed to the analyzed components. The contribution of analyzed compounds to the measured antioxidant activity of fresh Jonagold apple, apple pulp, and pomace was total catechins > quercetin glycosides > chlorogenic acid > cyanidin galactoside >> phloridzin. In the raw juices, the contribution was total catechins > chlorogenic acid > quercetin glycosides > cyanidin galactoside >> phloridzin. It was hypothesized that the remainder of the apple antioxidant activity can be ascribed to procyanidins, vitamins, and carotenoids (not analyzed) that contribute to apple antioxidant activity as well.

To produce a ‘healthier’ apple juice, with a higher polyphenol content and a higher antioxidant activity, it is important to use the obtained knowledge about the influence of the chosen cultivar, cultivation methods, and processing methods on the final product, not losing sight of regular quality aspects, such as taste and color. A novel production process was developed in order to produce an apple juice with an enhanced concentration of polyphenolic compounds (Chapter 5). This was achieved by applying an alcoholic extraction on the pulp or on the pomace. An extract containing high levels of polyphenols was obtained, which could be added to the regular apple juice. In such an enriched apple juice more than 50% of the antioxidant activity present in fresh apples was found. Antioxidant activity of enriched juice was 5 times higher than that of conventional apple juice The levels of flavonoids and chlorogenic acid in enriched juice were between 1.4 (chlorogenic acid) and 9 (quercetin glycosides) times higher than in conventional apple juice. The novel

processing method had similar effects for three apple cultivars tested (Elstar, Golden Delicious, and Jonagold), furthermore for all three cultivars enriched juice yield was at least 2 times higher than conventionally (non-enzymatically) produced juice yield. The taste and color of enriched juice were different from those of conventional juice.

Apple juice, manufactured by one of the previous described methods, can be further processed before it reaches the consumer. Treatments are for example clarification/filtration, concentration, pasteurization, and packaging. The effect of heat treatments and storage conditions (temperature and oxygen concentration) on the stability of polyphenolic compounds present in enriched apple juice and the antioxidant activity is described and modeled in Chapter 6. The most (thermally) sensitive compounds were the various quercetin glycosides and epicatechin, whereas phloridzin and chlorogenic acid were more stable. Accelerated shelf-life testing of enriched apple juice during 4 days at 80 °C showed a decrease in antioxidant activity of 20–40%. It was shown for the first time that at elevated temperatures quercetin glycosides were hydrolyzed and that quercetin aglycon appeared in apple juice. The formed quercetin aglycon was not stable but was further degraded. Model predictions showed that polyphenolic antioxidant concentrations and antioxidant activity of enriched apple juice should remain quite stable at ambient or refrigerated storage conditions up to half a year.

Chapter 7 provides a general discussion of the findings. It was hypothesized that the novel processing method can be applied during cider production as well, because of the fact that in cider production apple juice is used and fermented and a pomace is produced. Monte Carlo simulations were used to assess dietary intake of flavonoids and chlorogenic acid from apple and apple products. The changes in flavonoid intake as a result of replacing normal apple juice by enriched apple juice were estimated with this method. Two scenarios were compared: scenario 1 in which conventional apple juice is consumed and scenario 2 in which the same amount of apple juice is replaced by enriched apple juice. In both cases intake of flavonoids and chlorogenic acid from apple and apple sauce consumption were included in the estimations. Replacing conventional apple juice consumption by the consumption of the enriched juice would have the largest effect on quercetin glycoside intake. A twofold increase is expected to result from it (from 9 to 18 mg/day). For the other components smaller increases in daily intake figures were estimated.

Although minimum or optimal intake levels are missing, the possibilities and restrictions of a procedure to obtain an enriched product and how to evaluate the effects on dietary intake from polyphenolic compounds present in such a product, is illustrated. Also the importance of knowledge about the relation between an industrial raw material and processing technology as a source for a successful implementation of product optimization from a chain perspective was shown. Summarizing it can be concluded that the research objectives were reached: the main effect of the production chain on polyphenolic antioxidants occurs during conventional processing into juice, where more than 90% of the antioxidant activity is lost, and a process yielding a product with a higher polyphenolic content as well as a higher antioxidant activity was developed.

Knowledge on what happens to polyphenolic compounds in the production chain can be used in improved estimations of dietary intakes, which is needed to establish possible associations between fruits and vegetables, the polyphenols they contain, and the occurrence of aging diseases.

SAMENVATTING

De laatste jaren bestaat er een groeiende belangstelling voor de mogelijke gezondheidsbeschermende werking van stoffen in ons voedsel. Deze stoffen werden voorheen beschouwd als ‘non-nutriënten’, dit zijn stoffen die nauwelijks tot niet bijdragen aan de voedingswaarde van het product. Het betreft hier met name secundaire plantenmetabolieten. Uit bevolkingsonderzoek is naar voren gekomen dat deze verbindingen van belang kunnen zijn bij het verkleinen van de kans op het krijgen van verouderingsziekten, zoals hart- en vaatziekten en kanker. Het werkingsmechanisme is nog niet duidelijk, maar een mogelijke verklaring ligt in hun antioxidantactiviteit (het wegvangen van schadelijke radicalen in het lichaam) of via stimulering van natuurlijke beschermingsmechanismen (bijvoorbeeld ontgiftigingsenzymen of het immuunsysteem). Daarom staan levensmiddelen met hoge gehalten aan gezondheidsbeschermende stoffen steeds meer in de belangstelling van de consument. De productie van dergelijke voedingsmiddelen vereist kennis over de invloed van processen in de hele productieketen van een voedingsmiddel: teelt, veredeling, industriële verwerking, distributie, opslag en consumentenverwerking. Dit proefschrift beschrijft het proces van kennisverwerving, -verwerking en toepassing vanuit een productgerichte benadering, en wel aan de hand van polyfenolische antioxidanten in appels. Polyfenolen komen van nature in appels voor en dragen bij aan de kleur en de smaak ervan. Deze verbindingen zijn voor de gezondheid interessant, ondermeer vanwege hun antioxidatieve eigenschappen.

Het doel van het onderzoek dat beschreven is in dit proefschrift is het onderzoeken van de appelproductie en –verwerkingsketen (oogst, opslag, verwerking en verdere opslag van het product), met een focus op de gevolgen ervan op de gehaltes aan polyfenolen en de antioxidantactiviteiten van de producten.

Ter inleiding geeft Hoofdstuk 1 een overzicht van polyfenolische verbindingen, hun biochemische eigenschappen, hun voorkomen in appels en ook andere aspecten betreffende teelt, opslag en de verwerking naar appelsap worden beschreven. Verder wordt er aandacht besteed aan de mogelijke relatie tussen polyfenolen en gezondheid. De inname van polyfenolische verbindingen uit het dieet wordt geschat op 1 g/dag en over het algemeen is na consumptie van 10–100 mg van een

polyfenolische verbinding de maximum concentratie die wordt waargenomen in menselijk plasma ongeveer 1 µM. Optimale plasmaniveaus voor polyfenolen en flavonoïden zijn nog niet beschikbaar en er zijn ook geen gegevens bekend over aanbevolen dagelijkse inname van deze stoffen.

Een methode om antioxidantactiviteit *in vitro* te meten (met behulp van rattelever microsomen) is geoptimaliseerd om grote aantallen monsters te kunnen analyseren (Hoofdstuk 2). De antioxidantactiviteiten van standaardverbindingen en van extracten van appelproducten zijn bepaald. Het quercetine aglycon was een sterkere antioxidant dan zijn overeenkomstige glycosiden in de gebruikte methode. De antioxidantactiviteit van polyfenolen aanwezig in appels vertoonde de volgende rangorde: Q > Q-3-xyloside ≈ epicatechine > catechine ≈ Q-3-galactoside ≈ Q-3-glucoside ≈ Q-3-arabinoside ≈ Q-3-rhamnoside > Q-3-rutinoside > cyanidine galactoside >> chlorogeenzuur >>> phloridzine. Wanneer de antioxidantactiviteit van appelsap werd vastgesteld m.b.v. deze methode, werd er geen interferentie van de suikers die in het sap aanwezig zijn waargenomen.

Concentraties van polyfenolen verschilden aanzienlijk tussen appelrassen en appelweefsels (Hoofdstuk 3). Vier appelrassen (Jonagold, Golden Delicious, Cox's Orange, en Elstar), die als vers product gegeten kunnen worden of die kunnen worden verwerkt tot appelproducten werden vergeleken. Van deze rassen had Jonagold het hoogste flavonoïdengehalte en de hoogste antioxidantactiviteit. De antioxidantactiviteit van de andere drie rassen nam af in de volgorde: Elstar > Cox's Orange > Golden Delicious. Om het effect van seizoensinvloeden te bestuderen zijn appels van drie verschillende oogstjaren vergeleken, maar bij drie van de vier rassen werd er geen verschil in flavonoïdenconcentratie waargenomen, alleen in Cox's Orange waren er significante verschillen waarneembaar. Op de antioxidantactiviteit waren geen seizoensinvloeden zichtbaar. Verder is het effect van langdurige bewaring, zowel bij koelkasttemperatuur als onder 'controlled atmosphere' (CA) condities onderzocht, en bij beide opslagprocedures bleven de polyfenolgehalten tamelijk stabiel, slechts kleine verschillen tussen individuele componenten werden waargenomen: b.v. in Jonagold appels waren de gehalten totaal quercetineglycosiden, phloridzine, en cyanidine galactoside stabiel, echter de afnames in de gehalten chlorogeenzuur en totaal catechine waren significant. De waargenomen afnames waren niet erg hoog (respectievelijk 18 en 40%), en na 52

weken opslag waren er nog steeds aanzienlijke hoeveelheden aanwezig. De antioxidantactiviteit van de vier rassen bleef stabiel gedurende de opslagperiode.

Bij conventionele appelsapbereiding bleek dat het grootste deel van de polyfenolen verloren ging (Hoofdstuk 4). Pulpenzymering van Jonagold appels (2 uur bij kamertemperatuur, met roeren) veroorzaakte een verlies aan phloridzine, chlorogeenzuur en totaal catechine, echter er werd geen verlies aan cyanidine galactoside en totaal quercetineglycosiden waargenomen. De polyfenolen bleven voornamelijk in de perskoek achter en in het sap werd slechts 50% van het chlorogeenzuur en 3% van de catechines teruggevonden. Van de geanalyseerde polyfenolen in appelsap was chlorogeenzuur in de hoogste concentraties aanwezig, het is ook het best wateroplosbare. De antioxidantactiviteit van conventioneel bereid appelsap bedroeg slechts 3–10% van die van de appels waaruit het bereid was. Massabalansen zijn gebruikt op verschillende plaatsen in het sapbereidingsproces om de belangrijkste verwerkingsstappen te identificeren die van invloed zijn op de gehalten aan polyfenolen die bijdragen aan de antioxidantactiviteit van het uiteindelijke product. Het bleek dat naast het partitiefenomeen, wat flinke verliezen aan antioxidantverbindingen tijdens de verwerking veroorzaakte, ook een ander proces, waarschijnlijk oxidatie (vooral van de catechines), resulteerde in aanzienlijke verliezen tijdens de persstap. Drie appelrassen (Jonagold, Golden Delicious, en Elstar) werden vergeleken als grondstof voor de verwerking, en bij alle drie de rassen had conventionele verwerking overeenkomstige gevolgen. Analyse van de antioxidantactiviteit liet zien dat ongeveer 35% van de antioxidantactiviteit van verse appels, pulp, en perskoek en 45% van de activiteit van het ruwe sap kon worden toegeschreven aan de geanalyseerde componenten. De bijdragen van de geanalyseerde componenten aan de gemeten antioxidantactiviteit van verse Jonagold appels, appelpulp, en perskoek was totaal catechines > totaal quercetineglycosiden > chlorogeenzuur > cyanidine galactoside >> phloridzine. In de ruwe sappen was de bijdrage: totaal catechines > chlorogeenzuur > totaal quercetineglycosiden > cyanidine galactoside >> phloridzine. De hypothese werd opgesteld dat de rest van de antioxidantactiviteit van appels kan worden toegeschreven aan procyanidinen, vitaminen, en carotenoïden (niet geanalyseerd) die ook aan de antioxidantactiviteit van appels kunnen bijdragen.

Om een ‘gezonder’ appelsap te kunnen produceren, met een hoger polyfenolgehalte en een hogere antioxidantactiviteit is het belangrijk om de verworven kennis op het

gebied van de invloed van ras, teelt- en verwerkingsmethoden op het uiteindelijke product te gebruiken, terwijl tegelijkertijd reguliere kwaliteitsaspecten (zoals smaak en kleur) niet uit het oog worden verloren. Een nieuw productieproces is ontwikkeld met als doel een appelsap te produceren met een verhoogd polyfenolgehalte (Hoofdstuk 5). Dit is bereikt door het toepassen van een alcoholische extractie op de pulp of op de perskoek. Er werd een extract verkregen dat hoge gehalten aan polyfenolen bevatte en dat kon worden toegevoegd aan het conventioneel gewonnen appelsap. In zo'n verrijkt appelsap werd meer dan 50% van de antioxidantactiviteit aanwezig in verse appels aangetoond. De antioxidantactiviteit van verrijkt sap was 5 maal hoger dan die van conventioneel bereid appelsap. In verrijkt sap waren de gehalten aan flavonoïden en chlorogeenzuur tussen de 1,4 (chlorogeenzuur) en 9 (quercetineglycosiden) maal hoger dan in conventioneel bereid appelsap. De nieuwe verwerkingsmethode had overeenkomstige resultaten voor drie onderzochte rassen (Elstar, Golden Delicious, en Jonagold). Bovendien was bij alle drie de rassen de sapopbrengst van verrijkt sap ten minste 2 maal hoger dan de sapopbrengst van conventioneel (niet-enzymatisch) geproduceerd sap. De smaak en de kleur van verrijkt sap verschilden van die van conventioneel geproduceerd sap.

Appelsap dat met een van de eerder beschreven methoden is gemaakt, kan verder worden verwerkt, voordat het door de consument genuttigd wordt. De volgende behandelingen zijn mogelijk: clarificatie/filtratie, concentratie, pasteurisatie, en verpakking. Het effect van warmtebehandelingen en opslagcondities (temperatuur en zuurstofconcentratie) op de stabiliteit van polyfenolische verbindingen die aanwezig zijn in verrijkt appelsap en de antioxidantactiviteit wordt beschreven en gemodelleerd in Hoofdstuk 6. De meest temperatuurgevoelige verbindingen waren de quercetineglycosiden en epicatechine, terwijl phloridzine en chlorogeenzuur stabieler waren. Versneld houdbaarheidsonderzoek van verrijkt appelsap gedurende 4 dagen bij 80 °C verlaagde de antioxidantactiviteit met 20–40%. Voor het eerst werd aangetoond dat bij verhoogde temperatuur quercetineglycosiden werden gehydrolyseerd en dat quercetine aglycon verscheen in appelsap. Het gevormde quercetine aglycon was niet stabiel maar werd verder afgebroken. Modelvoorspellingen lieten zien dat de concentraties aan polyfenolische antioxidanten en de antioxidantactiviteit van verrijkt appelsap redelijk stabiel zullen blijven bij kamertemperatuur of gekoelde opslag gedurende een half jaar.

Hoofdstuk 7 levert een algemene discussie van de bevindingen. Het is te verwachten dat de nieuwe verwerkingsmethode ook in ciderproductie kan worden toegepast, omdat in ciderproductie appelsap gefermenteerd wordt en er een perskoek wordt geproduceerd. Monte Carlo simulaties werden toegepast om een schatting te maken van de dagelijkse inname van flavonoïden en chlorogeenzuur uit appels en appelproducten. De veranderingen in flavonoïdeninname die op kan treden als gevolg van vervanging van conventioneel appelsap door verrijkt appelsap werden geschat met deze methode. Twee scenario's werden vergeleken: scenario 1 waarin conventioneel appelsap wordt geconsumeerd en scenario 2 waarin dezelfde hoeveelheid appelsap wordt vervangen door verrijkt sap. In beide gevallen werd de inname van flavonoïden en chlorogeenzuur uit appel en appelsap meegenomen in de schattingen. Vervanging van conventioneel appelsap door de consumptie van verrijkt appelsap zou het grootste effect hebben op de quercetineglycosideninname. Deze inname werd geschat twee keer zo hoog te worden (en ging van 9 naar 18 mg/dag). Voor de andere componenten werden kleinere toenames geschat wat betreft de dagelijkse inname.

Hoewel minimum- of optimale innameniveaus niet beschikbaar zijn, werden de mogelijkheden en beperkingen van een methode om een verrijkt sap te verkrijgen geïllustreerd, evenals een manier om de gevolgen te evalueren op de dagelijkse inname van polyfenolen die aanwezig zijn in zo'n product. Bovendien is het belang van kennis over de relatie tussen een industriële grondstof en verwerkingstechnologie als bron van succesvolle implementatie van productoptimalisatie vanuit ketenperspectief aangetoond. Samengevat kan geconcludeerd worden dat de onderzoeksdoelstellingen gehaald werden: het belangrijkste effect van de productieketen op polyfenolische antioxidanten lag in de conventionele verwerking tot sap, waarbij meer dan 90% van de antioxidantactiviteit verloren ging, en een methode waarbij een product werd verkregen met een hoger polyfenolgehalte en een hogere antioxidantactiviteit was ontwikkeld.

Kennis over hetgeen gebeurt met polyfenolische verbindingen in de productieketen kan gebruikt worden voor verbeterde schattingen van dagelijkse inname ervan, wat nodig is om eventuele associaties tussen de polyfenolgehalten in groenten en fruit en het voorkomen van verouderingsziekten aan te kunnen tonen.

NAWOORD

Hier ligt het dan eindelijk, mijn proefschrift. Vier jaren waarin ik met veel plezier heel veel materiaal heb verzameld, volgden door een lange periode worstelen om de resultaten netjes op papier te krijgen, zodat de ‘rest van de wereld’ ook kennis kon nemen van de resultaten.

Veel mensen hebben aan de tot stand koming bijgedragen en hen wil ik op deze plek bedanken.

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During this PhD-research I spent two periods at other laboratories, and I have very good memories of that. Gary Williamson, thanks for the opportunity to visit your laboratory at the Institute of Food Research, Norwich, United Kingdom. One week I was able to test there four different methods for measuring antioxidant activity. I found it very impressive to be surrounded by so many flavonoid specialists, and that all my questions about sampling, sample storage, extraction, HPLC-analysis and antioxidant activity, were answered by different persons.

Furthermore I would like to thank Grete Skrede for the opportunity to work for six weeks at Matforsk, Ås, Norway. Now it was possible to see that apple juice with a higher antioxidant activity and a larger content of flavonoids and chlorogenic acid could be produced at pilot plant scale. Two papers are based on the work that was

performed here. I still remember the midwinter blue sky's, with lots of sun shining on the white snow. Your company and that of your collegues and our discussions were very much appreciated.
Heel veel studenten hebben in het kader van hun afstudeervak bijgedragen aan dit onderzoek en ik wil ze daarvoor hartelijk bedanken. Natasja Essed, Andrea Mertens, Maud Vissers, Cissy Warmerdam, Francoise van Heeswijk, Margot van Soelen, Marjon Matser, René Monderen, Annemarie Renkens, Sanne Hoogerwerf, Katrien van Scherpenzeel en Dorine Molenaar, ik vond het heel leuk om met jullie samen te werken. Jullie waren allemaal erg enthousiast om aan een 'echt' product te werken.
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Lieve Edwin, eindelijk is het dan zo ver. De lange jaren waarin het proefschrift altijd wel in mijn achterhoofd speelde, zijn voorbij. Dank je wel dat je al die tijd achter me bent blijven staan, de ene keer met meer, de andere keer met minder geduld. Jouw relativeringsvermogen is steeds verfrissend geweest. Vanaf nu is vrije tijd weer echt vrije tijd en ik hoop dat we daar samen met onze wondertjes Sybe, Marijn en Wessel heerlijk van zullen genieten.

CURRICULUM VITAE

Adriënne Albertine van der Sluis werd in Nijmegen geboren op 25 februari 1969. Haar Atheneum B-diploma behaalde ze in 1987 in haar geboorteplaats aan het Stedelijk Scholengemeenschap Nijmegen. In datzelfde jaar begon ze haar studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. Deze studie rondde ze in 1993 af, met als specialisatie Levensmiddelenmicrobiologie. Ze volgde ook diverse voedingskundige en kwaliteitskunde vakken.

Na haar studie was ze enige tijd werkzaam als vrijwillig medewerkster in de adviesgroep voeding en voedselverwerking bij Agromisa te Wageningen en vervolgens verrichtte ze in het kader van een na-doctoraal onderzoeksproject bij ATO-DLO literatuuronderzoek naar bruinverkleuring in appelproducten.

In 1995 werd ze aangenomen als assistent in opleiding bij de sectie Zuivel- en Levensmiddelennatuurkunde, later Geïntegreerde Levensmiddelentechnologie en nog weer later leerstoelgroep Productontwerpen en Kwaliteitskunde genaamd, aan de Wageningen Universiteit. Het onderzoek dat ze daar verrichtte resulteerde uiteindelijk in dit proefschrift. In dit onderzoek kwam haar belangstelling voor wat er bij productie en verwerking gebeurt met de in een levensmiddel aanwezige voedingstoffen, hoe deze voedingstoffen in het menselijk lichaam omgezet worden en wat de gevolgen daarvan zijn voor de gezondheid, samen.

Sinds 2001 is ze werkzaam bij Agrotechnology and Food Innovations BV (voorheen ATO) , in de groep Naoogstkwaliteit van Verse Producten, business unit Quality in Chains (voorheen Agro-Industriële Productieketens), Wageningen Universiteit en Researchcentrum.

PUBLICATIONS

FULL PAPERS

Van der Sluis, A. A.; Dekker, M.; van Boekel, M. A. J. S. Activity and concentration of polyphenolic antioxidants in apple juice. 3. Stability during Storage. *J. Agric. Food Chem.* **2005**, *53*, 1073–1080.

Van der Sluis, A. A.; Dekker, M.; Skrede, G; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple juice. 2. Effect of novel production methods. *J. Agric. Food Chem.* **2004**, 52, 2840–2848.

Van der Sluis, A. A.; Dekker, M.; de Jager, A.; Jongen, W. M. F. Polyphenolic antioxidants in apple: effect of storage conditions on four cultivars. *Acta Horticulturae* **2003**, 600. Proceedings of the Eight International Controlled Atmosphere Research Conference. Oosterhaven, J.; Peppelenbos, H.W.; Eds., pp 533–540.

Van der Sluis, A. A.; Dekker, M.; Skrede, G; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *J. Agric. Food Chem.* **2002**, *50*, 7211–7219.

Van der Sluis, A. A.; Dekker, M.; de Jager, A.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple; effect of cultivar, harvest year, and storage conditions. *J. Agric. Food Chem.* **2001**, *49*, 3606–3613.

Van der Sluis, A. A.; Dekker, M.; Verkerk, R.; Jongen, W. M. F. An improved, rapid in vitro method to measure antioxidant activity; application on selected flavonoids and apple juice. *J. Agric. Food Chem.* **2000**, *48*, 4116–4122.

Dekker, M.; Verkerk, R.; van der Sluis, A. A.; Khokhar, S.; Jongen, W. M. F. Analyzing the antioxidant activity of food products; processing and matrix effects. *Toxicology in vitro* **1999**, *13*, 797–799.

ABSTRACTS

Van der Sluis, A. A.; Dekker, M.; Jongen, W. M. F. Effect of processing on content and antioxidant activity of flavonoids in apple juice. In: *Natural Antioxidants and*

Anticarcinogens in Nutrition, Health and Disease. Kumpulainen, J. T.; Salonen, J. T.; Eds; Royal Society of Chemistry: Cambridge, U.K., **1999**; pp 209–211.

Dekker, M.; Verkerk, R.; Van der Sluis, A. A.; Awad, M.; Jongen, W. M. F. Modelling the healthiness of fruit and vegetable products from field to table. In: *Bioactive Compounds in Plant Foods, Health Effects and Perspectives for the Food Industry*, COST 916 Conference, Tenerife; **2001**, p25.

Dekker, M.; Van der Sluis, A. A.; Verkerk, R.; Jongen, W. M. F. Processing of foods containing flavonoids and glucosinolates; effect on composition and bioactivity. In: *Natural antioxidants and anticarcinogens in nutrition, health and disease.* Kumpulainen, J. T.; Salonen, J. T.; Eds; Royal Society of Chemistry: Cambridge; **1999**; pp 303–308.

Dekker, M.; Van der Sluis, A. A.; Verkerk, R.; Jongen, W. M. F. Effects of processing on the level and bioactivity of antioxidants in food products. In *AgriFood Quality II, Quality Management of Fruits and Vegetables*; Hägg, M; Ahvenainen, R.; Evers, A. M.; Tiilikkala, K.; Eds.; Royal Society of Chemistry: Cambridge; **1999**; pp 17–21.

Van der Sluis, A. A.; Dekker, M.; Jongen, W. M. F. Flavonoids as bioactive components in apple products. *Cancer Letters* **1997**, 114, 107–108.

Van der Sluis, A. A.; Dekker, M.; Jongen, W. M. F. Flavonoïden als bioactieve componenten in appelproducten. *Trends in voedingswetenschappen* (NVVL-congres samenvattingen), Ede; **1997**, p30.

Dekker, M.; Van der Sluis, A. A.; Khokhar, S. D.; Jongen, W. M. F. (1997) Effects of processing on the level and bioactivity of antioxidants in food products. *Trends in voedingswetenschappen* (NVVL-congres samenvattingen), Ede; **1997**, p9.

OTHER

Van der Sluis, A. A.; Dekker, M.; Linnemann, A. R.; Jongen, W. M. F. Verhoging van gezondheidsbeschermende stoffen in appelsap in ketenperspectief. In: *Werkende ketens. Management, kwaliteit, logistiek en ICT in de agro-industriële sector.* Keesing Noordervliet; **1999**; pp 87–99.

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DISCIPLINE SPECIFIC ACTIVITIES

- VLAG course Reaction kinetics, 1995.
- VLAG course System analysis in food and bioprocess engineering, 1996.
- VLAG course Ecophysiology of the GI-tract, 1996.
- MBA course Management of technology and innovation, 1997.
- Training period at other laboratories: Institute of Food Research, Norwich, United Kingdom, 1996.
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- Conference: Food and Cancer Prevention II, Ede, 1996.
- Second International Conference on natural antioxidants and anticarcinogens in nutrition, health and disease, Helsinki, Finland, 1998.
- Various meetings of 'learned societies'.

GENERAL COURSES

- Scientific writing in English, CENTA, 1995.
- VLAG PhD week, 1995.
- Successful functioning in organisations, VLAG, 1998.

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- PhD study tour France and Switzerland, 1997.
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The study described in this thesis was carried out at Wageningen University and Research Centre in the Netherlands; Product Design and Quality Management Group of Wageningen University. The thesis is the result of independent research by Ir. A.A. van der Sluis, under supervision of Prof. Dr. W.M.F. Jongen, Prof. dr. M.A.J.S. van Boekel, and Dr. Ir. M. Dekker. The work was part of the research programme of the Graduate School VLAG (Food Technology, Nutrition & Health Sciences). The project was financially supported by the Netherlands Ministry of Agriculture, Nature Management and Fisheries (LNV).

Cover: Photo on front page: Elstar apples on tree. Photo on back page: apple juice pressing and polyphenol rich extract.

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