VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

FACULTY OF CHEMISTRY
INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

STUDY OF QUALITATIVE PARAMETERS OF FRUIT JUICES AND POSSIBILITIES OF THEIR MODIFICATION VIA TECHNOLOGICAL MODIFICATIONS

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

AUTOR PRÁCE Bc. ZDENĚK FAJTL
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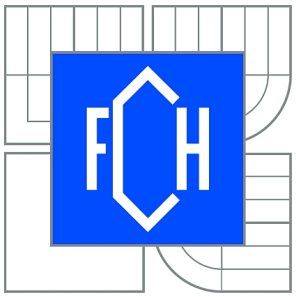

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Vysoké učení technické v Brně
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Zadání diplomové práce

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Konzultanti:	doc. Ing. Jiřina Omelková, CSc.		

Název diplomové práce:

Study of qualitative parameters of fruit juices and possibilities of their modification via technological modifications

Zadání diplomové práce:

Selected physico-chemical parameters of fresh fruity juices produced by conventional methods and their changes following from their long-term storage under the defined conditions (temperature, relative humidity, light conditions) will be monitored. Subsequently, the influence of changes in production conditions of fruit juices on their properties will be studied. Effect of production technology modification on selected physico-chemical parameters and final quality of the product will be assessed via monitoring the changes in the content of selected vitamins, polyphenolic compounds, antioxidant and radical-scavenging activity as well as color changes involving spectroscopic methods, dominantly EPR and UV-VIS. Additional analytical techniques (e.g., HPLC, GC) will be employed, when appropriate.
Multivariate statistics will be used for complex data evaluation and processing.

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Diplomová práce se odevzdává v děkanem stanoveném počtu exemplářů na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

Bc. Zdeněk Fajtl Student(ka)	Ing. Martin Polovka, Ph.D. Vedoucí práce	prof. RNDr. Ivana Márová, CSc. Ředitel ústavu

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Děkan fakulty

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ABSTRACT

Fruit juices belong to the most popular drinks worldwide. Besides vitamins and minerals they represent rich source of phenolic compounds recognized for their antioxidant activity. Increasing consumer's demands towards high quality and safe food products push their producers to increase the production and qualitative standards of the products. Recent innovations in juices production resulted in modification of production conditions, comprising the modification of composition (e.g., addition of fruit pulp and/or small fruit pieces) innovations in packaging materials and the production atmosphere modification. In the diploma thesis, the effects of production conditions of fresh orange juices with pulp and pineapple juices and their modification, as well as the effect of conditions of their long-term storage on selected qualitative characteristics (antioxidant activity, color changes and concentration of selected phytochemicals) are assessed and evaluated dominantly by EPR and UV-VIS spectroscopy. Results obtained were processed by multivariate statistics in order to evaluate the effects of sample origin and season or production technology on the monitored characteristics and overall quality of fruit juices. Results obtained indicated that pasteurization influence the quality of the product, although the slight pasteurization was applied, as in comparison to the fresh – non-pasteurized samples, slight decrease of values of the monitored parameters was observed. However, as regards the post-pasteurization changes, significant influence of the production atmosphere on the monitored qualitative parameters of juices was noticed, proving that replacement of oxygen by inert gases prolongs significantly the juices shelf live. Kinetic study of the changes of the monitored parameters upon the storage period indicate that in majority of cases, gradual worsening of all the monitored parameters was observed, without respect on technology of juice processing. In the samples processed under the modified atmosphere, generally the retardation of the decrease as a result of inert atmosphere application in dependence on type of production gas was noticed. This trend was particularly obvious for longer storage periods, whereas in the beginning of storage (6 - 7 weeks after production), the observed trends were unambiguous. Thus, it can be supposed that this "protecting" effect of inert atmosphere is influenced by the physico-chemical properties of gases, mostly by their solubility in the liquid juice medium. It was also proved, that the properties of juices, are significantly affected by the effects of season and raw juice origin. As follows from the results of statistical analysis, the year of production is strong discriminant factor as on the basis of all the monitored characteristics, absolute discrimination of the samples by canonical discrimination analysis was achieved. As regards raw material origin, only partial differentiation, although with high classification score was obtained. As regards the effect of production technology, the partially successful differentiation of samples according to the inert gas applied was obtained. The obtained results will help the juice producer in optimization of the juice production conditions in order to obtain the product with maximum beneficial properties kept during the whole expiration period. Its length can also be optimized on the basis of the presented results.

KEYWORDS

Fruit juices, modified atmosphere, stability, antioxidant activity, color changes, seasoning effect, statistics

ABSTRAKT

Ovocné šťávy jsou celosvětově nejoblíbenějšími nealkoholickými nápoji. Kromě vitamínů a minerálů jsou také bohatým zdrojem polyfenolů, které jsou považovány za látky přispívající k celkové antioxidační aktivitě. Zvyšující se požadavky konzumentů na kvalitu a bezpečnost potravin nutí výrobce k zvyšování výrobních a kvalitativních standardů potravin. Nedávné inovace ve výrobě ovocných šťáv vedly k modifikacím výrobních podmínek, které zahrnují změny ve složení šťávy (např. přídavek ovocné dužiny nebo malých ovocných kousků), vylepšování vlastností obalových materiálů a modifikace produkční atmosféry. Cílem diplomové práce bylo pomocí metod EPR a UV-VIS spektroskopie posoudit vliv různých výrobních postupů na vybrané kvalitativní znaky (antioxidační aktivita, změna barvy a koncentrace vybraných fytochemikálií) pomerančových šťáv s dužinou a ananasových šťáv, a charakterizovat jejich změny v průběhu dlouhodobého skladování. Získané výsledky byly zpracovány metodami multivariační statistické analýzy s cílem posoudit vliv původu suroviny, roku produkce a použitých výrobních podmínek na sledované parametry a celkovou kvalitu ovocných džusů. Výsledky jasně prokázaly, že pasterizace ovlivňuje kvalitu výrobku; ačkoli byla použita „šetrná“ pasterizace, byl u těchto vzorků pozorován mírný pokles prakticky všech monitorovaných parametrů v porovnání s čerstvými – nepasterizovanými šťávami. Avšak pokud jde o po-pasterizační změny, byl prokázán významný vliv produkční atmosféry na monitorované kvalitativní parametry šťáv, prokazující, že náhrada kyslíku inertními plyny může výrazně prodloužit trvanlivost šťáv. Kinetické studie změn sledovaných parametrů na době skladování ukazují, že ve většině případů dochází k postupnému zhoršování všech monitorovaných parametrů, a to bez ohledu na technologii zpracování šťávy. Ve vzorcích vyrobených použitím modifikované atmosféry bylo, v závislosti na typu inertního plynu, pozorováno zpomalení poklesu jednotlivých kvalitativních parametrů šťáv jako výsledek aplikace inertní atmosféry. Tento trend byl zřejmý především pro delší skladovací období, zatímco na začátku skladování (6 - 7 týdnů po výrobě dané šťávy) byly pozorované trendy nejednoznačné. Lze proto předpokládat že tento „ochranný“ efekt inertní atmosféry je ovlivňován fyzikálně-chemickými vlastnostmi jednotlivých plynů, zejména jejich rozpustností v kapalném médiu. Výsledky také prokázaly že vlastnosti šťáv jsou ,bez ohledu na druh ovoce, výrazně ovlivňovány také sezónními vlivy a původem surové šťávy. Z výsledků statistické analýzy vyplývá, že rok výroby je silným diskriminačním faktorem. Na základě všech monitorovaných charakteristik, bylo dosaženo absolutní diskriminace vzorků pomocí kanonické diskriminační analýzy. Pokud jde o původ surovin, byla dosažena pouze částečná diferenciace, i když s poměrně vysokým klasifikačním skóre. Pokud jde o technologii výroby, byla dosažena pouze částečná diferenciace vzorků podle typu použitého inertního plynu. Získané poznatky pomohou výrobcům ovocných šťáv s optimalizací výrobních podmínek s cílem získat výrobek s maximem prospěšných vlastností, které by si udržel po celou dobu expirace, jejíž délka může být také optimalizovány na základě prezentovaných výsledků.

KLÍČOVÁ SLOVA

Ovocné šťávy, modifikovaná atmosféra, stabilita, antioxidační aktivita, barevná změna, sezónní vlivy, statistika

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DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, VUT.

…….....
Zdeněk Fajtl

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CONTENT

1 INTRODUCTION

Fruit juices are consumed worldwide and are preferred by consumers due to their palatable taste, color, freshness, nutritional value and beneficial health effects. The last mentioned beneficial effects are frequently attributed to bioactive phytochemicals, such as vitamins, carotenoids, phenolic compounds, etc. contained in fruit and fruit juices. Beside their other positive effects, these compounds reveal frequently also antioxidant activity; it is widely accepted, that the concentration of phytochemicals is in direct relation to the antioxidant capacity of fruit/fruit juices and can be influenced by the processing technology, pasteurization and/or long-term storage of fruit and fruit juices.

Oxygen plays important role in the oxidation of phytochemicals and other compounds in fruits and fruit juices, thus decreasing their concentration and/or forming new, oxidative products. Consumers nowadays expect juices which preserve maximum of their original high nutritional value and are microbiologically safe. Thus, innovations in fruit juices production which are recently spreading out would lead to fulfillment of these expectations. The novel production methods comprise the application of e.g., modified atmosphere in the production steps to minimize negative effects of oxygen, flash pasteurization techniques to obtain microbiologically safe product or utilization of innovative – functional packaging materials with UV-filters or oxygen scavengers. Use of oxygen scavengers is one way of reducing amount of oxygen in fruit juices and container headspace. Combination of the active packaging, modified atmosphere production and subsequent cold-chain storage are recognized as most efficient tools for extension of shelf-life and for improvement of storage conditions.

Analytical methods which are the most frequently used for quantification of antioxidant activity of diverse samples are electron paramagnetic resonance (EPR) and UV-VIS spectroscopy. UV-VIS spectroscopy is also often used for quantification of total polyphenols content. Changes in color during storage are negatively perceived by consumers. These changes can be objectively measured by use of colorimetry.

In this diploma thesis, the effects of production conditions of fresh fruit juices and their modification as well as the effect of conditions of their long-term storage on selected qualitative characteristics (antioxidant activity, color changes and concentration of selected phytochemicals) are assessed, dominantly by EPR and UV-VIS spectroscopy. Results obtained were also processed by multivariate statistical methods in order to evaluate the effects of sample origin, season or production technology on the monitored characteristics and overall quality of fruit juices.

2 THEORETICAL PART

2.1 Fruit juices

Production of fruit juices is one of many ways how to process horticultural products. In small scale, fresh juices for immediate consumption are produced locally by squeezing fruits of appropriate ripeness. Industrially produced juices needs to follow the specific legislation regarding the hygiene, sanitation, composition and production to maintain the nutritional values, quality and microbial safety of the product. Directive 2012/12/EU of the European parliament and of the Council of 19 April 2012 relating to fruit juices and certain similar products intended for human consumption gives the definitions of the products, its names and characteristics.

Fruit juices are defined herein as the fermentable but unfermented product obtained from the edible part of fruit which is sound and ripe, fresh or preserved by chilling or freezing of one or more kinds mixed together having the characteristic color, flavor and taste typical of the juice of the fruit from which it comes [1].

2.1.1 Ingredients

2.1.1.1 Pineapple

Pineapple (*Ananas comosus*) is the most important species of the *Bromeliaceae* family grown commercially for its fruit. Pineapple is native to South America, where wild relatives occur. Commercially grown cultivars (Cayenne, Queen, Spanish, Pernambuco, and Perolera) are nearly or completely seedless. This is an advantage for production of fresh juices with pulp [2]. The fruit is used as fresh or processed. Processed products are most frequently juice, canned slices, dried pieces, jam and glaze.

Pineapple is non-climacteric fruit; thus it is incapable of continuing ripening process once the fruit is harvested from the plant. Non-climacteric fruits must be harvested mature but not ripe, so they can withstand transportation without damage. Maturity can be determined physically by judging size, shape, weight, external and internal color, firmness, surface texture and skin morphology. Chemical methods of indicating maturity are sugar/acid ratio, total soluble solids and juice content. Pineapple with appropriate ripeness is harvested and stored at 7 °C - 13 °C for 2 - 4 weeks. Non-climacteric fruits produce small amounts of ethylene; pineapple produces 0.1 - 1.0 ppm of ethylene. Respiration rate is less than 35 mg $CO_2 \cdot kg^{-1} \cdot h^{-1}$. Pineapples, as many other tropic fruits, are highly sensitive to chilling injury, which occurs if pineapple is held at temperatures below 8 °C - 10 °C for prolonged storage. Chilling injury symptoms are surface and internal discoloration, uneven ripening, off-flavor, increased acidity, loss in ascorbic acid content, surface molds and decay. Pineapples are transported to Europe mainly by sea. Marine transport is relatively inexpensive, and facilities for refrigeration, modified and controlled atmosphere are available [3].

Pineapple is rich source of various phytochemicals, such as vitamins A, B and C, carotenoids, flavonoids, isoflavones, flavones, anthocyanins, catechins and other phenolic compounds. Flavonoids are present as coloring pigments and also function as antioxidants [4]. Pineapples also contain significant amount of bromelain, proteolytic enzyme with varied use. Bromelain is used for tenderizing meat, preventing cold haze in beer and for medicinal purposes.

2.1.1.2 Orange

Sweet oranges (*Citrus sinensis*) are most abundant citrus species produced worldwide. Citrus originated in China and spread mostly throughout the Asian continent. Nowadays, citruses are grown in subtropical regions all around the world [2]. Oranges are non-climacteric fruit. Oranges with

appropriate ripeness are harvested mostly by hand and can be stored at 5 °C - 10 °C for 6 - 8 weeks. Minimum safe temperature for storing oranges is 3 °C - 5 °C. During the storage, oranges produce very small amount of ethylene, less than 0.1 ppm. Respiration rate is 7 - 13 mg $CO_2 \cdot kg^{-1} \cdot h^{-1}$ [3].

Analyses of oranges have revealed the presence of approximately 224 phytochemicals, including 23 monoterpenoids, 15 sesquiterpenoids, 13 diterpenoids, 32 flavones, 13 flavanones, 6 flavanols, 9 anthocyanins, 3 chaconnes, 4 phenolic acids, 15 carotenoids and 4 coumarins [5]. Citruses are rich source of phenolic compounds, mainly flavonoids. Orange contains high amount of flavonoid hesperidin and small amounts of flavonoids narirutin and didimin. Occasionally, aromatic and aliphatic acids, methylenedioxyl or isoprenyl groups also attach to the flavonoid nucleus and their glycosides. Other flavonoids that can only be found in citruses are polymethoxyflavones and glycosylated flavones [6]. Orange also contains vitamin C and folic acid. Most health promoting carotenoids in oranges are α-carotene, β-carotene, lutein, β-cryptoxanthin and zeinoxanthin. Color of orange is mainly influenced by its carotenoid content [7].

2.1.2 Production of fruit juices

Fruit and vegetable cells consist of parenchyma cells, which consist of vacuoles, cytoplasm and other cell compartments surrounded by the cell walls. Vacuoles contain all water-soluble components and their precursors such as sugars, acids, salts, etc. Juice can be described as cell sap, which is obtained by disruption of the membrane. Disruption of membrane can be achieved by any of the following way:

- Mechanical cell disruption by pressing;
- Enzyme liquefaction - enzymatic removal of cell walls;
- Diffusion treatment - denaturation of the membranes in diffusion processes with hot water or alcohol.

2.1.2.1 Concentrated fruit juice, fruit juice from concentrate

In order to reduce transportation and storage cost, juices may undergo the processing step in which, part of water is removed, following the respective legislation. The so obtained concentrated juices can be sold at their destination as such or frozen.

European legislation defines concentrated fruit juice as the product obtained from fruit juice of one or more fruit species by the physical removal of a specific proportion of the water content. Where the product is intended for direct consumption, the removal shall be at least 50 % of the water content. Flavor, pulp and cells obtained by suitable physical means from the same species of fruit may be restored to the concentrated fruit juice [1].

The commercial processes of concentrating orange juice usually involves the removal of water at high temperature with vacuum for short time followed by recovery and concentration of volatile aromas and their addition back to the concentrated product. In cheaper juices of lower quality, original volatile content is not completely recovered due to economic reasons. The concentrated juice is stored at standard temperatures or frozen [2].

Concentrate can be shipped to a distant distribution point where it is diluted, pasteurized and packaged. This type of juice is labeled as fruit juice from concentrate [2]. According to the legislation, fruit juice from concentrate is the product obtained by reconstituting concentrated fruit juice with potable water that meets the criteria set out in Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. The soluble solids content of the finished product shall meet the minimum Brix level for reconstituted juice specified in Annex V. If a juice from concentrate is manufactured from a fruit not mentioned in Annex V, the minimum Brix level of the reconstituted juice shall be the Brix level of the juice as extracted from the fruit used to make the concentrate. Flavor, pulp and cells obtained by suitable physical means from the same species of fruit may be restored to the fruit juice from concentrate. The fruit juice from concentrate is prepared by

suitable processes, which maintain the essential physical, chemical, organoleptic and nutritional characteristics of an average type of juice of the fruit from which it comes. The mixing of fruit juice and/or concentrated fruit juice with fruit purée and/or concentrated fruit purée is authorized in the production of fruit juice from concentrate [1].

The aroma of fruit juices from concentrate is noticeably different from that of freshly squeezed oranges. Frequently, off-flavor odor is observed most commonly in canned fruit juices from concentrate, because of double heating, first time during the concentration process and subsequently, after the reconstitution with water and pasteurization. These thermal treatments (high temperature/times) induce chemical changes in orange juices, which may significantly affect the volatiles present in the original fresh orange juice and lead to the generation of the off-flavors [8].

2.1.3 General schema of citrus and soft fruit processing

Because of the differences in soft and citrus fruits, two main processing/production methods of the juices are used. General schema for the citrus fruit and soft fruit processing is depicted on *Figure 1* and *Figure 2*, respectively. Most significant difference in processing of both types of fruit is in the juice extraction step. Soft fruit generally do not contain bitter substances in the peel so it can be milled before press. On the other side, possible extraction of bitter substances from the citrus fruit peel requires either removal of the oils from the peel or use of processing methods which avoids extraction of oils. Remaining steps in most cases are the same.

2.1.4 Inspection and washing

Incoming fruit is often discharged into pits filled with water, which provides gentle means of transportation to the processing facility. This transportation also acts as first washing step that removes dust and debris. In the processing facility, fruit is typically inspected and sorted (e.g., classification of orange according to size). Small scale operations uses manual inspection, large scale operation uses automatic inspection. Damaged fruit is removed since it can be partly fermented and thus can affect aroma of juice. After the inspection step, fruit is washed using spray of clean water with mild sterilant. Washing of fruit also lowers microbial load and thus, more gentle pasteurization can be used [9].

2.1.5 Milling

Fruit is milled to the appropriate size before pressing to improve release of juice. Different methods are used for various fruits. These methods are influenced by structure of raw material. Most common disintegrator is the hammer mill, or variation of the hammer mill with fixed or free swinging hammers which force the fruit through a screen. The degree of disintegration is varied by two types of hammer used. Blunt hammers disintegrate fruit on principle of blunt disintegration and sharp hammers cut fruit into pieces. Hammer speed and diameter of the screen holes also affect the result of the disintegration process.

Fixed knife mill represents alternative system used for milling. Fruit is forced against fixed knives by rotating three-armed spider. Usually, fruits are gravity fed into the fixed knife mill from the top. Mash falls from mill through small holes under the knives. Milling is essential for relatively hard fruit such as apples or pineapple whereas relatively soft fruits like grapes, berries and oranges needs only light crushing [10].

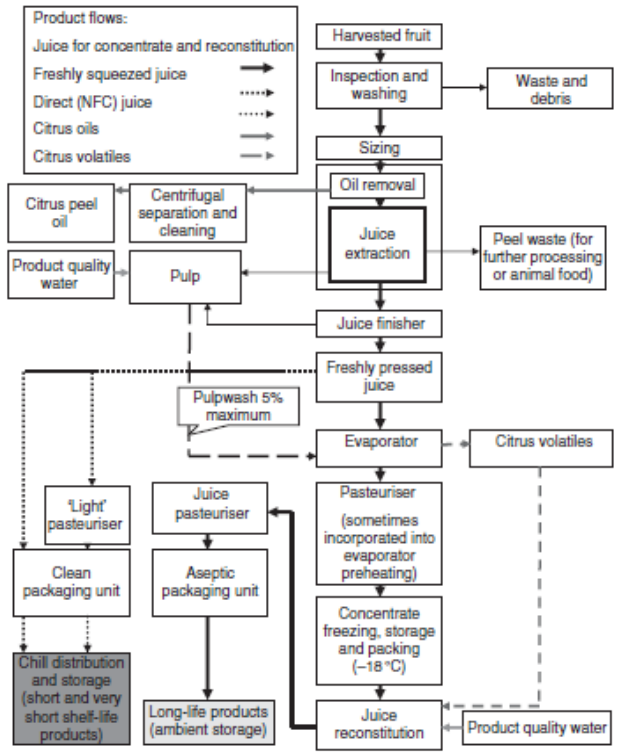

Figure 1: *Typical processing schema of citrus fruits (product diagram) [10].*

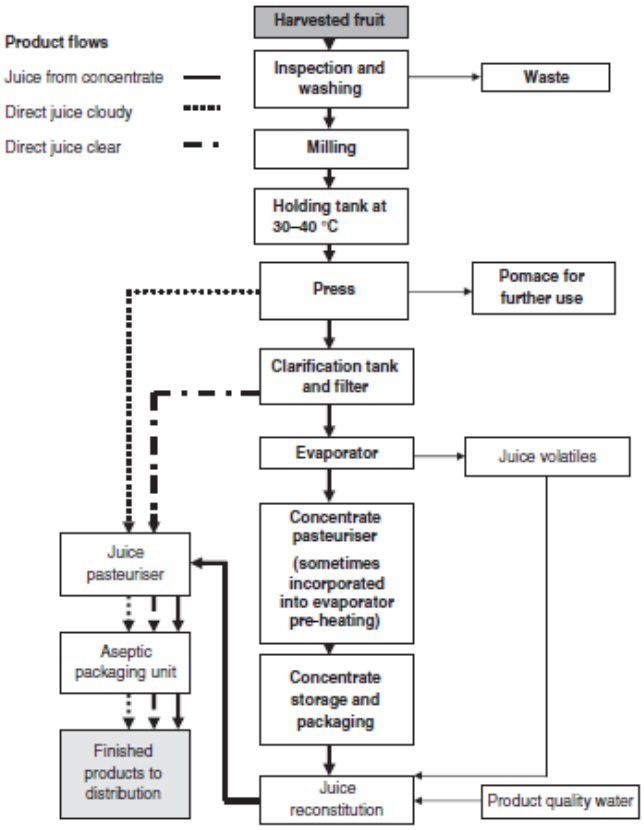

Figure 2: *Typical processing schema of soft fruit (product diagram) [10].*

2.1.6 Holding tank

After milling, fruit pulp is stored in the holding tank. In this step, use of modified atmosphere can prevent oxidation. In addition, use of enzyme or diffusion treatment can enhance yield of fruit juice.

2.1.6.1 Enzyme treatment

Fruits juice production can be enhanced by use of liquefaction enzymes during the storage of the milled fruit in the holding tank. Enzymes degrade cell wall polysaccharides and release soluble compounds. Enzymatic liquefaction therefore can enhance the yield and improve the clarification of juice. Combinations of pectinase, celullase and hemicellulase enzymes are generally used as the liquefaction enzymes. Pectinases are responsible for degradation of pectins, structural polysaccharides in the middle of the lamella and in the cell wall of plant cells [11]. Juice yield from pineapple pulp treated with enzymes is significantly higher than that of untreated pulp. Also the flow characteristics, filtration speed and clarity of pineapple juice are improved as a result of enzymatic treatment. Enzymes can also be utilized in processing of fruit by-products Treatment of orange peel with combination of polygalacturonase and cellulose provides natural clouding agents, which are then used in juice and juice-type beverages [2].

2.1.6.2 Diffusion treatment

In the diffusion process, the cell walls and membranes are heat - denatured to make them permeable and in order to allow diffusion of cell sap constituents from the vacuole into the extracting medium. It uses the counter-current principle, in which hot water (50 °C -70 °C) is used as the extraction medium [12]. Higher extraction yields are achieved by proper chopping of fruit before the diffusion treatment [13].

2.1.7 Press, juice extraction

The traditional way of raw fruit juice production is the pressing fruit of appropriate ripeness through the suitable press. Different techniques are used for clear and cloudy juices pressing. Compartmentalization of the fruit tissue is destroyed by pressing. As a consequence of the cell disruption, many chemical, biochemical and physical changes occur [12]. Juice of higher quality is produced while lower pressure is applied because higher pressure causes contamination of juice with undesirable compounds contained in the peel, skin and seeds of the plant. Generally, fruit should be pressed after removal of the seeds, as the substances from seeds can cause bitter taste [3].

Pineapples are inspected and washed. Next step is milling to the appropriate size and then pulp is stored in holding tank, where additional steps can take place, such as enzyme treatment of pulp. Juice is then pressed from pulp [2].

Modern extractors for the orange juice production extract juice by pressing half of orange against spinning serrated reamer. Orange is cut in half by stationary knife and then is held by synthetic rubber cups. Rotating reamer exerts increasing pressure and expresses the juice and pulp. The oil and pulp contents in the juice increase with rising reaming pressure When pressure is accurately adjusted, there is practically no risk of extracting bitter oil substances from orange peel [2].

In another type of extractor, fruit is located between two cups having sharp-edged metal tubes at their base and metal intermeshing fingers. The upper cup descends and the metal fingers on each cup mesh to express the juice as the tubes cut holes in the top and bottom of the fruit. On further compression, the rag, seeds and juice sacs are compressed into the bottom tube between the two plugs of peel. A piston moves up inside the bottom tube forcing the juice through perforations in the tube wall. A simultaneous water spray washes the peel oil expressed during the extraction away from the peel as an oil–water emulsion; the peel oil is recovered separately from the emulsion, usually by a

centrifugal process. Each extractor unit has several cups of a single size and in a typical factory installation; banks of extractors processing different fruit sizes are located. The recoverable oil is removed in a separate step prior to juice extraction. Needle-sharp spikes prick the peel of the whole fruit, releasing oil that is washed away with water and recovered from the oil - water emulsion. Most citrus juice extraction equipment is based on these two types of extractor [10].

2.1.8 Clarification

The finely suspended solids, which give juice its turbid appearance, are referred to as cloud. These solids retain many juice volatiles, partitioned between the insoluble pulp/cloud and aqueous serum. Hydrocarbons (mono and sesquiterpene) are almost exclusively (80 - 90 %) associated with pulp, whereas oxygenated compounds (esters, alcohols, and aliphatic aldehydes) are more closely associated with the serum. If the juice is clarified to remove this finely suspended material, quantity of aroma compounds are eliminated, and the flavor of the clarified juice is altered. It has been reported that the volatile compounds associated with suspended solids (pulp and cloud) from a freshly squeezed orange juice represent about 80 % of the total juice volatiles. Because pulp content and particle size of commercially produced juices can be controlled, such juices will have a different pulp content and physical distribution than the hand squeezed ones [8].

Extracted fruit juices are usually viscous liquids with persistent cloud of insoluble plant particles (e.g., fibers, cellulose, hemicellulose, protopectin, starch, lipids) and colloid macromolecules (e.g., pectin, proteins, soluble-starch fractions and polyphenols). Depending on the manufacturer practices, type of product or on consumer expectations, these particles can be partially or entirely eliminated to avoid precipitation and to improve the sensory attributes. Juice clarification can be performed by physicochemical methods, mechanical procedures or by their combinations. Physicochemical methods include the employment of clarification agents (enzymes, mineral clarifying agents, gelatin, polyvinylpolypyrrolidone), which form precipitates from insoluble particles and macromolecules [13]. The enzymes normally used in clarification processes are pectinesterase, polygalacturonase, pectin-transeliminase, cellulase and hemicellulase [3]. Precipitate is separated by mechanical methods afterwards. Mechanical clarification is used for removal of suspended fibers and precipitation. This process is usually carried out in centrifuges and filtration devices [13].

Juice turbidity is the result of complex system involving pectins, gums, cellulose, hemicellulose and proteins. Colloid particles are composed from the protein particles inside and from the soluble or insoluble pectin particles on the outside. The particles appear from the outside as negatively charged, but within their structure they poses positive charge [13].

2.1.9 Filtration and finishing

Juice is composed of an aqueous phase and water insoluble phase. Aqueous phase contains soluble compounds and the serum. Water insoluble phase contains pulp and cloud [14]. Filtration is voluntary step performed to adjust the amount of pulp in fruit juice. The most widely used equipment for the filtration of fruit juices is the hydraulic press, horizontal filters, vacuum filters, ultrafiltration and microfiltration techniques. From general point of view, the filtration rate is affected by the filter area, the amount of filter aid material, the filtering time, the pressure applied to the system, the type of fruit, viscosity of juice and its temperature [3].

Pulp content can be controlled in centrifuges or finishers. Centrifuges are more precise for low pulp content juices than the finishers. Juices with lower pulp content are less viscous and have more beverage-like sensory properties. Minimally processed fresh juices usually have the higher content of pulp, thus only finisher is used to reduce the amount of pulp.

During the finishing step, juice pass through a stainless steel screen to separate the extraneous cell and segment wall material, and embryonic seeds from the juice. Screw press is used to separate juice from the solid material. Juice composition is altered depending on the finisher pressure employed. Use

of high finishing pressure squeezes the liquid portion of the pulp to the juice. Solid particles that pass through the finisher screen are dispersed into smaller particles using homogenizers. At a later stage, some juice manufacturers add juice sacs to produce a pulpy juice product as the physical appearance and mouth feel is what some consumers consider close to what they might prepare at home.

The amount of pulp and insoluble solids has effect on the aroma composition of processed juices. Reduction of insoluble solids by centrifugation lowers the amount of aroma compounds in juice. Hydrocarbons (mono and sesquiterpene hydrocarbons) are associated with pulp and oxygenated compounds (ethyl butyrate and octanal) are mainly contained in the serum. Addition of natural pulp content to low pulp orange juice increases its fresh juice character [14].

As the pulp content and particles size can be controlled under the commercial production conditions, commercially produced orange juices have usually a different pulp content and physical distribution than hand squeezed juices [8].

2.1.10 Pasteurization

Juices are pasteurized after the centrifugation or finishing in order of eliminating the pathogenic and spoilage microorganism. Other reason is to inactivate enzymes, which are in whole fruit separated from cell sap. Enzymes, such as pectin esterase can cause cloud precipitation in clarified juices [2].

Salmonella, *Shigella* and *Escherichia coli* can potentially survive in fruit juices despite their low pH. Only freshly pressed juices for direct consumption does not require pasteurization. Flash pasteurization at 85 °C - 90 °C for 15 - 20 seconds or in-pack pasteurization at 70 °C for 20 minutes of filled bottles is used most frequently [9]. Juices with pulp are pasteurized in tubular pasteurizer. Clarified juices can be pasteurized using the conventional plate heat exchangers.

Pasteurization of the juice may result in alterations to the original flavor of fresh orange juice, whereas consumers are increasingly demanding juices with a flavor as close as possible to unpasteurized, freshly hand-squeezed juices. Pasteurization decreases the amount of the volatile compounds such as aldehydes and esters. Adding natural pulp prior to pasteurization protects the quality of juice aroma by restoring high quantities of these volatile compounds [14].

2.1.10.1 Hot packaging

In this process, juice is rapidly heated to 85 °C - 90 °C in heat exchanger. Hot juice is immediately filled into the prepared containers and closed. Residual heat is used to pasteurize the juice, package and closure. Containers are mechanically rotated to ensure hot juice comes into contact with all inner surfaces of container and closure. Hot packaging is very energy demanding, due to need of adequate cooling facilities after the filling [10].

2.1.10.2 Non-thermal pasteurization

Novelty approach in juice processing is non-thermal pasteurization. Non-thermal technologies are based on high hydrostatic pressure, microwaves, ohmic heating, pulsed electric fields, irradiation and supercritical carbon dioxide [2]. Heating foods by the use of electric fields and/or high pressure differs from conventional thermal processing because it uniformly penetrates several centimeters into the food. Heat is generated quickly and evenly throughout the mass, and steam generated heats adjacent areas by conduction [15].

High pressure pasteurization causes the rupture of microbial membranes, and thus inhibits growth of spoilage microorganisms. Products pasteurized by high pressure are available in the USA, Japan and European markets. Combination of high initial temperature with high pressure further improves this technology.

Microwaves, pulsed electric fields and irradiation use radiation energy, which changes foods properties as a consequence of absorption of energy. Microwave processing uses frequencies of either

915 MHz, or 2450 MHz. Water and other dipolar molecules in food oscillate to align themselves with alternating microwave current. These high-speed oscillations cause friction, which heats the food. Heating through the use of electric fields and/or high pressure differs from the conventional thermal processing because it is capable to uniformly penetrate several centimeters into the food. Heat is generated quickly and evenly throughout the mass, and generated steam heats adjacent areas by conduction [15].

Electrical current applied directly to food increases temperature in ohmic processing, a continuous pasteurizing process based on a series of low-frequency alternating electric currents of 50 Hz or 60 Hz. Both, container and liquid heats quickly, then are cooled and are packaged aseptically.

Pulsed electric field processing, which is currently applied to juices, involves the application of short pulses of a strong electric field on a flowing fluid in order to destroy the vegetative cells of microorganisms. In both, ohmic and pulsed electric field processing, the electric current is uniformly applied to the entire food product, which creates local heating and also causes the rupture of microbial and plant cells [15].

2.1.11 Packaging

The purpose of packaging is to protect its content against wide range of risks during the distribution and storage. Main risk for juices represents microbial contamination and potential oxidation. Material used for packaging should not interact with juice. Undesirable interactions are migration of toxic compounds and reactions between the juice and packaging material.

Three types of packaging are recognized nowadays: passive, active and intelligent. Passive packaging only serves as barrier for oxygen and light and is used as basis for active packaging. On the other hand, active packaging provides additional functions such as oxygen scavenging, antimicrobial activity, atmosphere control, edibility and biodegradability. Last but not least, intelligent packaging has the same functions as active packaging, but intercorporate also some new functions, e.g., monitoring of food properties (temperature, pressure, pH…) or indication of food freshness [16].

2.1.11.1 Plastic containers

Trend in the juices packaging is the use plastic containers. Most frequently used material for plastic containers is polyethyleneterephtalate (PET). PET has many advantages, such as its relative strength, low weight, recyclability and clarity. Main disadvantage of PET is its permeability to gases. Juices packed in PET are susceptible to oxidative deterioration because of the transportation of oxygen through the packaging walls during the storage. Use of multilayer PET material can eliminate this effect. However, cost of multilayer PET container is higher than that of single layer PET container. Thus, oxygen scavengers are often used to reduce the negative effect of oxygen penetration.

Another material used for plastic containers is polyethylene naphthalate (PEN). PEN due to its properties allows in-pack pasteurization. PEN is much less liable to distort at the temperatures used for pasteurization. Other plastics, such as high density polyethylene (HDPE) and polystyrene are used in production of freshly squeezed fruit juices as cup drinks [9].

2.1.11.2 Laminated board containers

Fruit juices can be filled into laminated board packs known under the commercial name TetraPak, Combibloc and others. Materials used in these packs are usually polyethylene (PE), paper board and aluminum foil. Layers forming a laminate, starting from inside, are PE/aluminum foil/PE/paper board/polyethylene. Laminate is sterilized in bath and containers are formed to the required shape. After the filling, container is sealed and inspected for leaks. Advantages of these packs are their light weight, high level of protection, easy distribution (thanks to standard size), and large outside surface for decoration and labelling. On the other hand, juices cannot contain substances such as Sulphur

dioxide, which would react with aluminum foil. Another disadvantage is the maximum capacity of about 2 liters per container [9].

2.1.12 Exclusion of oxygen, oxygen scavengers

Air with oxygen is naturally dispersed in intercellular spaces of fruits. During the fruit processing cells are crushed, the cell wall is disrupted and the air is mixed into the juice. Juices contain air as dissolved gas in solution, or associated with the pulp particles. During the cell disruption, metabolites and enzymes that are normally separated in different compartments are mixed together, producing chemical and biochemical reactions. Juice is saturated by oxygen and the juice can undergo oxidation reactions that often result in browning, changes in aroma, loss of nutritional value or other indications of spoilage. These reactions are initiated/accelerated by the increase of temperature, usually during the pasteurization and have significant influence on the overall quality of the product during the storage/juice expiration period. Preventive steps, such as deaeration can be employed during fruit processing [17].

Oxidation is major problem in juice quality and shelf life. Oxidation, compared to microbial spoilage is slower process. The oxidation reaction starts in the presence of (usually) proper catalysts, such as iron, copper, enzymes, heat, or light. Particularly important is the presence of metallic ions, occurring in juice either naturally, or as a result of fruit processing. Once started, the oxidation is self-accelerated (autocatalytic) process. Through the stages of oxidation, various compounds are produced that give the juice rancid odors and flavors. Oxidation can be inhibited e.g., by the use of active packaging with oxygen scavengers. Oxygen scavengers chemically combine with free oxygen and thus remove it from the container. Exclusion of oxygen during the production and packaging combined with oxygen scavengers can help to protect juices from oxidation and improve both, quality and shelf life of fruit juices [16].

The most frequently commercially used oxygen scavengers represent ferrous compounds, catechol, ascorbic acid and its analogues, ligands, oxidative enzymes such as glucose oxidase, unsaturated hydrocarbons and polyamides. Most commonly used oxygen scavengers for beverages are based on ferrous iron – as a consequence of its oxygen scavenging action, from ferrous state the iron is oxidized to the ferric state. They are convenient for beverages, as they require high moisture for iron to be oxidized. A level of 0.01 % oxygen in the closed container is achievable via scavengers application. Ferrous iron oxide powder is usually intercorporated into layer of polymer which is part of the cap. Disadvantage is, that containers must be stored with cap upwards, so the liquid is not in contact with cap [18].

Removal of oxygen from the container headspace inhibits the oxidation and thus also helps to prevent the formation and accumulation of oxidation reaction products such as off-flavor compounds. Inhibition of lipid oxidation with antioxidant agents or active packaging is of great importance in protecting foodstuffs with high amounts of unsaturated fatty acids from possible quality deterioration and providing the required shelf life.

2.1.12.1 Deaeration

Deaeration is the process of removing or reducing the dissolved air from the juice. Deaeration is performed prior to thermal treatment, and can affect juice quality. Dissolved air can cause foaming problems during the packaging and accelerate vitamin C degradation during the storage. Deaeration is employed only for those juices that will not undergo concentration as the concentration process removes entrained air along with water and most aroma volatiles [8]. Deaereation is usually accomplished by vacuum, centrifugation, nitrogen-sparging, membrane deaerators and enzyme based deaerators [17]. During the deaeration, the content of volatile alcohols, aldehydes, and terpene hydrocarbon concentrations can be significantly reduced. Therefore, the process must be carefully controlled in order to prevent the negative effects on flavor quality [8].

2.1.13 Application of modified atmosphere

Modified atmosphere packaging (MAP) is used to preserve the freshness of product, as well as to ensure the color stability and inhibit microbial growth during the storage. MAP is based on modification of the gas composition in the package (inner atmosphere), which is different than gas composition in the (outer) atmosphere. These changes allow controlling the biochemical metabolism of packaged product. Usually either single gas or mixture of gases is used for MAP, frequently nitrogen, carbon dioxide, carbon monoxide, inert gases (e.g., argon) or their combination. Vacuum packaging is also one type of MAP, used for solid state products [16].

Modified atmosphere is often used in juice production in order to achieve the decrease of dissolved oxygen in juice during milling of fruit, storing of mash in holding tank, pasteurization and packaging.

2.2 Oxido-reduction characteristics

Free radicals are reactive species of most frequently oxygen (reactive oxygen species, ROS), nitrogen (reactive nitrogen species, RNS, carbon or sulphur (reactive sulphur species, RSS), such as superoxide ($O_2^{\bullet -}$), the hydroxyl radical •OH), hydrogen peroxide (H_2O_2), nitric oxide, or lipid peroxide radicals. Free radicals naturally emerge in living organisms during many biological reactions and can damage crucial biomolecules, if their concentration is above the required quantities and is not controlled by the respective elimination mechanism. Such conditions are frequently assigned as the oxidation stress. Negative effect of free radicals can be partially balanced out by antioxidants, which are capable of scavenging these radicals and thus eliminate their action. Most frequently, ROS mechanisms of formation, reactions and elimination are studied.

The oxidation starts by the reaction of ROS with unsaturated fatty acids in the presence of a proper catalyst. Oxidation reaction can generally be divided into three phases, initiation, auto-oxidative propagation and final termination. After the initiation, the process is self-accelerated. The oxidation of juices result in conjugated dienes, hydroperoxides, alkanes, alkenes, aldehydes, and ketones production, which give rancid odors and flavors and are capable of subsequent reaction with other functional groups and, worsening of the physical and sensorial properties of foods. Particularly, cross-linking of aldehydes with amino groups in proteins may cause structural damage and textural change [16].

Mechanism of lipid autoxidation [19]:

Initiation	$RH \rightarrow R^{\bullet} + H^{\bullet}$	(1)
Propagation	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$	(2)
	$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$	(3)
Termination	$2\ ROO^{\bullet} \rightarrow$ non-radical products	(4)
	$ROO^{\bullet} + R^{\bullet} \rightarrow ROOR$	(5)
	$2\ R^{\bullet} \rightarrow R\text{-}R$	(6)

Metal-catalyzed decomposition of hydroperoxides [19]:

$$ROOH + M^n \rightarrow RO^{\bullet} + HO^- + M^{n+1} \quad (7)$$

$$ROOH + M^{n+1} \rightarrow ROO^{\bullet} + H^+ + M^n \quad (8)$$

Food antioxidants are substances that can slow-down or prevent the development of food rancidity induced by oxidation. Naturally occurring antioxidants in foods can inhibit lipid peroxidation and improve the quality and storage time of food product. Free radicals can be inhibited either by direct or by indirect scavenging. Primary antioxidants scavenge radicals directly, secondary antioxidants indirectly. By definition, antioxidants are various compounds that counteract the effects of reactive oxygen species [5]. Antioxidants deactivate or immobilize free radicals by stabilizing them, or by

reacting them to form new, stable or inactive compound. Phytochemicals, metal binding proteins, antioxidant enzymes and non-enzymatic antioxidants can show antioxidant activity [20]. Antioxidants forms diverse group of compounds, thus can be divided into groups according to various criteria. Thus, the antioxidants can be classified e.g., as:

- Primary, Secondary, Tertiary
- Endogenous, exogenous, natural, artificial
- Hydrophilic, lipophilic, amphofilic
- Extracellular, intracellular
- High-molecular weight /low molecular weight antioxidants

Phytochemicals are referred to as compounds which possess antioxidant properties in plant-based foods. The main ROS scavenging antioxidants in fruits are vitamin C, carotenoids and flavonoids. Carotenoids and flavonoids also provide fruits with their orange, yellow, pink, red and purple colors. Amount of vitamin E in fruits is generally low, but seeds and nuts have high vitamin E content. Fruits also contain antioxidant enzymes, but these protein-based catalytic antioxidants are usually destroyed during the pasteurization step. Phytochemicals a when present in a living organisms possess also physiological benefits related to promoting health and preventing effects of a disease [5].

2.2.1 Polyphenols

Polyphenols are a structurally diverse class of phytochemicals, which occur as plant secondary metabolites. Polyphenols are divided into four basic groups: flavonoids, phenolic acids, stilbenes and lignans. Basic division of polyphenols is depicted on *Figure 3*. Phenolic compounds contain at least one aromatic ring bearing with one (phenols) or more (polyphenols) hydroxyl substituents and their functional derivates (esters and glycosides) in their structure. Polyphenol content of fruits is influenced by several factors, of which the most important are cultivation and harvesting conditions [5].

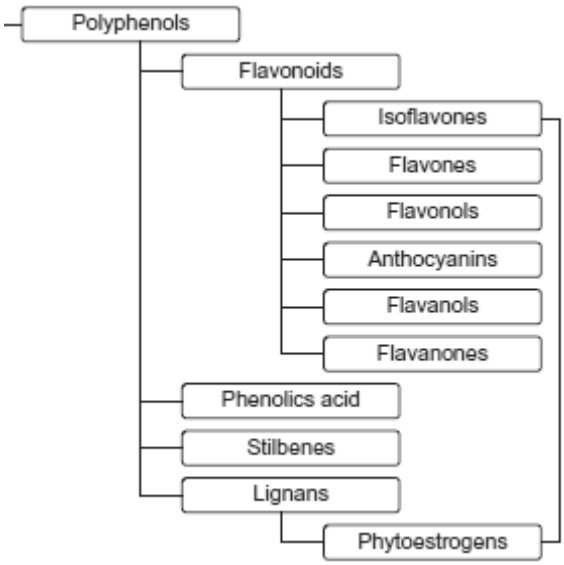

Figure 3: *Classification of polyphenols [5].*

Polyphenols have ability to directly inactivate ROS or bind pro-oxidant metallic ions by means of their hydroxyl groups. In the former case, the polyphenol transfers one hydrogen to the peroxyl radical as follows:

$$ROO^{\bullet} + ArOH \rightarrow ArO^{\bullet} + ROOH \quad (9)$$

The phenoxyl radical ($ArO^{\bullet}$) formed in this reaction is relatively stable and reacts slowly with other substrates, thus interrupting the chain of oxidative reactions. At high concentrations, the polyphenol may act as a pro-oxidant, since the amount of formed phenoxyl radicals is able to trigger the oxidative reactions [21].

2.2.1.1 Flavonoids

Flavonoids are one of the main bioactive compounds found in fruits. They generally consist of two aromatic rings, A and B, linked by an oxygenated heterocyclic C ring. According to the structure, oxidation state and functional groups of C ring, they can be further classified as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids. When flavonoids are linked to one or more sugar molecules, they are called flavonoid glycosides; otherwise they are designated as aglycones. Flavonoids usually occur as glycosides which are less effective than aglycones. There is a relationship between the structure and antioxidant activity. The groups, such as catechol moiety (showed within the dotted ellipse on *Figure 4*), the 2,3 double bond conjugation with a 4-oxofunction of a carbonyl group (showed within the solid ellipse on *Figure 4*), and presence of hydroxyl groups at the 3 and 5 positions (showed within the dotted ellipse on *Figure 4*), determine the free radical scavenging and oxidation potential [5].

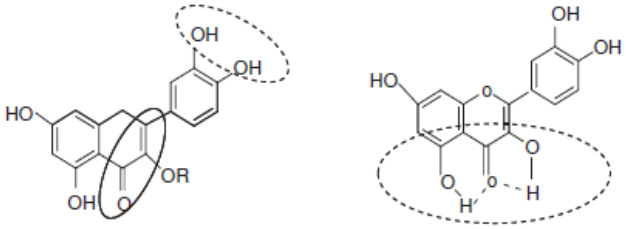

Figure 4: *Relationship between the antioxidant activity and structure of flavonoids [5].*

2.2.1.2 Phenolic acids

Phenolic acids comprise two main subgroups: benzoic acids and cinnamic acids. Natural phenolic acids occur in fruits and vegetables either in free or conjugated forms, usually as esters or amides. Benzoic acid creates basic structure of tannins (gallotannnins and ellagitannins) and cinnamic acid comprise basic structure of *p*-coumaric, caffeic, ferulic and sinapic acids [5].

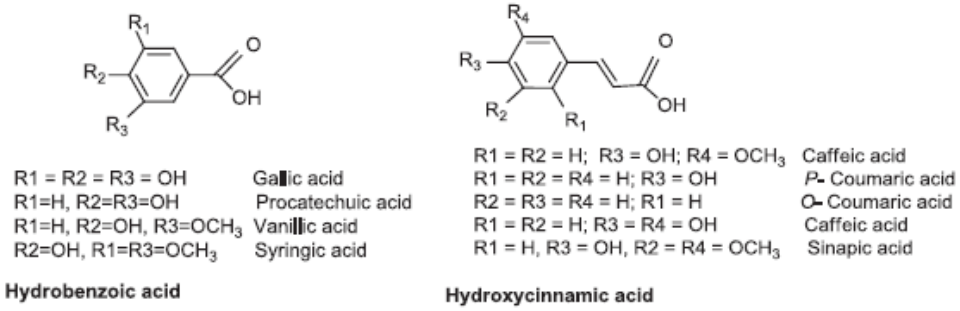

Figure 5: *Structure of phenolic acids [5].*

2.2.1.3 Stilbenes

Stilbenes are phenolic but non-flavonoid type of antioxidant compounds containing two benzenic rings linked by an ethane or ethene bridge. They are widely distributed in higher plants, acting as phytoalexins and growth regulators. Resveratrol (3,4,5-trihydroxystilbene) is the representative of stilbene antioxidants, found in grapes and wine. Preventing heart disease, primarily through the consumption of red wine is frequently attributed to resveratrol [21].

2.2.1.4 Lignans

Lignans are basically dimers of the cinnamic alcohol, which cyclizes in different ways, generating a wide range of molecules. The lignans are contained in woody tissues, cereals, and vegetables like carrots, broccoli, and berries. Together with isoflavones, the lignans belong to the class of phytoestrogens, which are protective factors of cardiovascular and immune systems [21].

2.2.2 Carotenoids

Carotenoids are fat-soluble pigments, produced by plants as secondary metabolites. They function as antioxidants protecting lipids against peroxidation by quenching free radicals, particularly the singlet oxygen. More than 600 different carotenoids have been identified [21]. They are responsible for the yellow, orange, red and violet colors of fruit. According to their structural properties, they are further sub-classified into carotenoid hydrocarbons (carotenes) and carotenoid alcohols (xanthophylls) [5].

Lycopene, a linear carotenoid with 11 double conjugated bonds, is the precursor of all carotenoids. Xanthophylls are yellow carotenoid pigments involved in photosynthesis and found in the leaves of most plants. Zeaxanthin and lutein are the xanthophylls that are absorbed and bioavailable Carotenes occur as α-carotene and β-carotene followed by γ-, δ-, and ε-carotene. β-Carotene is composed of two retinyl which are precursor of vitamin A [21].

Carotenoids color is influenced by the structure, i.e., mostly by the length of the chromophore, the arrangement of conjugated double bonds in the end ring and the form of geometrical (cis/trans) isomers, in which they appear. Most carotenoids absorb light between 400 nm and 500 nm. Carotenoids are prone to various degradation reactions because of their highly unsaturated structure. Degradation reactions also affect their color and their biological activity. The presence of oxygen in combination with light and heat leads to oxidative degradation and color loss. During various reactions, mainly during the oxidation and isomerization, epoxides and free radicals are formed. The stability of carotenoids is influenced by processing intensity, storage time and temperature, amount of dissolved of oxygen and light conditions [22].

2.2.3 Vitamins

Vitamins are heterogeneous group of substances that are vital to humans. They can be generally divided into water-soluble and fat-soluble ones. Most vitamins are relatively unstable in foods and their amount decreases during the processing and storage. Major factors affecting the stability of vitamins are temperature, moisture, oxygen, pH and light. Minor factors are the presence of oxidizing and reducing agents, metallic ions and Sulphur dioxide [19].

2.2.3.1 Ascorbic acid

Ascorbic acid (AA) is a natural antioxidant present in most fruits. AA content is mainly affected by the dissolved oxygen. Orange and pineapple juices are rich source of ascorbic acid. Its degradation in juice is a result of both, aerobic and anaerobic mechanisms that occurs simultaneously Main degradation reactions are oxidation and hydrolysis. Ascorbic acid in aqueous solution is easily oxidized to dehydroascorbic acid (DHA) in a 2-step process that produces monodehydroascorbate

(MDHA), also called ascorbate-free radical. As an intermediate, MDHA can be reduced back to ascorbic acid or form DHA, which has a pro-oxidant effect. The oxidation of AA to DHA is reversible process; the direction of which is affected by the presence of oxidizing or reducing agents. Dehydroascorbic acid also undergoes irreversible hydrolytic ring cleavage to produce 2,3-diketogulonic acid (2,3-DKG). Beside others, the oxidation of ascorbic acid to DHA lead to color and flavor changes [17].

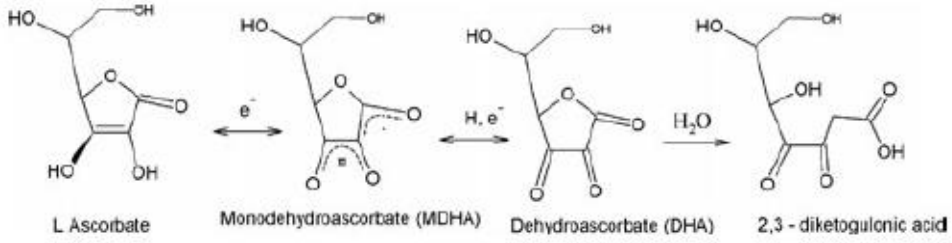

Figure 6: *Ascorbic acid degradation mechanism [17].*

2.2.3.2 Other water soluble vitamins

All group B vitamins are soluble in water. These vitamins are: B_1 (thiamine), B_2 (riboflavin), B_3 (nicotineamide), B_5 (pantothenic acid), B_6 (pyridoxine), B_9 (folic acid) and B_{12} (cyanocobalamin).

2.2.3.3 Fat soluble vitamins

Vitamin A, D_2 (ergocalciferol), D_3 (cholecalciferol), E (tocopherol), K_1 (phylloquinone) and K_2 (menaquinone) are fat soluble vitamins. These vitamins are non-polar, hydrophobic compounds. Their content depends on amount of oils in fruit juices.

2.2.4 Effects of oxygen on some physico-chemical properties of juices

2.2.4.1 Aroma

Volatile compounds with aroma activity are responsible for characteristic aroma of juices. In the presence of dissolved oxygen, formation of precursors such as dehydroascorbic acid and aldehydes occurs [17].

2.2.4.2 Nonenzymatic browning

During the pasteurization, heating promotes changes in color and flavor. Nonenzymatic browning, including Maillard type reactions, ascorbic acid, and sugar degradations are important reactions taking place in pasteurized fruit juices. Oxygen can promote browning, but is not directly responsible for it, as oxygen's indirect effect on browning is by ascorbic acid oxidation to dehydroascorbic acid with further formation of decomposition products such as furfural that has a brown color [17].

2.2.4.3 Enzymatic browning

Enzymatic browning is not a concern in most fruit juices because the pasteurization process, when applied correctly, inactivates the enzymes present in the raw fruit. Enzymatic browning consists of the oxidation of phenolic compounds to *o*-quinones that combine with amines and sulfur groups from proteins and reducing sugars to produce brown-colored polymers (melanines). Enzymatic browning occurs because of reaction between the phenolic compounds and the enzymes. Enzymes are located in plastids, polyphenols in the vacuole. In fruits and vegetables, the enzymes responsible for enzymatic

browning are polyphenol oxidases (PPO). There are 2 types of PPO: catechol oxidase (EC 1.10.3.1) and laccase (EC 1.10.3.2). Both contain copper in their structure and use molecular oxygen as a secondary substrate. Catechol oxidase catalyzes two reactions referred to as having cresolase and catecholase activity. They differ in that cresolase converts monophenols into orthodiphenols and catecholase oxidizes *o*-phenols to *o*-quinones., while laccase activity oxidizes *p*-diphenols and also of *o*-diphenols [17].

2.3 Fundamentals of the applied techniques

2.3.1 Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) deals with the interaction between the electromagnetic radiation and magnetic momentum of electron. Magnetic moment is created mainly by unpaired electrons in paramagnetic sample. Basic principle of EPR is very similar to that of nuclear magnetic resonance (NMR) technique [23]. Because of its selectivity, EPR provides insight into the nature of the paramagnetic center while also revealing detailed information on its environment and the dynamical processes in which it is involved [24]. However, its application is limited to systems revealing paramagnetism.

2.3.2 Basic principles of EPR

Electron is elemental particle which possess negative charge and orbital angular momentum as it moves around the nucleus. The electron also possesses spin angular momentum *s* as it spins around its own axis. Value of spin angular momentum is quantized and its absolute value is given by the equation:

$$|s| = \hbar\sqrt{s(s+1)} \tag{10}$$

Where *s* = the spin quantum number (electron has *s* = 1/2), $\hbar$ = reduced Planck constant.

Unpaired electron has its magnetic moment uncompensated and the molecule itself therefore has magnetic moment other than zero. In the absence of external magnetic field, these magnetic moments points at random directions and thus all of the unpaired electrons have the same energy, and total energy of the system is E = 0, the system is used to assign as degenerated in energy. If a strong homogeneous external magnetic field is applied, the degeneracy is lifted, as the magnetic moments are subdivided into two groups of different energy levels, each. One group of magnetic moments line up parallel with the external magnetic field, other group line up antiparallel to the magnetic field as a result of a competitive process between the thermal motion and magnetic interaction. The new energy levels will be [25, 26]:

α state $$E_1 = E_0 + \frac{1}{2} g_e \cdot \beta \cdot B \tag{11}$$

for antiparallel alignment (Ms = + 1/2)

β state $$E_2 = E_0 - \frac{1}{2} g_e \cdot \beta \cdot B \tag{12}$$

for parallel alignment (Ms = - 1/2)

This phenomenon is called Zeeman effect and the newly emerged energy levels are called Zeeman levels. An unpaired electron in β state can absorb quantum of electromagnetic radiation energy which is equal to energy difference between Zeeman levels and pass onto the higher energy level. The difference between two Zeeman levels is: [24]

$$\Delta E = E_1 - E_2 = g_e \cdot \beta \cdot B = h \cdot \nu \tag{13}$$

Where ν is radiation frequency, h is Planck constant; B is the induction of magnetic field and β is the Bohr magneton ($\beta = 9.2740154 \cdot 10^{-24}$ J·T^{-1}), g_e is the g-factor of free electron ($g_e = 2.00231$).

This equation is fundamental equation of EPR spectroscopy, also known as the resonance condition.

At thermal equilibrium and under the influence of the homogeneous external magnetic field, the spin population is split between the two Zeeman levels. The ratio of populations N_1 and N_2 of both levels is a function of temperature and of the difference E_1 - E_2 according to the Maxwell – Boltzmann law:

$$\frac{N_1}{N_2} = e^{-\frac{E_1 - E_2}{kT}} \tag{14}$$

Where k is the Boltzmann constant, T is the absolute temperature, N_1 and N_2 are spin populations of individual states. Since the net magnetization of the sample is proportional to N_1-N_2, it follows that it will increase with decreasing temperature and will tend to vanish at sufficiently high temperature [27]. At 298 K in a field of about 3000 G (300 mT) the distribution shows that:

$$\frac{N_1}{N_2} = e^{-\frac{E_1 - E_2}{kT}} = e^{-\frac{\Delta E}{kT}} = e^{-\frac{g_e \mu_e B}{kT}} = 0.9986 \tag{15}$$

The populations of the two Zeeman levels are therefore almost equal, but the slight excess in the lower level gives rise to a net absorption. If the populations of unpaired electrons in both Zeeman levels were equal, there would be no absorption of the energy. Slight excess in population with lower energy is maintained by the relaxation processes. Otherwise, phenomenon known as saturation occurs and the signal intensity decreases [25]

2.3.3 Relaxation processes

Unpaired electron releases quantum of energy equal to $h\nu$ during the relaxation processes and returns to lower energy state. The release of energy occurs via spin-lattice relaxation and spin-spin relaxation. During the spin-lattice relaxation, energy is dissipated within lattice as vibrational, rotational and translational energy. Loss of energy is characterized as exponential decay of energy as function of time. Spin-spin relaxation is based on energy exchange between the spins without transfer of energy to the lattice [25].

2.3.4 g - factor

External magnetic field (B_{ext}) applied to the sample is not an actual field that affects the magnetic moment. In addition to the external magnetic field, there also exist local magnetic fields (B_{local}) which produce effective magnetic field altogether with external magnetic field. B_{local} adds vectorially to the B_{ext} to form the total field B_{eff}.

$$B_{eff} = B_{ext} + B_{local} \tag{16}$$

Local magnetic field can be permanent or induced by external magnetic field. Permanent field is independent on external magnetic field except in their orientation. Induced magnetic field has a magnitude dependent on external magnetic field. Therefore, B_{eff} is:

$$B_{eff} = (1 - \sigma)B = \frac{g}{g_e} B \tag{17}$$

Where g is the so called effective Zeeman g factor, g_e is g factor of free electron and δ is EPR equivalent of the chemical shift parameter used in NMR spectroscopy. Many free radicals and some transition ions have $g \approx g_e$. There are many systems that show deviations from g. The g factor of free electron is 2.0023. Every paramagnetic sample has its specific g-factor value. By study of g-factor, important information about electron structure of studied paramagnetic sample can be acquired. With anisotropic systems, g is dependent on orientation of B. Values of g factor are given in matrix form.

Systems with isotropic properties are for instance liquid systems of low viscosity. G factor of the paramagnetic species in isotropic system is not dependent on orientation of B.

$$g_x = g_y = g_z \tag{18}$$

Value of g factor is expressed as an effective value averaged over all orientations. In isotropic systems, g-factor is identical with center of EPR spectrum [23].

2.3.5 Hyperfine splitting

The source of hyperfine splitting is the interaction between nuclei possessing intrinsic spin angular momentum and the magnetic moment of the unpaired electron. Proton is the source of magnetic field; newly-created local magnetic field is either higher or lower than the external magnetic field, in dependence on orientation of nuclei spin. Total local magnetic field is:

$$B_{local} = B_r + \alpha \cdot m_I \tag{19}$$

Where α is hyperfine interaction constant, m_r is nuclear spin quantum, B*r* is the value of magnetic field induction at which the resonance condition is satisfied. Hyperfine splitting is source of hyperfine structure observable in EPR spectrum. Generally, nucleus with spin *I* splits spectrum in to 2*I*+1 hyperfine lines with identical intensity [28]. Chemically equivalent nuclei with spin *I* cause 2n*I*+1 hyperfine splitting of all lines in spectrum.

Dipole-dipole interaction and Fermi contact interaction affect the interactions between nuclei and electron, affecting thus hyperfine interactions.

Dipole-dipole interaction is typically anisotropic i.e. sample orientation in magnetic field does change the interaction. Dipole-dipole interaction is observable only in solid state free radicals with p-orbitals because there is none dipole-dipole interaction at free moving radicals.

Fermi contact interaction is isotropic, i.e. it is non-affected by radical orientation. Electron in s-orbital is spherically dislocated around nucleus; therefore it has zero dipole-dipole interaction with nucleus. Fermi constant interaction becomes evident even in fast and chaotic moving molecules if pattern of spin density have s-character.

2.3.6 Spin traps

Free radicals in samples usually undergo fast termination reaction. Thus, their direct identification is only possible if there is sufficient concentration of short-lived radicals. There are two solutions to obtain steady-state, detectable, concentrations of free radicals. First is to physically stabilize radicals by freezing sample to temperature below 175 K. Second approach is chemical stabilization of radical via reaction between the radical and diamagnetic compound, spin trap, leading to the production of different new type of radicals (spin adducts) with longer lifetime. This approach is widely used in medically-oriented and food-related research involving EPR spectroscopy.

Spin trap is originally diamagnetic compound of appropriate structure that reacts with radical and form stable paramagnetic spin adduct. Analysis of hyperfine interaction constant and g-factor from EPR spectrum enables characterization of the trapped radical. Variety of spin traps of different structure and application have been synthesized. Conventionally, they include nitroso functional group (-N=O) and nitrones functional group (>C=N-O) in their structure, which react with short-lived radicals R$^{\bullet}$ to produce persistent nitroxyl radicals (*Figure 7*). Spin traps are usually organic compounds based on pyrolinoxide or nitrone compounds [29].

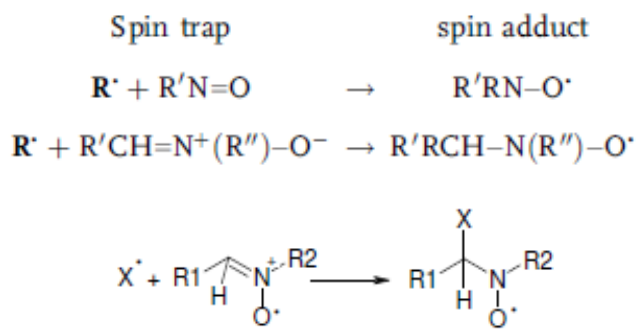

Figure 7: *General schema of principle of the spin trapping technique [29].*

2.3.6.1 Nitrone based spin traps

Free radicals react with alpha carbon in the structure of nitrone-based spin traps. Stable nitroxide radical is formed by addition of radical $X^{\bullet}$ to the double bond of $–CH=N^{+}O-R_2$ fragment. Nitrone spin traps form long-lived adducts with a wide range of radicals (e.g. carbon-, oxygen-, sulphur-, and nitrogen - centered species). Scheme of spin adduct formation is showed on *Figure 9*.

α-phenyl-N-tert-butyl nitrone (PBN) (showed on *Figure 8*) is lipophilic nitrone spin trap. Its low solubility in water limits its use in water containing systems. On the other way, it is often used in spin trapping of radicals emerging during the oxidation processes in cell walls. PBN can trap radicals generated in both the extracellular and intracellular compartments, without toxic effects associated with other lipophilic spin traps. *α-(4-Pyridyl N-oxide)-N-tert-butylnitrone* (POBN) (showed on *Figure 8*) is more hydrophilic than PBN, thus is used more often in water solutions. The spin trap, POBN is often used to detect carbon centered radicals because of good stability of the adducts [30].

Although the nitrone spin traps and the resulting nitroxyls are much more stable than their nitroso counterparts, identification of the primary radical $R^{\bullet}$ is less straightforward. This is because, in addition to the three-line pattern of the ^{14}N nucleus, the ESR spectrum of the nitroxyl adduct exhibits substantial splitting due to the methine β proton of the nitrone spin trap, and the nuclei of the added group R give rise to only minor hyperfine features [31, 32].

Figure 8: *Structure of α-phenyl-N-tert-butyl nitrone (PBN) and of α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN) spin traps [24].*

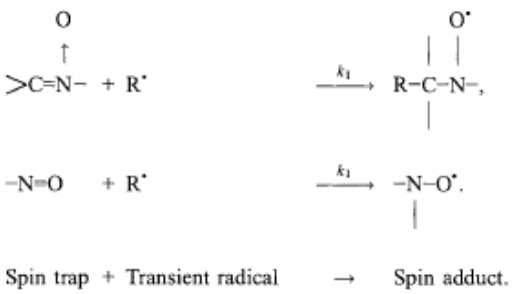

Figure 9: *Schema of spin trapping principle by means of nitrone spin trap [24].*

2.3.6.2 Pyrolinoxide based spin traps

Most used pyrolinoxide based spin trap is 5,5-dimethyl-1-pyrolin-N-oxid (DMPO). DMPO is based on the pyrolline-N-oxide moiety and is mostly used for detection of superoxide and oxygen-centered radicals such as $^{\bullet}$OH, HOO$^{\bullet}$, RO$^{\bullet}$, ROO$^{\bullet}$. Detection of $^{\bullet}$DMPO-OOH nitroxide spin adduct is limited by its short half-life. $^{\bullet}$DMPO-OOH rapidly decay to $^{\bullet}$DMPO-OH spin adduct [33]. $^{\bullet}$DMPO–OH adduct has a half-life in the range of 12–156 min in neutral solutions. $^{\bullet}$DMPO-OOH EPR spectrum contains distinctive features which differentiate it from $^{\bullet}$DMPO-OH EPR spectrum [30].

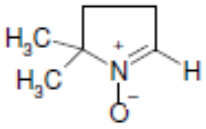

Figure 10: *Chemical structure of 5,5-dimethyl-1-pyrolin-N-oxid (DMPO) spin trap [24].*

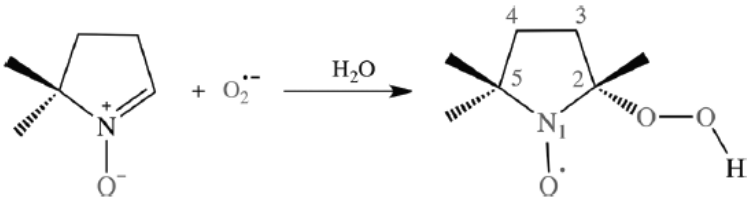

Figure 11: *Spin trapping of the superoxide radical by DMPO pyrolinoxide [33].*

2.3.7 Spin labels

Spin labels (also called spin probes) are stable paramagnetic radicals. They are intentionally added to the sample in order to determine both the qualitative and quantitative properties of reaction system [29]. For instance, kinetics of radical reaction can be observed on the basis of hyperfine interaction constant and g-factor changes.

Spin labels advantages are:

- Sensitivity to local environment properties
- Ability to measure molecule motion
- EPR signal is not influenced by surrounding diamagnetic compounds
- Great variability of available spin labels for different systems

Observed EPR spectrum is dependent on two parameters:

- Relative ease of orientation and movability of spin label nitroxyl moiety
- Hydrophobicity or hydrophylicity of an environment

Although the spin label is often a stable nitroxide group, virtually any free radical or paramagnetic metal or even a group with a long-lived triplet state ion can be used for the labelling purposes [24].

Nitroxyl compounds contain the C-N$^{\bullet}$-C moiety typically stabilized by three to four methyl groups, e.g., $(CH_3)_2$-RC-N$^{\bullet}$-CR-$(CH_3)_2$. This basic building block can be modified in many ways to change its aqueous solubility, its two-phase partition coefficient, its bulkiness, its side-chain reactivity, etc. The >N-O$^{\bullet}$ radical is a reporter group and, therefore, is not intended to undergo chemical reaction itself. However, single-electron reduction to the diamagnetic hydroxylamine is a not uncommon interfering side-reaction in biological samples [29]. The most persistent nitroxyls radicals lack H atoms at the C atoms linked to the NO group. That is why such paramagnetic species are favored as spin labels and spin adducts.

Small organic molecules, when inserted into biological macromolecules, can provide information about the molecular structure and biological function of these macromolecules. The so-called labels can be incorporated into biological macromolecules covalently or by diffusion [31].

The prominent hyperfine feature of nitroxyls is a three-line pattern due to the ^{14}N nucleus, in which the shape and width of the individual lines are governed by the combined contributions of the g_e and hyperfine anisotropies. In case of molecules containing two nitrogen atoms have five line patterns due to spin-electron interactions and subsequent hyperfine splitting. It is used to study the structure of lipids and membranes , proteins and nucleic acids, enzymes, and polymers Applications to both *in vitro* and *in vivo* systems are possible under the appropriate conditions [31].

2.3.7.1 TEMPOL

2,2,6,6-tetramethyl-4-oxopiperidinyl-1-oxyl (TEMPOL) is the only alkylnitroxyl containing a heteroatom outside the NO group. TEMPOL is hydroxyl-derivate of 2,2,6,6-tetramethyl-4-oxopiperidinyl (TEMPO) It is presumably the best known and the most persistent nitroxyl radical and has been widely investigated [31]. TEMPO is often used in quantitative EPR spectroscopy to measure the number of EPR active species in sample. Tempol, in dependence on reaction conditions, mostly the presence of certain low-molecular organic acids, can act as pro-oxidant and antioxidant [34, 35].

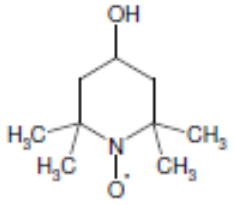

Figure 12: *Tempol structure[36].*

TEMPOL can be used for determination of ascorbic acid (AA) content, as it reacts with AA and produce dehydroascorbic acid and diamagnetic hydroxylamine according to the following reaction scheme [37]:

$$AH_2 \rightarrow AH^- + H^+ \quad (20)$$

$$AH^- + RNO^{\bullet} \rightarrow R\text{-}NOH + A^{\bullet -} \quad (21)$$

$$2\ A^{\bullet -} + H_2O \rightarrow AH^- + DHA \quad (22)$$

Loss of paramagnetic species can be detected by EPR spectroscopy and thus, it can be quantified.

2.3.7.2 •DPPH

2,2-diphenyl-1-picrylhydrazyl (•DPPH) belongs to the group of hydrazyls and has become particularly prominent. This highly persistent radical is available in the crystalline state [31] The •DPPH solution has deep purple color, with two absorption peaks at 517 nm and 320 nm. The 517 nm absorption peak is used for identification of •DPPH and determination of its concentration. Absorbance is highly influenced by the used solvent. •DPPH can accept hydrogen atom, but also can act as H-donor. In the presence of the radical scavenger (e.g., antioxidant) in the reactive system, •DPPH undergoes reduction and color of solution turns to light yellow or disappears completely. During the reduction, •DPPH accepts hydrogen atom from scavenger [38, 39].

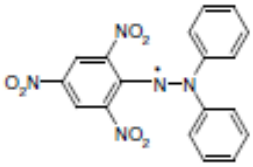

Figure 13: *Structure of 2,2-diphenyl-1-picrylhydrazyl (•DPPH) radical [23].*

Scavenging reaction between ˙DPPH and the antioxidant A is represented by equation (23). The less reactive A^- anion can undergo interactions with another ˙DPPH molecule as well as with A˙ according to the equations (24) and (25). These secondary reactions may be of limited occurrence [39].

$$\text{˙DPPH} + HA \rightarrow \text{DPPH-H} + A^{\bullet} \quad (23)$$

$$\text{˙DPPH} + A^- \rightarrow \text{DPPH}^- + A^{\bullet} \quad (24)$$

$$A^{\bullet} + X^{\bullet} \rightarrow \text{termination products} \quad (25)$$

2.3.7.3 ABTS$^{\bullet+}$

Radical cation of 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) is nitrogen-centered radical with blue/green color with maximum absorption at 734 nm. $ABTS^{\bullet+}$ is generated by oxidation of ABTS. Often used oxidizing agents are ammonium persulfate, manganese dioxide and system composing of ABTS/H_2O_2/peroxidase [37, 40].

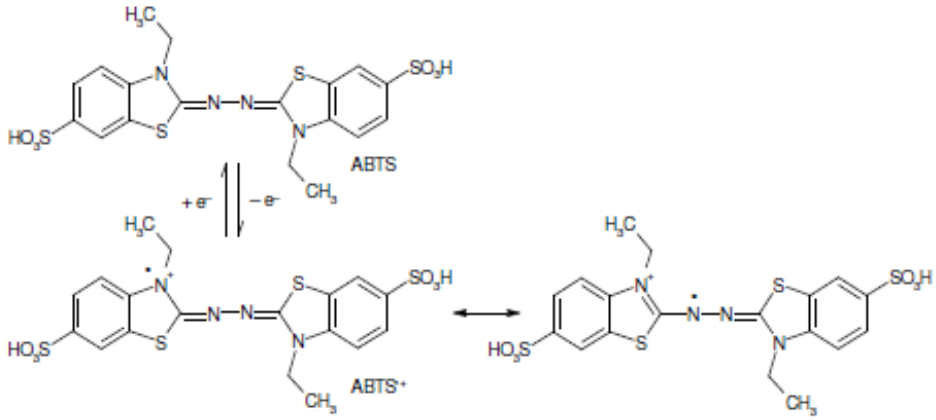

Figure 14: *Structure of 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) - ABTS and scheme of ABTS$^{\bullet+}$ formation [41].*

2.3.8 Methods used for antioxidant activity assessment

Two main types of methods are regularly used for antioxidant activity assessment of samples: methods assessing ability of sample to eliminate radicals and methods assessing redox ability of sample. EPR, UV-VIS and HPLC techniques are often used for characterization of antioxidant activity, the last mentioned via formation or diminution of specific compounds to which antioxidant activity is traditionally attributed.

Methods based on DPPH, ABTS and DPMD decay/discoloration assays utilize sample ability to eliminate these artificially synthesized free radicals. ORAC is based on ability of sample to eliminate oxygen radicals. Decrease in the radical concentration correlates with antioxidant activity of the sample.

2.3.8.1 DMPD assay

DMPD (N,N-dimethyl-p-phenylene diamine dihydrochloride) assay is used for measurement of the antioxidant activity in food and biological samples. This assay is based on the reduction of buffered solution of colored DMPD in acetate buffer and ferric chloride by sample antioxidants. Decrease in the absorbance of DMPD measured at 505 nm can be expressed as antioxidant activity of sample. The results are most frequently expressed as Trolox equivalent from relevant calibration curve [42].

2.3.8.2 •DPPH assay

The •DPPH radical scavenging assay is one of the most frequently used methods for evaluating the antioxidant activity. This assay is based on neutralization of •DPPH radical by antioxidants through donation of electron and formation of $DPPH_2$. The reaction is accompanied with discoloration of the •DPPH which acts as an indication of the antioxidant properties. The •DPPH assay is a simple technique and requires only a UV-VIS spectrophotometer or a spectrometer [38, 40, 43].

2.3.8.3 $ABTS^{\bullet+}$ assay

The cation radical $ABTS^{\bullet+}$ is used for Trolox-equivalent antioxidant capacity (TEAC) assay, which measures the ability of antioxidants to scavenge the stable $ABTS^{\bullet+}$. Resulting antioxidant capacity is compared to TROLOX, which is synthetic water soluble vitamin E derivate. Decrease of $ABTS^{\bullet+}$ concentration can be measured by UV-VIS or EPR spectroscopy. The assay has been used to measure the total antioxidant activity of pure substances, body fluids and plant materials [40, 44].

2.3.8.4 ORAC assay

The ORAC assay measures the ability of antioxidants to break the peroxyl radical induced chain reaction. Peroxyl radicals cause mainly oxidation of fats in foods and biological systems under the physiological conditions. The generated peroxyl radicals react with the fluorescent probe, which leads to the loss of fluorescence recorded with a fluorimeter. Sample scavenging capacity can be calculated from decrease of fluorescence. Results are usually expressed in relation to standard antioxidant, e.g. as Trolox-equivalent. ORAC assay can be used for detection of both, lipophilic and hydrophilic antioxidants by altering the radical source and solvent [40].

2.3.8.5 FRAP assay

The FRAP (Ferric reducing activity of plasma) assay measures the reduction of ferric ion (Fe^{3+})–ligand complex ferrous (Fe^{2+}) complex by antioxidants in acidic pH conditions (pH = 3.6) Antioxidant acts as electron donor and Fe^{3+} ions act as electron acceptor. Ferrous complex has intensive blue color, thus antioxidant activity is determined as increase of absorbance at 593 nm, and results are expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard. The FRAP assay is simple, fast and cost-effective and does not require specialized equipment. Original use of FRAP was to measure reducing power in plasma, but its use has been extended for assessing antioxidant activity in other biological fluids, foods, and plant extracts [40, 45].

2.3.8.6 CUPRAC assay

The CUPRAC assay is a variant of the FRAP assay. CUPRAC is based on copper reduction instead of iron. This method measures the reducing power of antioxidants to convert cupric ions (Cu^{2+}) to cuprous (Cu^{+}) ones. An advantage of this method is that the chemical reaction is very fast and pH of solution is close to physiological conditions (pH = 7). Absorbance of Cu^{+} ion complex is measured by at 450 nm UV-VIS spectroscopy and concentration is calculated from calibration curve [40, 46].

2.3.8.7 Phosphomolybenum assay

This assay is used for the determination of antioxidant capacity through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo^{VI} to Mo^{V} by the sample and formation of a green phosphate Mo^{V} complex at 95 °C and acidic pH for 90 minutes. After that, absorbance of cooled sample is measured at 695 nm. Antioxidant capacity is often expressed as equivalents of α-tocopherol [42].

2.4 UV-VIS

UV-VIS techniques are based on observation of transitions between different molecule energy levels during the absorption or emission of radiation in diluted solution. Absorption of energy in UV-VIS part of the spectrum (200-800 nm) is related to the transition of valence electron between two or more energy levels in the molecule. Absorption can be observed in all states of the sample, liquid, solid or gaseous.

2.4.1 Basic principles

UV-VIS absorption methods are based on measuring of transmittance. Light radiation can be absorbed, reflected or scattered. If the absorption occurs, intensity of radiation decreases. Transmittance (T) is defined as ratio of intensity of light emerging from sample (Φ) to intensity of light entering the sample (Φ_0) [47]:

$$T = \frac{\Phi}{\Phi_0} \tag{26}$$

Absorptivity is ratio of absorbed radiation:

$$\alpha = \frac{\Phi_0 - \Phi}{\Phi_0} = 1 - T \tag{27}$$

Absorbance is defined as negative decadic logarithms of transmittance:

$$A = \log \frac{\Phi_0}{\Phi} = -\log T \tag{28}$$

Bouguer-Lambert-Beer law mathematically describes the basis of light-absorption measurements on gases and solutions in the UV-VIS and IR-region. Absorption at specific wavelength is influenced by the molar absorptivity coefficient (ε_λ), concentration of solution (c) and cell path length (l).

$$A_\lambda = \varepsilon_\lambda \cdot c \cdot l \tag{29}$$

The Bouguer-Lambert-Beer law is a limiting law for dilute solutions, i.e. the assertion that the extinction coefficient (ε_λ), is independent on the concentration of a substance at the given wavenumber only in diluted solutions[48].

Molecules are under the normal conditions in ground electronic, vibrational and rotational energy states. Total energy of molecule is equal to the sum of its electronic, vibrational and rotational state energies.

$$E_{total} = E_{electronic} + E_{vibrational} + E_{rotational} \tag{30}$$

The amount of energy a molecule possesses in each form is not a continuum but a series of discrete levels or states. The differences in energy among the different states are in the order:

$$E_{electronic} > E_{vibrational} > E_{rotational} \tag{31}$$

When a molecule absorbs a photon from the UV/Vis region, the corresponding energy is captured by one or more of its outermost electrons. As a consequence there occurs a modification of its electronic energy, E_{elec}, a component of the total mechanical energy of the molecule along with its energy of rotation, $E_{rotational}$, and its energy of vibration, $E_{vibrational}$. A modification of E_{elec} will result in alterations for both, E_{rot} and E_{vib}, resulting in vast possibilities of transitions between the respective energy states, obtained in all three cases [47, 49].

Transition from the lower to the higher electron state can take place to various vibrational and rotational sublevels. Increase of total energy causes the excitation of molecule. Molecule has large number of energetically similar electron transitions. These close energy transitions cannot be differentiated as single lines and final absorption spectrum corresponds to a band type curve that

envelops the individual transitions. Difference in the energies of the excited and ground state must be equal to absorbed quantum of energy according to equation [47]:

$$h \cdot \nu = E_1 - E_0 \quad (32)$$

The energy captured during the course of photon absorption can be released through a variety of processes. During the absorption of energy, molecule electric dipole moment is altered. This alteration changes an arrangement of charge in molecule compared to the ground state. Molecules remain in excited state for limited amount of time (~ 10^{-9} s). The energy captured during the course of photon absorption can be released through a variety of processes. Equilibrium between the excited and ground state molecules is preserved. One line in absorption spectrum is equal to one type of electron transition to excited state. Absorption line has maximum of absorption at specific wavelengths λ_{max}. Shape of absorption line is approximately symmetric. Complex molecules absorption spectrums have more absorption lines which commonly overlap and create curve lines with indistinctive maximums. Position of absorption line determines between what molecule orbitals the excitations occur. Bonds between the organic molecule orbitals are constituted by hybrid orbitals. Hybrid orbitals are formed by interactions between s and p atomic orbitals. Organic molecules provide the following types of electron transitions within the orbitals [47, 48]:

- $\sigma \rightarrow \sigma^*$ transitions
- $n \rightarrow \sigma^*$ transitions
- $\pi \rightarrow \pi^*$ transitions
- $n \rightarrow \pi^*$ transitions
- $\pi \rightarrow \sigma^*$ transitions

There are three types of molecule transitions to the excited state:

- Allowed transition
- Spin forbidden transition
- Symmetrically forbidden transitions

2.4.2 UV-VIS methods for quantification of polyphenols

2.4.2.1 Folin - Ciocalteu method

The Folin - Ciocalteu (F-C) method is based on chemical reduction of the reagent (phosphotungstic - phosphomolybdenum complex) with polyphenols in sample. The products of the metal oxide reduction have a blue color that has absorption maximum at 765 nm. The intensity of the absorption line (usually expressed in terms of absorbance at the specific wavelength) is proportional to the concentration of phenols according to Bouguer-Lambert-Beer law. F-C method requires the use of a proper reference substance usually in the form of calibration curve, then this method gives the total concentration of phenolic hydroxyl groups. Maximum of absorption depends on the alkaline solution and the concentration of phenolic compounds. However, this reagent rapidly decomposes in alkaline solutions. Thus, it is necessary to use an enormous excess of the reagent to obtain a complete reaction. On the other hand, excess of the reagent concentration may result in precipitates and high turbidity formation, making spectrophotometric analysis impossible. To solve this problem, lithium salts is included in the reagent, which prevented the turbidity [50]. A disadvantage of the F-C method is that it is non-specific and can be affected by other nonphenolic reducing molecules. This method depends on the selective oxidation of similar easily-oxidized substances that, when present, contribute to the apparent total phenol content. Other easily-oxidized substances besides phenols include aromatic amines, Sulphur dioxide, ascorbic acid and endiols. Sugars break down in alkali to give endiols, which are readily oxidized. The F-C reagent also oxidizes proteins [51].

2.4.3 Colorimetry

Color is defined as the impact of the light (typically of wavelengths 350 nm – 780 nm) in the visible spectrum that can be detected and perceived by human eyes. Observed color is the result of interaction between the object and electromagnetic light wave. Human eye can distinguish about ten million different colors; perception of color is subjective and thus inappropriate for visual analysis because it can be biased, moreover it can be influenced by the source of light interacting with object and by the properties of object itself. Colorimetry is instrumental method used to describe and quantify color attributes according to the human color perception. There are three basic attributes of color, brightness, hue and colorfulness. Relative perceptual attributes of colors are lightness and chroma.

Brightness is an attribute of a visual perception, according to which an area appears to exhibit more or less light. Hue is an attribute of a visual perception according to which an area appears to be similar to one, or to proportions of two of the perceived colors - red, yellow, green, and blue. Colorfulness is an attribute of a visual perception according to which an area appears to exhibit more or less of its hue. Lightness is the brightness of an area judged relative to the brightness of a similarly illuminated area that appears to be white or highly transmitting. Chroma is the colorfulness of an area judged in proportion to the brightness of a similarly illuminated area that appears to be white or highly transmitting [52].

The chroma and the hue are the quantitative and qualitative attributes of color, respectively. More specifically, the chroma provides information on the vividness of a color, whereas the hue is the attribute according to which colors have been traditionally defined as reddish, orange, yellowish, etc. The hue and the saturation are considered to be the main attributes of chromaticity. Moreover, as the real world consists of mixtures of colors, saturation is the color attribute that is essential for describing the infinite and subtle variations in color. The hue is the degree value that corresponds to the three-dimensional color diagram (i.e., 0 for red, 90 for yellow, 180 for green and 270 for blue) as seen by the human eye [53].

Figure 15: *Representation of hue [54].*

2.4.3.1 Color space

Color space is the way of describing color mathematically, using three coordinates which define it in three-dimensional space. Color space is three dimensional because of different color intensities. Common color spaces are RGB, CMY and CIE. Each color space uses specific method of describing color. RGB and CMY are device dependent color spaces, CIE is device independent [54].

*2.4.3.2 CIE L*a*b* color space*

The CIE system is an internationally approved method of specifying color numerically, especially for foods, because it uniformly covers the full visible spectrum of the human eye. Within the CIE L*a*b* space, a psychometric index of lightness (L*) and two color coordinates (a* and b*) are defined. The L* index is related to the luminosity; according to this property, each color can be considered as equivalent to a member of the grey scale, i.e., between black (L* ~ 0) and white (L* ~ 100). The a* coordinate has negative values for greenish colors and positive values for reddish

colors. The b* coordinate has positive values for yellowish colors and negative values for bluish colors [53].

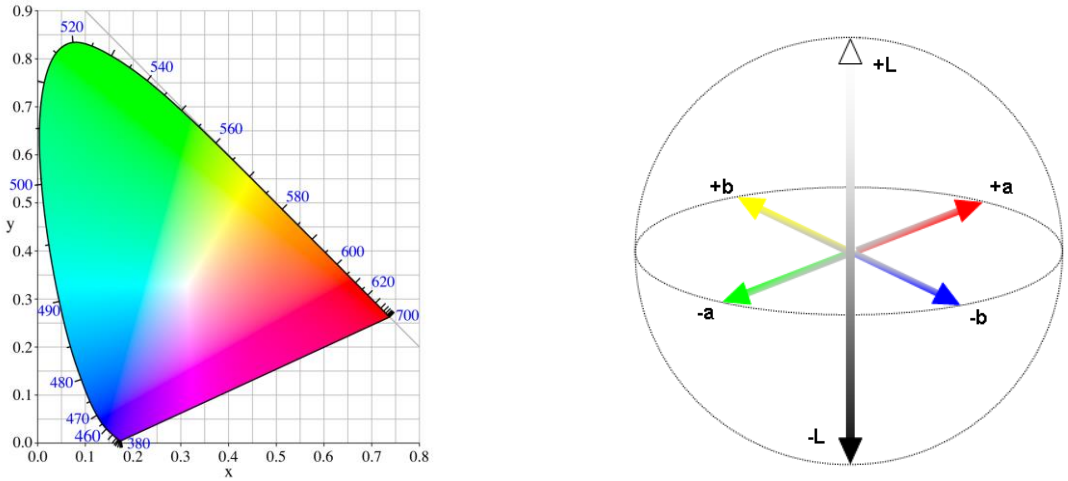

Figure 16: *CIE xyz and CIE L*a*b* color space [55].*

2.4.3.3 CIE 10° standard observer function

The perception of color depends also on the angle at which the object is observed. Thus, in order to quantify the perception of colors mathematically, the term standard observer was defined. There are two common standard observers: one at 10° and other at 2°. CIE proposed 10° Standard Observer in 1960 based on matching data for a matching field of 10° angular subtenses, ignoring the central area because of its difference in appearance from the rest of the field. A 10° visual field represents a diameter of about 90 mm at a viewing distance of 0.5 m. These color-matching functions are given in the standard as values from 360 nm to 830 nm at 1 nm intervals with six significant figures, and they define the CIE 1964 standard colorimetric observer. Alternative is the 2°standard observer, used in other color systems [53].

2.4.3.4 CIE Standard illuminants, illuminant D65

There are different types of illuminants, and each illuminant is composed of various wavelengths of visible light. The most common illuminants include illuminant A (intended to represent typical, domestic, tungsten-filament lighting, with correlated color temperature of approximately 2856 K.), illuminant C (north sky daylight, tungsten illumination that simulates the average daylight, with a temperature of 6774 K), D55, D65, and D75 (series of distribution curves of the illuminant spectral power based on the measurements of the diurnal light natural). The values are defined for the wavelength ranging from 300 to 830 nm. The illuminant D65 is the most commonly used daylight illuminant and the average of noon daylight from around the world, with correlated color temperature of 6504 K.

The illuminant D65 and the 10°Standard Observer are considered to be references. The CIE has recommended replacing the illuminant C with the illuminant D65, which is a more appropriate representation of daylight, and using the CIE 1964 supplementary standard observer (10° observer), representing the best average spectroscopic response from human observers [53].

2.4.3.5 Total color difference

Additionally, the values from the total color difference (ΔE*), the chroma (C*ab) and the hue (hab) provide valuable information on respective color characteristics. The ΔE* is important when evaluating the relationship between the visual and the numerical analyses; it is calculated as the

Euclidean distance between two points in a three-dimensional space defined by L*, a* and b*. It is numerically defined by the equation:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \tag{33}$$

Depending on the value of ΔE, the color difference between the treated and untreated samples could be estimated such as not noticeable (0 – 0.5), slightly noticeable (0.5 – 1.5), noticeable (1.5 – 3.0), well visible (3.0 – 6.0) and great (6.0 – 12.0) [56].

2.4.3.6 Use of colorimetry in food industry

Color of foods is one of the main attributes that is strongly associated with the concept of quality. The color of orange juice is moreover, directly related to the consumer's perception of flavor, taste (sweetness, sourness) and, thus, overall acceptability. Although the color can be evaluated through the visual analysis, it is pertinent to determine it objectively, since visual assessment is subjective and can be biased. Moreover, color measurements are valuable in estimation of the measure of color degradation, thus, predicting both chemical and quality changes of foods [22].

2.5 HPLC

High performance liquid chromatography is analytical separation method, where mixtures of sample components are separated on column in the flowing system. Liquid mobile phase is mechanically pumped through a column filled with stationary phase. The separation is based on interaction of the mixture components between the stationary and mobile phase. Separated components are analyzed by the proper detector on the way out of column. Types of HPLC are classified by the mechanism of interaction between the sample components in mobile phase and stationary phase. In this approach, HPLC is generally divided into five separation mechanisms: adsorption (with normal and/or reverse phase), partition, size exclusion, affinity and ion exchange [57].

In normal phase chromatography, analyte is adsorbed to the stationary phase by hydrogen bonding or polar interactions. Stationary phase is more polar then the mobile phase. Silica and alumina are mainly used as stationary phases, as they are compatible with non-aqueous mobile phase. These phases are suitable for separation of organic compounds [58].

In reverse phase chromatography, analyte is adsorbed to stationary phase by hydrophobic effect. Stationary phase is less polar then the mobile phase. Stationary phase is usually modified silica.

Size exclusion chromatography uses gel with pores of defined size distribution as stationary phase. Stationary phase acts as molecular sieve. Analytes larger than pores are excluded from entering the pores and travel through the colon faster compared to smaller molecules [58].

Affinity chromatography is based on binding of analyte to specific stationary phase. Extremely specific stationary-phase-analyte interactions are described as molecular recognition to indicate that the stationary phase binds one compound to the virtual exclusion of all others. However, capability of individuating more compounds is lower compared to the conventional HPLC methods. For this reason, affinity chromatography is mostly used as preparative technique [58].

In ion exchange chromatography, analyte is adsorbed on stationary phase of opposite charge due to the electrostatic interactions. Ions from mobile phase selectively displace components of the analyte from adsorption to the stationary phase. Ion exchange chromatography is used to separate charged analytes [57].

2.5.1.1 Pumps

Pumps with appropriate performance characteristic (i.e., flow rate reproducibility, flow rate range, pressure stability) are required for HPLC. There are two commercially available types of pumps suitable for HPLC. First type is reciprocating piston pump which use one or two pistons to create

pressure. Pistons are usually made from abrasion resistant material (sapphire). Use of two pistons allows one cylinder to recharge while other cylinder maintains the pressure. Two pistons setup requires use of check valves to ensure that flow is unidirectional and to prevent drop in pressure during the recharge cycle of one cylinder. Main disadvantage of this setup is pulsating flow [58]. To minimize pulsations, pulse dampers are often used. These devices are long narrow tubes folded on them many times. Energy is stored in volume of pulse dampers during the mobile phase delivery stroke. Mobile phase is accumulated and discharged this way and flow fluctuations are smothered out [57].

Second type of pump is syringe pump, which is basically large cylinder with piston. This type of pump creates pulseless flow of mobile phase. Main disadvantage is limitation by the volume of cylinder, because no flow occurs during refill the cycle.

2.5.1.2 Columns

There are generally two types of columns used in HPLC: packed bed and open tubular. Packed bed columns are most used in HPLC, since they have more general use than the open tubular columns. Column must be constructed of material that withstands pressures used in HPLC and is chemically resistant to mobile phase.

Packed bed columns usually have diameter of 1 to 10 mm, size of particles is usually 3 to 10 μm. Inner diameter affects the sample load, peak dilution and flow rate. The larger the inner diameter is, the greater is the loading capacity and higher is the flow rate. On the other side, peak dilution increases with internal diameter, thus the sensitivity decreases. Column length affects efficiency and speed of the separation. The longer the column is, the better efficiency, but analysis requires more time to be completed. The particles are typically made of incompressible materials, such as silica, alumina, graphitic carbon or rigid polymers. Type of material also affects the selectivity, capacity and efficiency of column [57, 58].

2.5.1.3 Detectors

Detector transforms the changes in column effluent into an electrical signal that is recorded by the data system. Depending on their properties, detectors are classified as selective or universal. Detectors can be also classified as destructive or non-destructive. Selective detectors measure the physical or chemical property that is characteristic to the analyte in mobile phase. On the other hand, universal detectors measure the physical property of the mobile phase.

Most of commonly used detectors in HPLC are based on refraction of light and absorption of UV-VIS light. If post-column reactor is employed, analyte undergoes the chemical reaction, and product can be detected by light absorbance or fluorescence. Other types of detectors are based on light scattering or viscosity measurements. Electrochemical detectors measure the current across the electrodes in flow cell and conductivity detectors measure the change in conductance of mobile phase between the electrodes. Mass spectrometer can be used as universal detector, if the mobile phase is evaporated on the way out of the column [57, 58].

3 AIMS OF THE DIPLOMA THESIS

Increasing consumer's demands towards high quality and safe food products push their producers to increase the production and qualitative standards of the products. Recently, innovations in juices production resulted in modification of production conditions. They comprise the modification of composition, as the modern trends in fruit juices production are based on the addition of fruit pulp and/or small fruit pieces, and also innovations in packaging materials/packaging technology in order to maintain the maximum of nutritional quality and valuable components and to prolong the shelf life and sensorial quality of juices. Beside the above-mentioned modifications, it is presupposed that the production atmosphere may also influence the qualitative parameters of juice products.

In this diploma thesis, the effects of modified production atmosphere application (argon, nitrogen, CO_2) on selected qualitative parameters of fresh orange and pineapple juices will be studied. Beside the effects of production atmosphere, also the effects of long-term storage of the juices at defined – controlled conditions (temperature, light conditions) will be assessed.

Main attention will focus on antioxidant properties, the concentration of ascorbic acid, total polyphenols and selected flavonoids. Beside these, color characteristics will be studied in CIE L*a*b* system, involving several EPR, UV-VIS and HPLC assays.

The results will also be processed by means of multivariate statistical methods in order to consider the mutual relationships between the experimental characteristics as well as the effects of modified atmosphere application, samples origin and seasoning effects on quality of fruit juices.

4 EXPERIMENTAL PART

4.1 Samples

Fruit juices were provided by McCarter a.s. Dunajská Streda, reputable Slovak fresh fruit juices producer. Juices were cold-processed and treated by light pasteurization (95 °C, 20 s). Analyzed juices were processed both with, and without the modified atmosphere application during the processing. After the pasteurization, juices were aseptically filled into the PET bottles and closed with cap containing oxygen scavengers (*SK38/16-O2S;* BeriCap, Budenheim, Germany).

Table 1: *Overview and basic characteristics of the analyzed samples.*

Sample label	Raw material	Year of production	Modified atmosphere	Country of origin	Manufacturing date	Expiration date
CA13	Orange	2013	Ar	Mix of Brazil and Costa Rica	11.11.2013	11.3.2014
CO14	Orange	2014	O_2	Costa Rica	21.5.2014	24.9.2014
CN14	Orange	2014	N_2	Costa Rica	21.5.2014	24.9.2014
CC15	Orange	2015	CO_2	Costa Rica	16.1.2015	15.5.2015
CN15	Orange	2015	N_2	Costa Rica	16.1.2015	15.5.2015
AN14	Pineapple	2014	N_2	Brazil	20.5.2014	16.9.2014

4.1.1 RIO Fresh 100% juice from freshly pressed oranges with pulp

Manufacturer declared RIO Fresh 100% juice from freshly pressed oranges with pulp average nutrition facts in 100 ml.

Table 2: *Nutritional and compositional characteristics in 100 ml of orange juice with pulp declared on the label.*

Parameter	Value
Energy value	188 kJ / 45 kcal
Protein	< 1.0 g
Total carbohydrates	10.4 g
Sugars	10.4 g
Total Fats	< 0.5 g
Saturated fats	< 0.5 g
Dietary fiber	< 3 g
Sodium	< 0.025 g
Vitamin C	36 mg

4.1.2 RIO Fresh 100% juice from freshly pressed pineapples

Manufacturer declared RIO Fresh 100% juice from freshly pressed pineapples average nutrition facts in 100 ml

Table 3: *Nutritional and compositional characteristics in 100 ml of pineapple juice declared on the label.*

Parameter	Value
Energy value	216 kJ / 51 kcal
Protein	< 0.4 g
Total carbohydrates	11.6 g
Sugars	11.6 g
Total Fats	< 0.1 g
Saturated fats	< 0.1 g
Dietary fiber	< 1g
Sodium	< 0.05 g

Bottled samples of respective fruit juice samples (from the same batch) were delivered to the laboratory immediately after their production in sufficient number and stored at defined conditions (7 ± 2 °C) in darkness in the refrigerator between the experiments,

Generally, kinetic of changes of the individual parameters was evaluated, on the basis of repeated measurements performed in regular time intervals (2 times a week, at minimum). For the purposes of quantitative comparison of the observed changes, the experimental characteristics of the samples recorded immediately after the juice production, at the time of their expiration at 120 ± 5 days (4 months, as declared by the producer), and 1 month after the expiration were taken into consideration, respectively. However, for practical reasons, and recognizing that part of the experiments are still in progress at the time of the diploma thesis finalization, the values of TPC (but also of other evaluated parameters) recorded at $90^{th} \pm 5$ days after the juice production were taken into consideration. These data were also taken for comparison of the evaluated effects.

4.2 Materials and equipment

4.2.1 Chemicals

- Gallic acid (Merck, Germany)
- Folin - Ciocalteu´s reagent (Microchem, Slovak Republic)
- Sodium carbonate anhydrous p.a. (Slavus, Slovak Republic)
- Deionized water (Rodem 6, average conductivity up to 1 μScm^{-1})
- 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) − ABTS (Polysciences, United Kingdom)
- L-ascorbic acid, p.a. (Lachema, Czech Republic)
- ethanol (AFT, Slovak Republic);
- 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL, Sigma Aldrich Ltd, Germany)

4.2.2 Instrumentation

- Refrigerator with freezer (Electrolux, Sweden)
- Analytical scale AP-110 (Ohaus, Sweden)
- Cooled laboratory centrifuge 2-16 KC (Sigma, Germany)
- UV-VIS-NIR spectrophotometer Schimadzu 3600 with accessories (Schimadzu, Japan)
- X-band portable EPR spectrometer e-scan with accessories (Bruker, Germany)

4.3 Preparation of samples prior the measurement

Respective fruit juice samples were centrifuged at 10 000 g and at 5 °C for 10 minutes in order to separate the solid matter before the analysis. The supernatants were stored at 7 °C in the darkness between the experiments. Sample was always prepared from new bottle/bottles just prior the measurement. All experiments were performed in duplicates, if not specified distinctively.

4.4 UV-VIS experiments

UV-VIS experiments were performed on UV-VIS-NIR spectrophotometer Schimadzu 3600 with accessories (Schimadzu, Japan). Total polyphenolic compounds (TPC) concentration was estimated by modified Folin - Ciocalteu method. Color characteristics in CIE L*a*b* color system were calculated by processing the absorbance spectra of respective sample by ColorLite Panorama Shimadzu software (LabCognition Analytical Software, Köln, Germany).

4.4.1 Total polyphenols

Total polyphenols concentration was estimated following the modified Chaovanalikit and Wrolstad method with application of Folin -Ciocalteu reagent [59]. Calibration curve was constructed from standard solutions of gallic acid. Results were expressed as gallic acid equivalent per liter (GAE).

Briefly, 200 µl of sample, 15.8 ml of deionized water and 1 ml of Folin - Ciocalteu reagent was mixed in vial. Mixture was gently stirred and left for 10 minutes to undergo the reaction. After that, 3 ml of 20 % (v/v) sodium carbonate in water was added and color reaction. After one hour, the absorbance of samples was measured at 765 nm against water, serving as the reference. Calibration curve with concentration range of 0–2000 $mg \cdot l^{-1}$ was constructed using the same method, using solutions of gallic acid of known concentration instead of sample. Total polyphenols were determined from gallic acid calibration curve, depicted on *Figure 17* automatically, by means of the UV-probe measurement software. The regression coefficient of the calibration curve was R^2=0.99902.

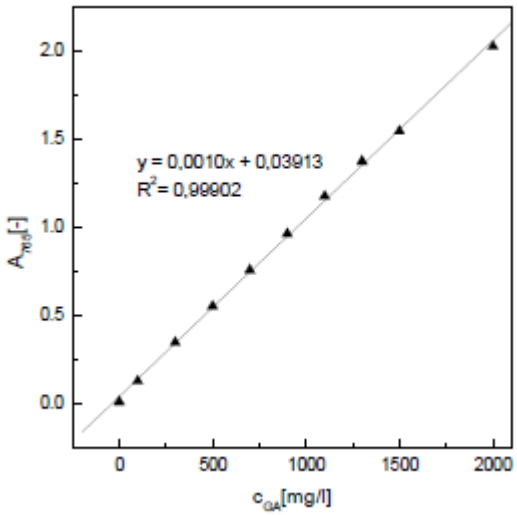

Figure 17: *Gallic acid solutions calibration curve - dependence of absorbance at 765 nm on gallic acid solution's concentration.*

4.4.2 Color characteristics in CIE L*a*b* color space

Samples were measured in quartz cuvettes (Hellma 100-QS-Suprasil, Sigma Aldrich; 1 cm optical path). CIE L*a*b* color characteristics of prepared samples were obtained by measurement of visible range spectra (380-780 nm) against deionized water as a blank. The measurements were performed in spectral range from 380 nm to 780 nm, sampling interval was 2 nm, slit width was 0.1 nm. Color

space descriptors were calculated from the measured absorbance spectra by ColorLite Panorama Shimadzu software (LabCognition Analytical Software, Köln, Germany). The D65 day light illuminant and 10° standard observer angle software settings were used for calculation of color descriptors. CIE L*a*b* values, chromaticity and hue angle were used for evaluation of color characteristics of samples and its changes following from different production atmosphere application and/or during the post-production storage. CIE *L*a*b** values were subsequently used to calculate the total color difference values.

4.5 EPR experiments

4.5.1 Parameters of EPR analysis

For EPR measurements, portable EPR spectrometer e-scan (Bruker, Germany) with accessories was used. Sample preparation procedure is specified above, details on individual radical solutions composition are given bellow at individual assays description.

The entire measurements were realized with flat quartz cell designed for EPR experiments with polar solutions. Syringe with internal volume of 2 ml was fitted to the top of the flat cell for improved and accurate filling of the cell with the measured sample. Sample preparation procedure is specified above, details on individual radical solutions composition are given bellow at individual assays description.

When filled, the cell was placed into the cavity of EPR spectrometer, fixed rigorously and measurement parameters were set. Recording of EPR spectra started exactly 3 minutes after mixing the sample with respective radical solution and series of 10 EPR spectra were recorded for total of 15 minutes. Every single spectrum represents an accumulation of 30 individual scans.

Typical parameters of EPR measurements:

- Central field: 346.5 mT ($ABTS^{\bullet+}$)/346.1 mT (TEMPOL)
- Magnetic field sweep width: 10 mT
- Modulation amplitude: 0.05 mT
- Receiver gain: $3.99 \cdot 10^3$
- Microwave radiation power: 6 mW
- Microwave frequency: 9.71 GHz
- Time difference between the spectra: $\Delta t = 1.5$ min
- Time constant: 10.24 ms
- Length of 1 scan: 2.62s
- Number of scans: 30
- Number of scanned spectra: 10
- Total time of measurement: 15 min

4.5.2 Evaluation of EPR spectra

The recorded time-evolutions of EPR spectra were processed as follows: As the EPR spectrum represents 1st derivation of an absorption signal, in order to quantify the concentration of radical species, the recorded spectra were double-integrated by means of WinEPR (Bruker) software after their transformation into the absolute values form (standardized procedure offered by the software).

For the purposes of quantification of radical-scavenging action of respective sample, the value of double integral of 5th EPR spectrum, which was recorded exactly 10.5 min after mixing the respective reagents. This spectrum was selected as the compromise – in the middle of the time series, to the lowest measure influenced by potential problems with apparatus or measurement itself.

Spectra or integral values in case of kinetic measurements were depicted by means of Origin (MicroCalc) software. The fit of experimental data (double integrals of the spectra) to the respective

kinetic equations was performed by means of Scientist (MicroMath) software, or by fitting it to the proper mathematical function directly in Origin software.

4.5.3 Ascorbic acid equivalents determination

In TEMPOL assay, 200 µl of respective sample (or distilled water, in case of reference measurement) was mixed with 900 µl of 1.10^{-5} mol.l^{-1} TEMPOL solution and the mixture was purged with 2ml of air, in order to mix the reagents perfectly. Then the solution was placed into EPR cell and the measurement was performed as indicated above.

Ability of fruit juices to terminate TEMPOL radical was evaluated in terms of ascorbic acid equivalents (AAE). AAE values were evaluated using the calibration curve constructed from standard solutions of AA with concentration ranges from 0 to $1.8 \cdot 10^{-3}$ mol·l^{-1}. The calibration curve with fitting parameters is depicted on *Figure 18*. For AAE evaluation, double-integrated fifth EPR spectrum of the respective sample, recorded exactly 10.5 minutes after the measurement start was used.

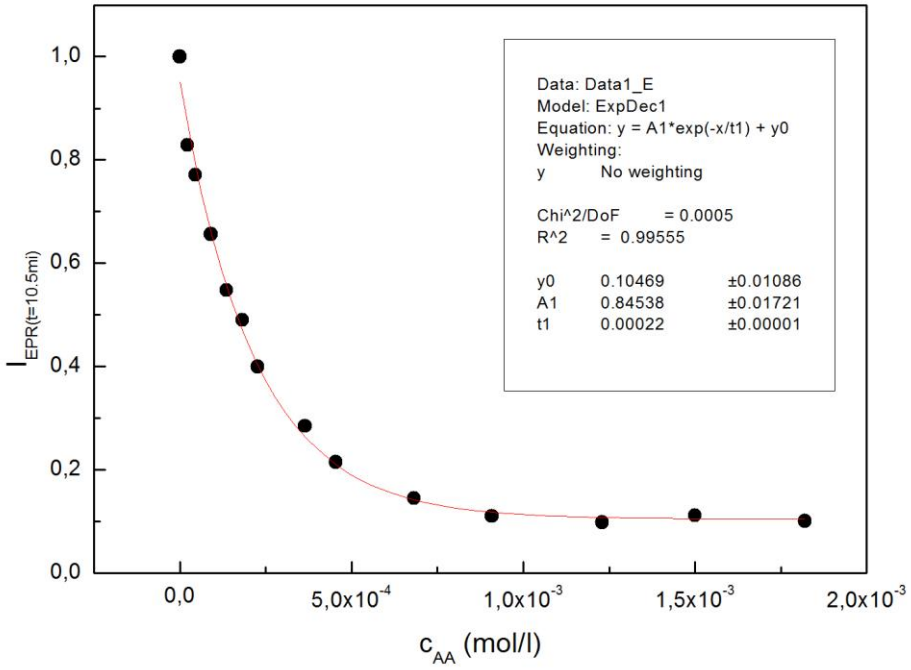

Figure 18: *Ascorbic acid calibration curve, used for ascorbic acid equivalents evaluation in TEMPOL assay.*

4.5.4 TEAC assay

The ability of fruit juice samples to terminate $ABTS^{\bullet+}$ cation radical was tested as follows: 300 µl of the respective juice sample (distilled water in case of reference) was mixed with 700 µl $ABTS^{\bullet+}$ solution with concentration of 0.1 mmol·l^{-1}., concentration of $ABTS^{\bullet+}$ was checked by the measurement of its absorbance at 734 nm by means of UV VIS (molar absorption coefficient of $ABTS^{\bullet+}$ in water is $\varepsilon = 14.8$ mol^{-1}·dm^3·m^{-1}). Reaction mixture was afterwards purged with 2 ml of air and filled into the flat EPR cell.

ABTS$^{\bullet+}$ radical-scavenging activity of samples was expressed Trolox-equivalents ($TEAC_{ABTS\bullet+}$), using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid as the reference standard antioxidant as previously described by Polovka et al. (2010) [60]. For $TEAC_{ABTS\bullet+}$ values calculation, the following formula was used:

$$TEAC_{ABTS^{\bullet+}} = \frac{\left(c_{0(ABTS^{\bullet+})} - c_{t(ABTS^{\bullet+})}\right) \cdot V_{(ABTS^{\bullet+})}}{V_{sample}} \cdot \nu \cdot F \quad (34)$$

Where $c_{0(ABTS\bullet+)}$ is the initial concentration of ABTS$^{\bullet+}$, $c_{t(ABTS\bullet+)}$ is the concentration of ABTS$^{\bullet+}$ after mixing with sample at given time t (t = 10.5 min), $V_{(ABTS\bullet+)}$ is volume of ABTS$^{\bullet+}$ solution, V_{sample} is volume of sample, ν is the stoichiometric coefficient of the reaction between ABTS$^{\bullet+}$ and TROLOX (ν = 1/2), F is the dilution factor.

4.6 HPLC measurements

Ascorbic acid and hesperidin concentration were determined by means of HPLC, involving Agilent Technologies 1100 Series chromatograph (Agilent Technologies) equipped with diode array detector (DAD), quaternary pump, degasser, column thermostat and autosampler. Juice samples were diluted tenfold and twentyfold, filtrated through a 0.45 μm syringe filter and subsequently analyzed on HPLC. The separation conditions and conditions of analysis were identical to these, previously described by Sádecká et al. (2014) [61].

4.7 Statistical analysis

Statistical calculations were performed by means of Unistat v. 6.0 (Unistat, London, United Kingdom) statistical package. Analysis of variance (ANOVA) and multivariate statistics were used to compare, explore and discriminate the complex dataset of experimental characteristics.

Multiple comparisons were performed by ANOVA Tukey's HSD test at the level of significance of $P \leq 0.05$. The differences in means of individual compared characteristics were recognized as highly significant at $P < 0.001$.

Principal component analysis (PCA) and canonical discriminant analysis (CDA) were used in order to define, interpret and visualize the differences between the compared juices in terms of production atmosphere effects, seasoning effect and/or sample origin. Using the CDA, the recognition ability was calculated as the percentage of correctly classified samples in the original data set in which all the samples were of known properties for the classification model.

5 RESULTS AND DISCUSSION

5.1 UV-VIS experiments

5.1.1 Total polyphenol content

Total polyphenol content is traditionally accepted as a basic parameter of samples of natural origin characterization, particularly in connection with antioxidant or radical-scavenging properties of the evaluated samples. In the experiments, the effect of production atmosphere on TPC content as well as the effect of long-term post-production storage on the concentration of total polyphenols was characterized. Where the raw, non-pasteurized and pasteurized samples were available, also the effect of pasteurization on TPC content was assessed.

For the purposes of quantitative evaluation, in recognition of differences followed from variability in raw juices (different country of origin, seasoning variance), the data cannot be compared on the absolute basis, just on the basis of relative change - in relation to the values determined for freshly produced juices (at the time of start of the experiment). Thus, the difference in the concentration of TPC, Δ value, representing the difference in TPC content of freshly produced sample and sample stored for 90 days was calculated. In addition, *% change*, representing the ratio of Δ change to the TPC value of freshly prepared sample was evaluated as follows:

$$\%change = \frac{\Delta i}{V_{0i}} * 100 \tag{35}$$

Where Δi represents difference of value of respective parameter at 90th (V_{90i}) day of storage and its value in the beginning of the storing experiment ((V_{0i})

$V_t i$ is the value of respective characteristics/parameter – at chosen time *t*

Table 4: *GAE values of juice samples evaluated at the respective time of their storage.*

Sample label	GAE (mg·l^{-1})					
	Start of experiment	**90th day of storage**	**Expiration date**	**End of experiment**	**Δ**	**% change**
CA13	619.38 ± 1.96	511.33 ± 4.71	498.88 ± 6.44	487.06 ± 15.55	108.05	17.4
CN14	725.76 ± 18.54	642.61 ± 10.44	653.03 ± 1.23	599.89 ± 1.89	83.15	11.5
CO14	707.78 ± 16.69	595.91 ± 11.39	618.30 ± 5.31	551.89 ± 1.44	111.87	15.8
CN15	683.93 ± 4.72	609.06 ± 3.50	N/A*	N/A*	74.87	10.9
CC15	674.78 ± 4.46	610.91 ± 2.15	N/A*	N/A*	63.87	9.5
AN14	716.22 ± 7.84	682.95 ± 1.31	663.01 ± 4.49	650.22 ± 3.01	33.27	4.6

* Experiments are still in progress, to the date of diploma thesis completion, only the data for 90th day of storage have become available.

TPC values recorded for orange and pineapple juice samples of different production atmosphere at the respective time of their storage are summarized in *Table 4*. Gradual decrease of TPC during storage was observed, in accord with expectations, probably as a result of the oxidation and polymerization of polyphenols, thus reducing the number of free hydroxyl groups measured by the Folin-Ciocalteu assay [62].

The observed trends are in more details described below, separately for the orange juices and pineapple juices, respectively.

5.1.1.1 Orange juices with pulp

As follows from the overview of the sample characteristics summarized in *Table 1*, orange juices differs by several production factors, including year of production, origin and the production atmosphere applied.

Effect of the storage

Changes of TPC in orange juices produced in argon atmosphere resulting from their post-production storage are depicted on *Figure 19*. Orange juices were produced in 2013, exhibiting TPC of 619.38 ± 1.96 mg·l^{-1} at the start of experiment, while at the 90th day of storage, the decrease to 511.33 ± 4.71 mg·l^{-1} was noticed, representing 17.44 % decrease. In comparison to other production atmosphere discussed below, utilization of Ar modified atmosphere resulted into the largest decrease of TPC during the storage. The observed decrease is, however, in contradiction to other experiments aimed at the application of Ar modified atmosphere, since the application of argon generally leads to slowdown of the degradation of TPC in solid foodstuff [63-65]. Effect of argon on degradation of food components is based on its solubility in water and inhibition of enzymes related to browning and respiration [64]. Low effect of Ar modified atmosphere in this study can be caused by application of maximally atmospheric pressure during the processing of juices while the other studies used high pressure Ar treatment [63-65].

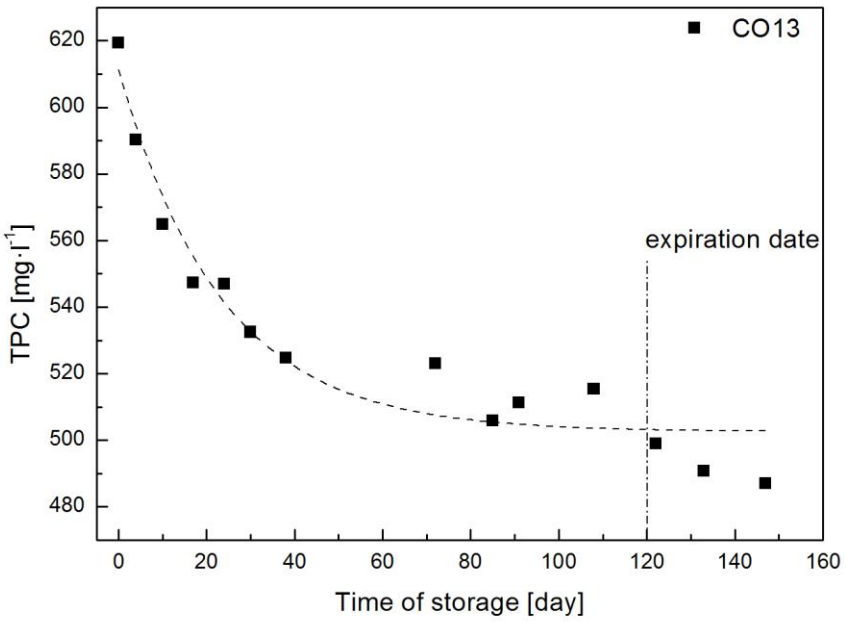

Figure 19: *Changes of TPC during the storage of orange juices processed under Ar modified atmosphere (CA13, Table 1). Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

Unfortunately, the producer did not provided the sample of identical quality produced under the conventional, air, conditions, thus the observed changes cannot be compared with any other sample under study.

In 2014 the orange juices were produced with the application of nitrogen atmosphere and under the air – i.e., with conventional unmodified procedure. Thus, the effect of long-term storage on their TPC content was assessed in terms of comparison of the effect of atmosphere modification on the monitored characteristics.

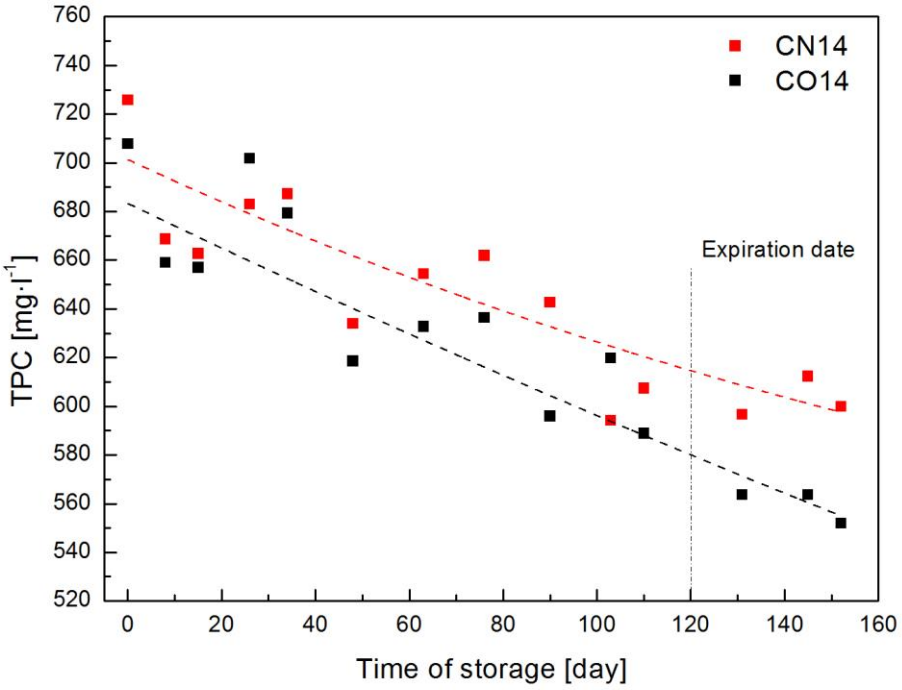

Figure 20: *Changes of TPC during the storage of orange juices CO14 produced under the conventional air atmosphere and orange juices CN14 processed under N_2 modified atmosphere. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

Results obtained are depicted on *Figure 20*. As clearly follows from the presented results, initial TPC concentration of juices produced by conventional technology reached the value of 707.78 ± 16.69 mg·l^{-1}, whereas that of samples produced under N_2 atmosphere 725.76 ± 18.54 mg·l^{-1}. These values were detected after the pasteurization (see below the pasteurization effect discussion). At 90th day of storage, taken as a reference day for the storage effects evaluation, TPC of juices produced under air and under N_2 modified atmosphere decreased by 15.8 % to 595.91 ± 11.39 mg·l^{-1} and by 11.5 % to 642.61 ± 10.44 mg·l^{-1}, respectively. In addition, at the end of the monitored period, TPC of juices processed under conventional atmosphere decreased to 551.89 ± 1.44 mg·l^{-1}, while TPC of juices processed under N_2 atmosphere decreased to only 599.89 ± 1.89 mg·l^{-1}. These values represent approx. 77% and approx. 83% of their initial values.

Thus, it is apparent, that the application of N_2 modified atmosphere resulted in slower and lower decrease of TPC (in other words, protection against their partial oxidation) during the storage then the decrease of TPC in conventionally processed juices. These results are in good agreement with the expected trends and also with our previous studies dealing with the effect of modified atmosphere on TPC of fruit juices [37, 61].

Juices produced in 2015 were as a pilot test study of the producer, prepared with application of CO_2 and N_2 modified atmosphere, while the conventionally produced samples were not provided due to practical problems with parallel production of samples under the three different atmospheres. Thus, in these experiments, the effectiveness of individual inert gases application in terms of the evaluated parameters changes was evaluated, taking into consideration also other factors, e.g., potential physical or physicochemical interactions of the samples with the inert gas was considered as discussed below.

Changes of TPC during the long-term storage of orange produced under N_2 and for those samples produced under CO_2 atmosphere are depicted on *Figure 21*.

Initial TPC concentration was found to be 683.93 ± 4.72 $mg \cdot l^{-1}$ for CN15 samples produced under N_2, and 674.78 ± 4.46 $mg \cdot l^{-1}$ for those produced under the CO_2. Fresh juices produced under N_2 modified atmosphere thus exhibited TPC concentration higher by 9.15 $mg \cdot l^{-1}$.

At the 90th day of storage, TPC concentration of CN15 samples decreased by 10.9 % to 609.06 ± 3.50 $mg \cdot l^{-1}$ and of C15 samples decreased by 9.5 % to 610.91 ± 2.15 $mg \cdot l^{-1}$. It is apparent, when considering also the measurement uncertainty (or the values of the standard deviations), that the values at the start of experiments and at the 90th day of storage are comparable or only small differences, statistically insignificant, were noticed. Thus, as regards the effect of modified atmosphere application on TPC values, both, N_2 and CO_2 revealed the comparable effectivity in protecting the polyphenols during the storage.

Comparison with other here-in presented results is problematic, as CN15 and CC15 samples had generally lower initial TPC concentration in comparison with CO14 and CN14 juices produced in year 2014. Respecting the fact, that all of these samples had identical country of origin, this decrease is probably caused by the seasoning effect, i.e., factors that occurred during the growth of orange fruit. These factors are partially discussed below in the statistical evaluation of the results.

As the general conclusion on the effects of production atmosphere and storing on TPC concentration of orange juices with pulp, it can be stated that:

- It is obvious that application of modified N_2 or CO_2 atmospheres positively influence the TPC in comparison to processing without the modified atmosphere.
- Our results indicated that the application of Ar atmosphere led to the most significant decrease in TPC, also when compared with conventionally produced juices.

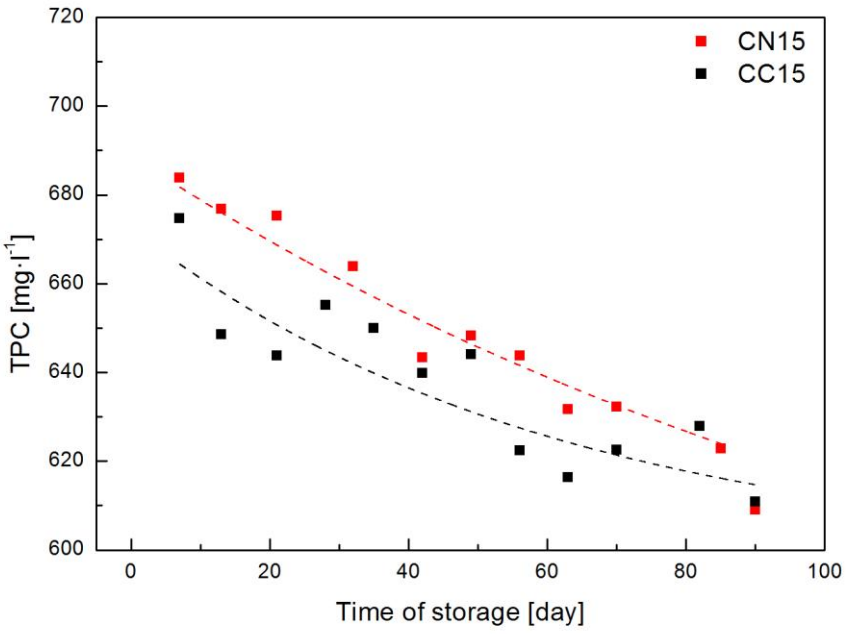

Figure 21: *Changes of TPC during the storage of orange juices CC15 processed under CO_2 modified atmosphere and orange juices CN15 processed under N_2 modified atmosphere. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

However, objective evaluation must consider also the effects of fruit origin, season of production and also the effects of price and other factors with effect on overall changes of juices properties, including the sensorial quality.

In the context of the last mentioned factors, application of N_2 modified atmosphere can be recommended in terms of TPC protection. N_2 has low solubility in water, and its effect on decreasing oxidative rancidness and inhibiting the growth of aerobic microorganisms is based on displacing the oxygen from the juice during processing [66]. Use of CO_2 modified atmosphere was very effective in preservation of TPC, practically comparable to the effects reached by N_2. However, organoleptic properties of juices processed under CO_2 are different from untreated juice, because in fact, carbonated juice is created, probably as the effect of good solubility of CO_2 in water systems and/or potential subsequent interactions of the dissolved gas with juice components.

Effect of pasteurization

As in case of orange juices of 2014 season the juice producer provided both, raw unpasteurized and pasteurized sample of juices, the impact of pasteurization on TPC was also studied at these juices. Influences of pasteurization on TPC are summarized in *Table 5*.

Table 5: *Impact of modified atmosphere production and pasteurization on GAE values of orange juices. Sample characteristics and labels correspond to those in Table 1.*

Sample label	GAE [mg·l^{-1}]	Δ_{GAE} [mg·l^{-1}]	% change
CO14-Raw	723.36 ± 5.94	15.58	2.15
CO14-Pasteurized	707.78 ± 16.69		
CN14-Raw	727.98 ± 9.15	2.22	0.30
CN14-Pasteurized	725.76 ± 18.54		

Raw juice samples were characterized by comparable content of total polyphenols, clearly proving that the samples comes from 1 common batch, as the results obtained are within the same confidence interval without respect on the production atmosphere. As a result of pasteurization, compared on absolute basis, the TPC concentration of conventionally produced juices (under the air) decreased by 15.58 mg·l^{-1}, i.e., in relation to the raw sample, by 2.2 %. On the contradiction, TPC of raw unpasteurized fruit juice produced under the nitrogen atmosphere decreased only by 2.22 mg·l^{-1}, i.e., by 0.30 %. Thus, partial conclusion may be done, that processing of juices under N_2 modified atmosphere revealed the protection effect on TPC content during the pasteurization.

5.1.1.2 Pineapple juices

The effect of modified atmosphere application was also evaluated on samples of pineapple juices. Unfortunately, the producer had not the capacity to provide the sample of the same quality produced simultaneously in different atmosphere. Only the samples prepared under the nitrogen were delivered. Thus the results obtained on pineapple juices presented in this diploma thesis are just descriptive aimed at the illustration of the effects of between-fruit variability on selected characteristics.

Changes of TPC during the storage of pineapple juices produced in 2014 under nitrogen atmosphere are depicted on *Figure 22*. As clearly follows from the depicted results, initial concentration of total polyphenols reached 716.22 ± 7.84 mg·l^{-1}, while at the 90th day, TPC decreased by 4.6 % to 682.95 ± 1.31 mg·l^{-1}. At the end of experiment additional decrease of TPC to 605.22 ± 3.01 mg·l^{-1} was noticed.

As regards the comparison on absolute values, pineapple juice had generally higher concentration of polyphenols than the orange juices under study, caused probably by different composition and concentrations of individual polyphenols in respective fruits. Also other factors, such as season, origin or way of fruit processing should be considered, what is due to the reasons described above, impossible.

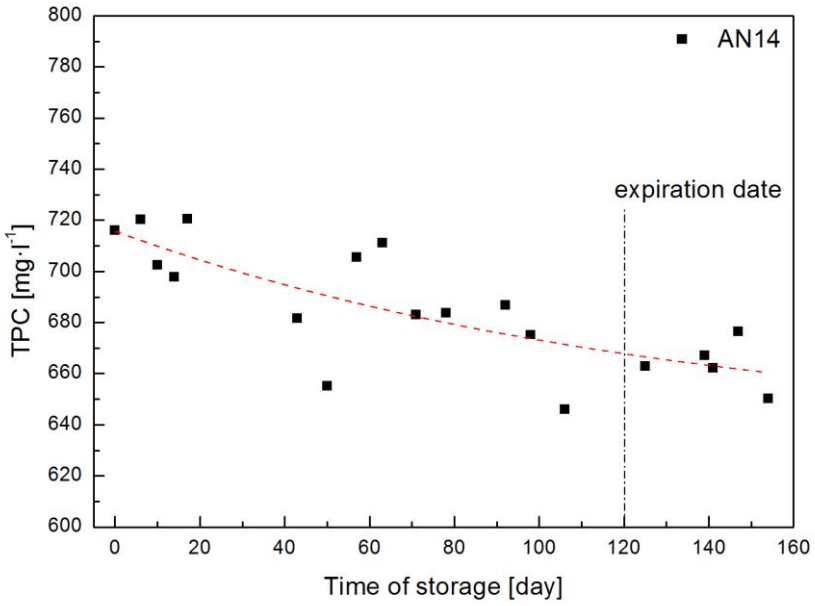

Figure 22: *Changes of TPC during storage - AN14 Pineapple juice processed under N_2 modified atmosphere. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

5.1.1.3 Kinetic evaluation

For evaluation of TPC content decrease, kinetic rate constant k_{TPC} was calculated by fit of experimental data to the model of 1st order kinetic reaction, using the equation:

$$c_t = c_0 \cdot e^{-kt} + b \tag{36}$$

Where c_0 is the initial concentration/value of the evaluated characteristics, c_t is the concentration/value of the characteristics at respective time of storage t, k is the formal first-order rate constant and b is the equation parameter. Half-life of TPC decrease ($t_{1/2(TPC)}$) was also calculated from k_{TPC} values, on the basis of first-order kinetics approach as follows:

$$t_{1/2} = \ln 2 / k \tag{37}$$

Calculated values of 1st order rate constants of TPC decrease of juice samples and $t_{1/2}$ are summarized in *Table 6*.

From all the kinetic models tested, the model of 1st-order kinetics seemed to be the proper option. In fact, the pseudo-1st order kinetic was evaluated in all the experiments presented in the diploma thesis, as there were at least two reactants in the mixture (esp. in antioxidant properties testing assays – antioxidant/s and radical). However, if one of the components concentration exceeds the concentration of the another, then the simplified model of pseudo-1st order kinetics may be used.

Table 6: *Calculated values from 1st order kinetic model of TPC decrease.*

Sample label	k_{TPC} [day^{-1}]	R^2	$t_{1/2(TPC)}$ [day]
CA13	0.043 ± 0.005	0.993	16.196
CN14	0.001 ± 0.001	0.964	652.405
CO14	0.001 ± 0.001	0.959	507.000
CN15	0.007 ± 0.007	0.989	92.541
CC15	0.015 ± 0.020	0.991	46.872
AN14	0.007 ± 0.010	0.988	92.442

Kinetic rate constants are in good correlation with changes in TPC observed during the storage of juices. However, kinetic evaluation is rather complicated by the fact, that the calculation is affected by the variability of the experimental characteristics. Also the factors as fruit origin, season of production must be considered.

5.1.2 Color characteristics

Color of fresh fruit juices is important qualitative parameter since consumers often associate color of juice with its freshness and quality. Color characteristic can also be used for evaluation of bioactive compounds responsible for juice color [22, 67]. As follows from the previously published papers, color characteristics are mainly influenced by changes in particle size during the processing and by heat accelerated non-enzymatic browning reactions (i.e. Maillard reactions) during the pasteurization step [68]. During the long-term storage, color characteristics are mainly influenced by non-enzymatic browning reactions [69].

Thus, the application of modified production atmosphere is employed with the aim of minimizing color changes during the production and long-term post-production storage of juices.

Effect of the storage

For the quantitative evaluation, total color difference (ΔE) was calculated from color parameters (L*, a*, b*) according to the *Equation* (33). Color characteristics of the freshly produced juices were always used as reference for TCD evaluation. Expression of TCD value on observable color change is showed in *Table 7* [56].

Table 7: *Expression of total color difference as observed by human eyes.*

ΔE	**Description of observed color change**
0 – 0.5	not noticeable
0.5 – 1.5	slightly noticeable
1.5 – 3.0	noticeable
3.0 – 6.0	well visible
6.0 – 12.0	great

TCD values recorded for juice samples of different production atmosphere at the respective time of their storage are summarized in *Table 8*. The obtained results are in more details described below.

Table 8: *Color characteristics of juice samples at the respective time of their storage.*

Sample label	Time of storage (day)	L*	a*	b*	Chromaticity	Hue Angle	ΔE
CO14	0	82.29	5.02	31.10	31.50	80.83	
	90	83.71	4.28	29.54	29.84	81.77	2.24
	124	85.20	3.60	29.06	29.29	82.95	3.83
	152	85.50	3.46	27.64	27.85	82.87	4.97
CN14	0	81.99	5.27	30.91	31.35	80.33	
	90	84.52	3.89	28.95	29.21	82.35	3.49
	124	85.54	3.40	27.07	27.28	82.85	5.55
	152	85.26	3.64	28.59	28.82	82.74	4.33
AN14	0	98.85	0.43	4.98	5.00	94.98	
	90	97.76	0.22	6.96	6.96	91.78	1.84
	125	97.36	0.31	7.63	7.64	92.32	2.62
	154	98.54	0.64	6.83	6.86	95.35	1.67
CA13	0	82.81	4.82	29.19	29.59	80.63	
	90	83.15	4.67	29.89	30.26	81.12	0.81
	122	81.81	5.29	31.67	32.11	80.51	2.73
	147	81.83	5.39	32.73	33.18	80.64	3.72
CN15	7	81.87	5.15	31.76	32.18	80.78	
	90	82.88	4.64	30.82	31.17	81.44	1.48
CC15	7	82.27	5.04	31.18	31.58	80.81	
	90	82.56	4.79	30.27	30.61	81.45	1.47

5.1.2.1 Orange juices with pulp

As follows from the data presented in *Table 8*, at the start of the storing experiment, lightness component L* of orange juice produced under Ar modified atmosphere (CA13) was 82.81, chromatic component a* was 4.82 and chromatic component b* was 29.19. At the 90^{th} day, L* and b* increased to 83.15 and 29.89, respectively, whereas values of a* decreased to 4.67. Total color difference calculated for 90^{th} day of storage reached 0.81 (slightly noticeable changes). Thus, slight lightening of samples accompanied by slight shift of the color perception to yellow/green color was noticed. Continuous storage resulted in gradual increase of ΔE up to 3.72 (well visible) at the end of experiment. Color values of orange juice samples produced in 2014 under the conventional conditions and under the N_2 atmosphere, evaluated from their VIS spectra by the ColorLite software at different time of their storage are also summarized in *Table 8*. We focused our attention on the description of just ΔE values as an effective parameter. However, as clearly follows from the data, reversed trend in ΔE was noticed than expected, as even after 90^{th} day of storage (i.e., within the expiration period), total color difference ΔE of sample produced conventionally, CO14, was 2.24 which represent noticeable changes. At the same time, ΔE of CN14 counterpart sample produced under N_2 was 3.49 (well visible). The identical phenomenon was observed for samples evaluated at 124^{th} day of storage (end of the expiration period) – samples produced under conventional air are characterized by the lower ΔE than of those produced under the nitrogen. This is in contradiction to the expected behavior. This could lead to false conclusion, that in comparison to unmodified conventional atmosphere, application of N_2 modified atmosphere was less effective in preservation of juice color. The only acceptable explanation of the observed trends is in fluctuations of the color parameter values between the experiments within day and/or between the individual measurements (data are not depicted). Fluctuation of color values and of ΔE was observed during the whole monitored period, probably caused by the inhomogeneity of the samples (presence of pulp in spite of sample centrifugation, or just turbidity coming from the proteins or solid particles…).

This explanation is consistent with subsequently calculated ΔE values of these samples at the end of the monitoring period. In both cases, well visible changes in color were detected, but numerical values indicated slightly higher color difference of conventionally produced samples. It can be also

presupposed, that as a result of the storage, also the pulp decomposition occur, along with viscosity and/or density of juice changes. These factors, although were not monitored, could also influence the properties of samples, quality of spectra and last but not least, calculation of the color parameters by spectra processing. Examples of typical color diagrams at respective time of storage of orange juices produced in 2014 evaluated from the absorbance VIS spectra, from which color characteristics were also calculated are showed on *Figure 23* and *Figure 24.*

Experiments performed with samples of orange juices produced in 2015 under N_2 and CO_2 atmosphere revealed that at 90th day of storage the evaluated color characteristics were practically identical or comparable. As regards TCD, $\Delta E = 1.47$ and $\Delta E = 1.48$ were calculated for samples produced under the CO_2 and N_2, respectively. These changes were in both cases assigned as slightly noticeable.

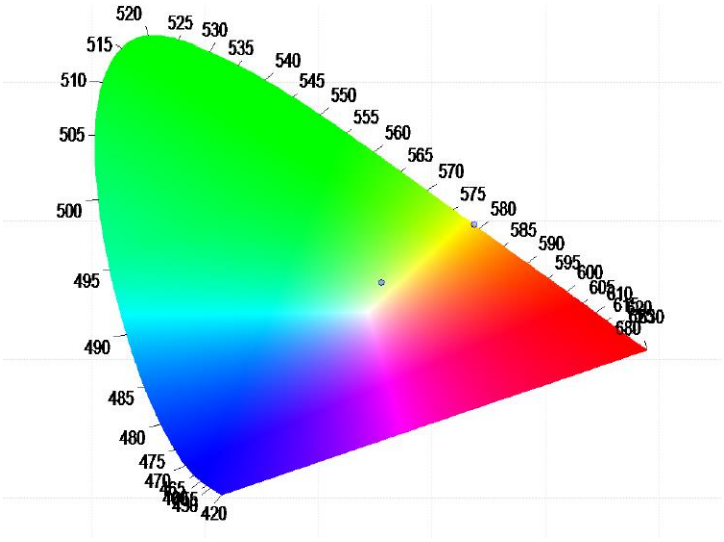

Figure 23: *Color diagram of CN14 orange juice sample processed from VIS spectra recorded at the 1st day of storage.*

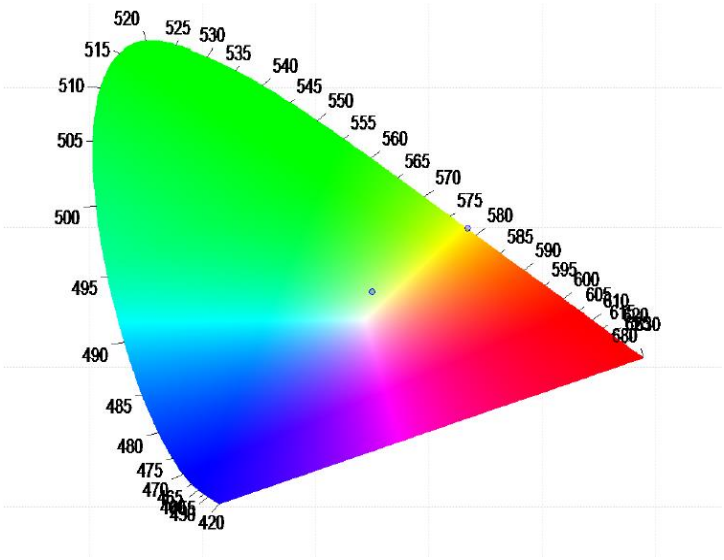

Figure 24: *Color diagram of CN14 orange juice sample processed from VIS spectra recorded at the 90th day of storage.*

Effect of pasteurization

Besides the effect of modified atmosphere on color characteristics during the storage, again the effect of pasteurization on color characteristics of orange juices produced in 2014 was assessed. The color parameters and also TCD of the samples are summarized in *Table 9*.

Table 9: *Effect of pasteurization and processing conditions on total color difference of orange juice samples produced in 2014.*

Sample label	L*	a*	b*	Chromaticity	Hue angle	ΔE
CN14 Raw	82.15	5.07	31.27	31.68	80.80	
CN14 Pasteurized	81.99	5.27	30.91	31.35	80.33	0.50
CO15 Raw	83.66	4.36	29.58	29.90	81.62	
CO15 Pasteurized	82.29	5.02	31.10	31.50	80.83	2.15

Due to practical reasons, TCD were taken for comparison of the effects of pasteurization. As clearly follows from the presented data, TCD of juices produced under N_2 atmosphere was 0.50, which represents change between not noticeable and slightly noticeable; whereas that of juices produced under conventional atmosphere reached 2.15, i.e., noticeable change. Application of N_2 modified atmosphere has therefore significant impact on color of juice during the processing and can prevent color changes, in terms of global prevention of the samples against the oxidative, thermal or storage conditions induced changes.

Thus, it can be partially concluded for orange juices under the study, that:

- The effect of the modified atmosphere production on changes in color characteristics of orange juices during their storage is influenced significantly by the sample homogeneity and the presence of solid particles in the juice solution, or the turbidity of the samples caused e.g., by the presence of proteins.
- Seasoning effect cannot be underestimated in this context, as well.
- From general point of view, the effect of modified atmosphere on color changes is rather positive.
- The comparison of TCD of samples produced under the respective atmospheres revealed the following positive effect (in decreasing order according to TCD values at 90^{th} day of storage): $Ar > (CO_2 \approx N_2)$.
- The TCD changes of CO_2 and N_2 produced samples are comparable, again in terms of what is written in the previous section, the further utilization of N_2 atmosphere would be a good compromise.
- Application of modified atmosphere has positive effects on juices color protection during the pasteurization.

5.1.2.2 Pineapple juices

Again just the description of the results for pineapple juices produced under N_2 are presented, as the corresponding conventionally produced counterpart was not available. In the beginning of the experiment, the initial value of lightness component L* was 98.85, and these of trichromatic components a* and b* were 0.43 and 4.98, respectively. At the 90^{th} day of storage, L* parameter, which represents the lightness of sample, decreased to 97.76, and at the expiration date to 97.36, which indicated slight continuous progressive darkening of the samples during the storage. The most significant changes during the storage were found for b* parameter, which increased to 6.96 at the 90^{th} day of experiment and to 7.64 at the end of expiration date. At the end of the monitored period (one

month after the juice expiration) value of the b* parameter decreased to 6.83. In correlation with darkening, this change could indicate the continuous browning of the samples, expected on the basis of our previous experiences, and observed in the end of the expiration period even by eye. The observed trends in individual parameters revealed the TCD values, which increased from the value of 1.84 evaluated for the 90th day of experiment to 2.62 at the expiration date. In both cases, the changes were assessed as noticeable in terms of TCD scale.

5.2 EPR experiments

5.2.1 Ascorbic acid equivalent

Ascorbic acid (AA) content is important qualitative parameter in fruit juices. AA content is mainly affected by the dissolved oxygen. Its degradation in juices is a result of aerobic and anaerobic mechanisms that occurs simultaneously. Main degradation reactions are oxidation and hydrolysis. Degradation of AA to dehydroascorbic acid also affects color characteristics of fruit juices. Detailed mechanisms of AA reaction pathways are described in the theoretical part of the thesis.

Changes in the ascorbic acid concentration in juices during the storage were evaluated by TEMPOL assay, as this stable free radical/ its water solution is sensitive to the presence of low-molecular organic acids of corresponding redox potential, forming EPR silent hydroxylamine. It was previously successfully applied e.g., for the indirect detection of the AA presence in fortified beers [70]. Results were expressed in terms of ascorbic acid equivalents, AAE, as is indicated in the experimental part.

For the purposes of quantitative evaluation of the individual assessed effects, the difference in the concentration of AAE, Δ value, representing the difference in AAE content of freshly produced sample and sample stored for 90 days was calculated, as well as % change analogously as in the case of TPC.

AAE values recorded for orange and pineapple juice samples of different production atmosphere at the respective time of their storage are summarized in *Table 10*.

Table 10: *Recorded AAE values of juice samples at the respective time of their storage*.

Sample label	**AAE [mmol·l^{-1}]**					
	Start of the experiment	**90th day of storage**	**Expiration date**	**End of the experiment**	**Δ**	**% change**
CA13	0.44 ± 0.01	0.17 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.27	61.4
CN14	0.30 ± 0.04	0.25 ± 0.03	0.26 ± 0.01	0.27 ± 0.02	0.05	16.7
CO14	0.35 ± 0.04	0.19 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.16	45.7
CN15	0.39 ± 0.03	0.31 ± 0.02	N/A*	N/A*	0.08	20.5
CC15	0.42 ± 0.01	0.36 ± 0.02	N/A*	N/A*	0.06	14.3
AN14	0.25 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	0.28 ± 0.02	0.01	4.0

* Experiments are still in progress, to the date of diploma thesis completion, data for 90th day of storage have become available.

During the storage, ability of samples to terminate TEMPOL radical gradually decreased, resulting into decrease of AAE values. In accord with expectation, during the storage, the ability of samples to terminate TEMPOL radical gradually decreased, resulting in the decreased AAE values. Detailed description of the observed changes is given bellow.

5.2.1.1 Orange juices with pulp

Effect of storage

Typical series of EPR spectra of TEMPOL radical in the presence of orange juice samples processed under conventional atmosphere at respective time of their storage are presented on *Figure 25*. Whereas the signal of reference sample (water instead of juice solution) remained stable during the experiment, the gradual decrease of the EPR spectra intensity is observable after the addition of sample to TEMPOL solution. It is apparent that ability of samples to terminate TEMPOL radical decreases as a result of storage.

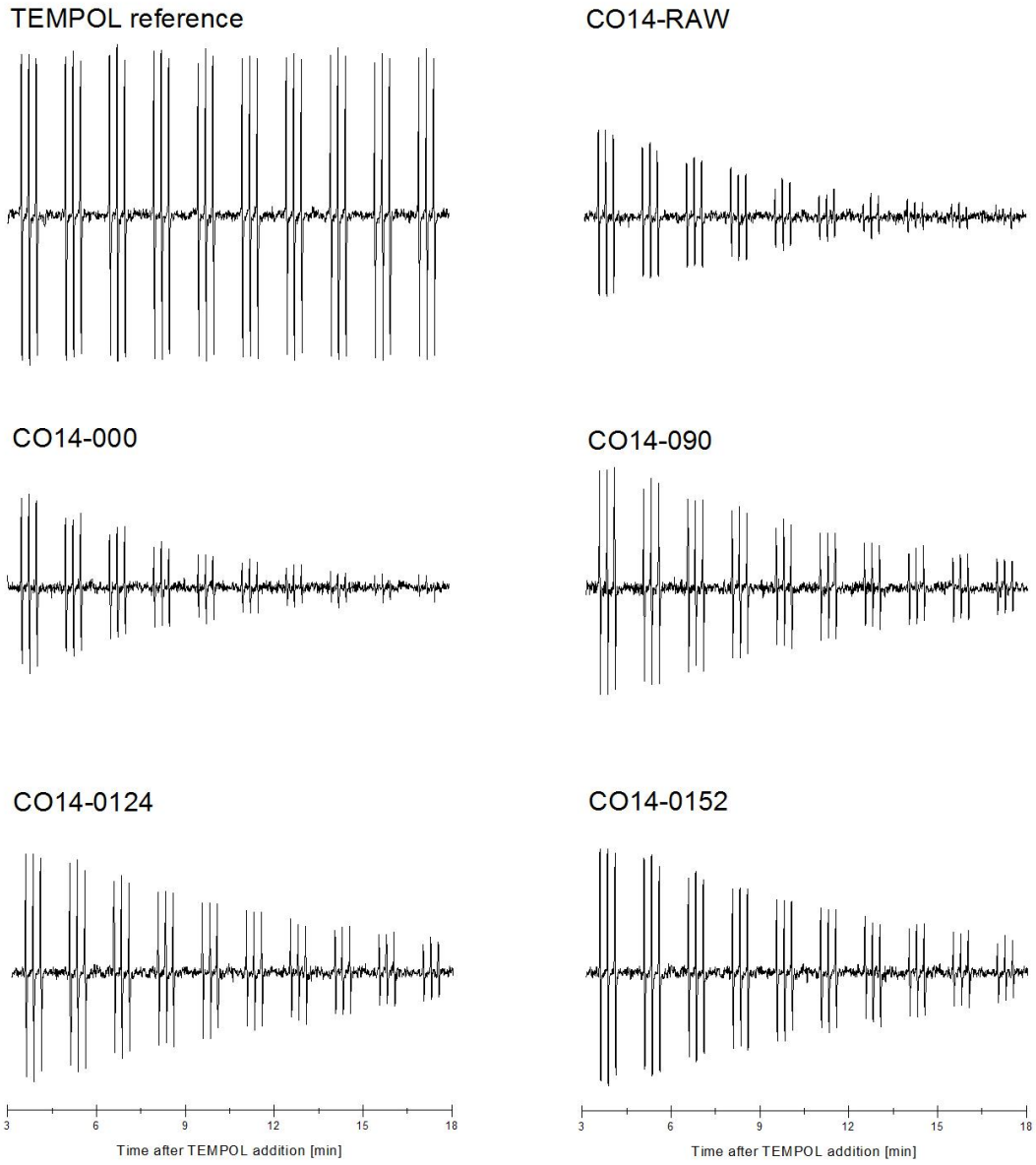

Figure 25: *Series of time evolutions of EPR spectra recorded for orange fruit juices with pulp at different time of their storage (0, 90, 124 and 152 days) and for raw orange juice sample in the presence of TEMPOL free radical (c_0TEMPOL=1.10^{-5} mol.l^{-1}). Distilled water was used instead of juice sample as the reference. Typical parameters of the measurements are defined in chapter 4.5.1.*

Evaluation of the recorded spectra in terms of AAE exhibited, that orange juices produced under Ar modified atmosphere (CA13) had the initial AAE concentration of 0.44 ± 0.01 mmol·l^{-1} which was the highest out of all the other measured samples. Even though, samples prepared under the Ar atmosphere exhibited the highest AAE values at the time of the start of experiment, but, in accord with the trends described for TPC, its concentration decreased surprisingly the most out of all the investigated samples. After 90th day of storage, AAE value decreased by 61.4 % to 0.17 ± 0.01 mmol·l^{-1} and at the end of the experiment, even to 0.10 ± 0.01 mmol·l^{-1}, i.e., to approx. 23 % of its initial concentration. Change of AAE values evaluated from the EPR spectra of argon-produced samples during their storage are shown on *Figure 26*. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation, which to the best describes the observed trend.

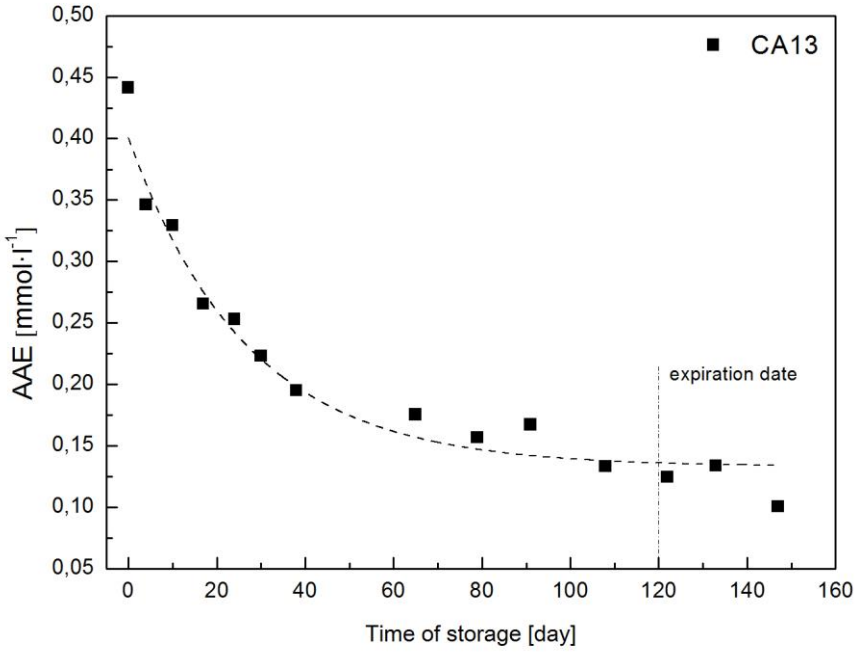

Figure 26: *Changes of AAE values in orange juice sample CA13 produced under the Ar atmosphere during the storage. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

Orange juices from 2014 produced under the conventional air (CO14) atmosphere and under the N_2 (CN14) atmosphere, exhibited on absolute basis the lowest but comparable initial concentrations of ascorbic acid, 0.35 ± 0.04 mmol·l^{-1} and 0.30 ± 0.04 mmol·l^{-1} for CO14 and CN14 samples. The differences in absolute values of individual samples are negligible, if the standard deviation values are taken into account. At the 90th day of storage, the AAE concentration of CO14 represents 45.7 % decrease, whereas that evaluated for CN14 samples decreased by 16.7 % only. It is apparent, that the application of N_2 modified atmosphere resulted in significantly lower decrease of AAE values during storage, in comparison to samples processed under the conventional conditions.

Change of AAE values during the storage of both juices is depicted on *Figure 27*, together with fits of the experimental data to the kinetic equation (dotted lines).

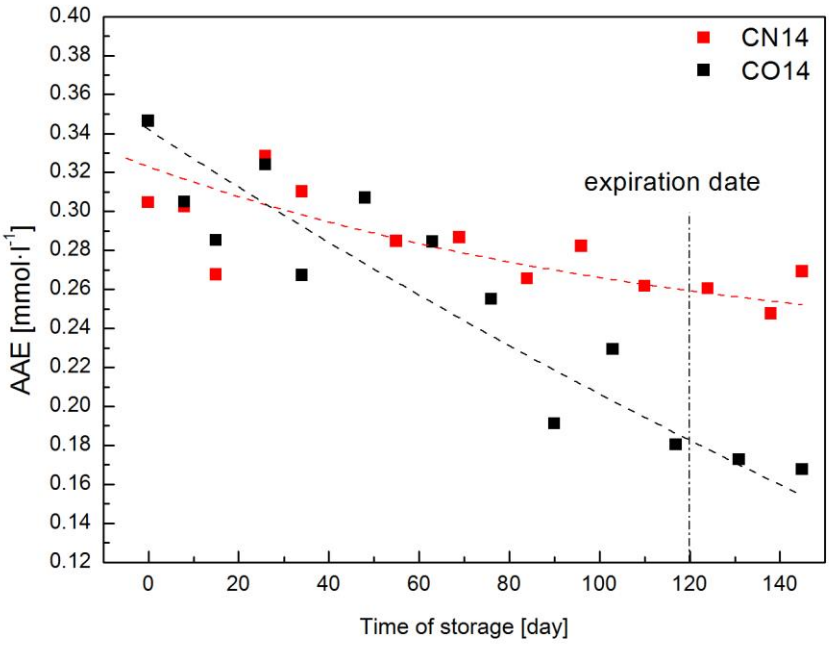

Figure 27: *Changes of AAE values in orange juice sample CO14 and CN14 produced under the conventional air and N_2 atmosphere during the storage. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

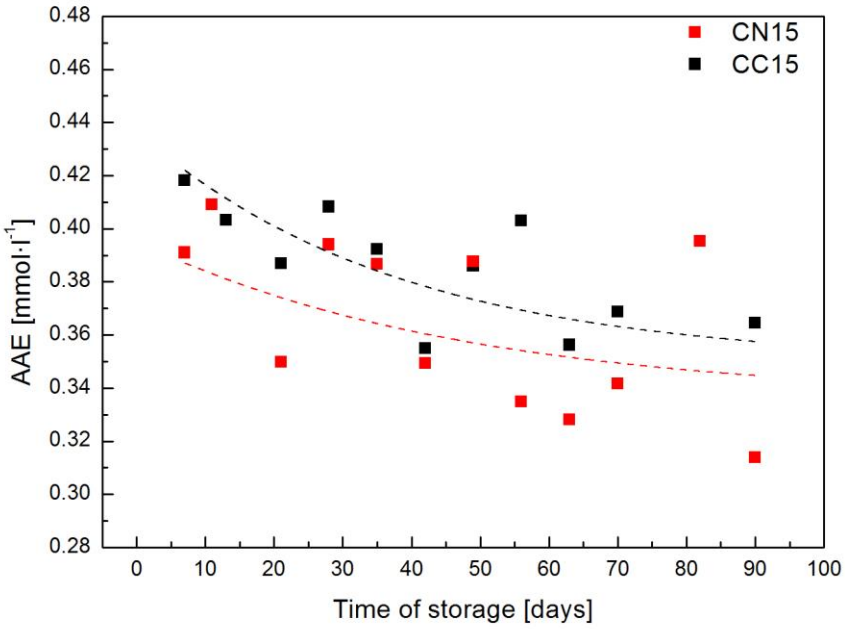

Figure 28: *Changes of the AAE values in CN15 and CC15 orange juice samples produced under N_2 and CO_2 atmosphere during the storage. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

Changes of the AAE values in CN15 and CC15 orange juice samples produced under N_2 and CO_2 atmosphere in 2015 during their 90-day storage are depicted on *Figure 28*. Again, as expected, gradual decrease of radical-scavenging activity of juice samples upon the storage was observed, expressed in terms of AAE values decrease. In the beginning of the experiment, AAE values of 0.39 ± 0.03 mmol·l^{-1} for CN15 and 0.42 ± 0.01 mmol·l^{-1} for CC15 were evaluated. At the 90th day of storage the AAE value of CN15 decreased to 0.31 ± 0.02 mmol·l^{-1}, i.e. 20.5 % decrease of AAE was noticed in comparison to the initial value evaluated for freshly prepared sample just after the pasteurization. In addition, CC15 sample produced under CO_2 exhibited AAE value of 0.36 ± 0.02 mmol·l^{-1}, which represents the 14.3 % decrease.

Thus, on the basis of the obtained results, it can be concluded that:

- The application of N_2 modified atmosphere lead to effective protection of low-molecular organic acids present in the orange juice, slowing the deterioration of the radical-scavenging activity of the samples in comparison to those produced under the conventional air atmosphere.
- As regards the efficacy of CO_2 and N_2 atmosphere, it is apparent that the application of CO_2 modified atmosphere proved to have similar effect as the N_2 modified atmosphere.

These results are in good correlation with the results described above for TPC and color value changes. It should also be noted here, that again the higher AAE values of orange juices processed in 2015 were noticed at the start of experiment and at the 90th day of storage, than for orange juices made in 2014. This is most probably caused by seasonal effect during the growth of orange fruit, mentioned also previously.

Effect of pasteurization

Impact of modified atmosphere and pasteurization on AAE values of orange fruit juices with pulp produced in 2014 is summarized in *Table 11*. As follows from the evaluation, the application of N_2 modified atmosphere has in terms of AAE negligible effect, as the values of AAEs of raw and pasteurized samples for both investigated atmospheres are comparable.

Table 11: *Effect of the modified atmosphere and pasteurization on AAE values.*

Sample label	AAE [mmol·l^{-1}]
CO14 Raw	0.36 ± 0.02
CO14 Pasteurized	0.34 ± 0.04
CN14 Raw	0.34 ± 0.04
CN14 Pasteurized	0.30 ± 0.04

5.2.1.2 Pineapple juices

Time dependence of AAE values of pineapple juices produced under N_2 modified atmosphere (AN14) is shown on *Figure 29*. It is obvious from the plot, that in case of pineapple, AAE value remained practically unchanged – unaffected – during the whole monitored period. These results indicate, that the behavior of pineapple juices differs from that prepared from orange fruit, what is in fact, not surprising. The previously published studies of the Food Research Institute on pineapple juice produced conventionally under the air atmosphere are in contradiction to herein presented results [71]. In the previously published study, during 26 weeks of storage at 7°C, i.e., at conditions to those used in the herein presented experiments, gradual worsening of AAE values by 25 % at 182nd day of storage was noticed.

As the conventional counterpart was not provided, one can only speculate, that the effect observed under the N_2 atmosphere is the effect of atmosphere modification, however, this assumption is more or less probable.

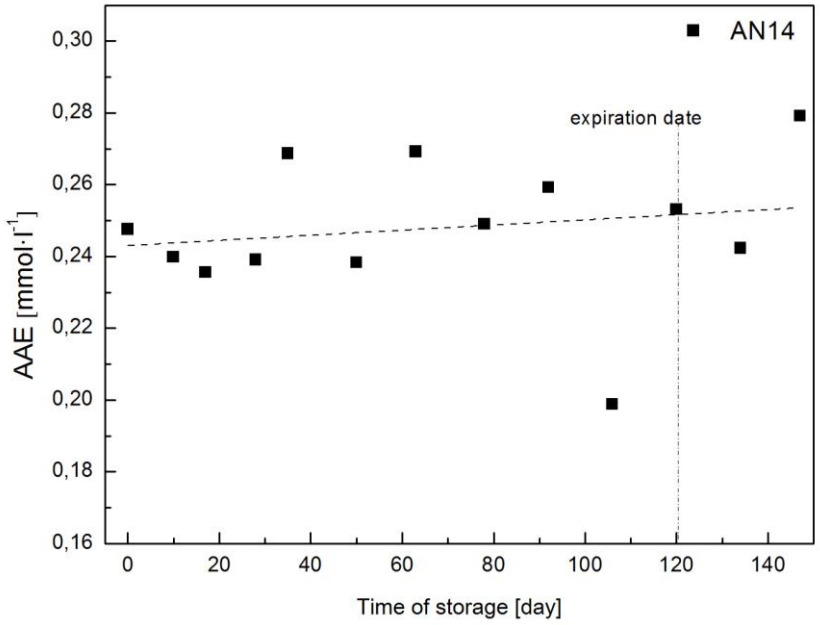

Figure 29: *Changes of the AAE values in AN14 pineapple juice samples produced under N_2 atmosphere during the storage.*

5.2.1.3 Kinetic evaluation

As indicated above, the time- dependent changes of individual monitored experimental characteristics were fitted to the model of the first-order kinetic equation, in order to evaluate the rate constants and half-lives of the deterioration of antioxidant/radical-scavenging properties of juice samples.

Values of 1st order rate constant of radical/scavenging activity decrease detected in fruit juices in terms of AAE values resulting from the long-term storage, together with the half-lives $t_{1/2}$ are summarized in *Table 12*.

Table 12: *Values of formal first-order kinetic parameters evaluated from the decrease of AAE values in fruit juices during the storage.*

Sample label	k_{AAE} **[day^{-1}]**	R^2	$t_{1/2}$
CA13	0.035 ± 0.003	0.995	19.986
CN14	0.002 ± 0.001	0.996	367.714
CO14	0.005 ± 0.001	0.991	134.408
CN15	0.006 ± 0.001	0.974	114.390
CC15	0.007 ± 0.001	0.980	102.495
AN14	N/A	N/A	N/A

Calculated rate constants and half-lives proved unambiguously the observed effects of modified atmosphere application in terms of AA and other low/molecular organic acids protection against oxidation and subsequent deterioration. It is obvious, that in case of orange juice with pulp, the application of modified atmosphere suppress the degradation processes, the effects of N_2 and CO_2 atmospheres are comparable. Of course, these values cannot be compared on absolute basis as the seasoning effect is obvious. Another factor is the variability of the experimental dataset obvious from graphs, caused by samples homogeneity, probably. However, in case of kinetic evaluation of AAE values the variability did not affect the calculations so dramatically like in case of TEAC values, discussed below.

In case of pineapple juice, it is obvious from the *Figure 29*, and also discussed above, that the values of AAE just oscillated around the average value, with only small fluctuations. Thus, any of the kinetic approach failed and thus, the values of kinetic parameters could not be calculated.

5.2.2 Trolox-equivalent antioxidant capacity

Trolox-equivalent antioxidant capacity (TEAC) expresses the ability of antioxidants to scavenge the $ABTS^{\bullet+}$ cation-radical, prepared from the neutral ABTS molecule by any of the methods described in the theoretical part of the thesis. The observed decrease in EPR spectra intensity resulting from the reaction of sample components with the cation-radical is the most frequently evaluated by comparing the radical-scavenging ability of the sample to that of TROLOX, synthetic water-soluble vitamin E derivate. TEAC value is often used for evaluation of the antioxidant and radical-scavenging properties of sample in a semi-quantitative scale.

Similarly like in the previously described assays, the effect of production atmosphere and long-term post-production storage on TEAC content was characterized. Where also the raw, non-pasteurized and pasteurized samples were available, also the effect of pasteurization on TEAC content was assessed.

For the purposes of quantitative evaluation, difference in the concentration of TEAC, Δ value, representing the difference in TEAC content of freshly produced sample and sample stored for 90 days was calculated. In addition, % change represents the ratio of Δ change to the TEAC value of freshly prepared sample. TEAC values evaluated for orange and pineapple juice samples of different production atmosphere at the respective time of their storage are summarized in *Table 13.*

Table 13: *TEAC values of juice orange and pineapple juice samples evaluated from EPR spectra at the respective time of samples storage.*

Sample label	**TEAC [mmol·l^{-1}]**					
	Start of the experiment	**90th day of storage**	**Expiration date**	**End of the experiment**	**Δ**	**% change**
CA13	1.71 ± 0.03	1.01 ± 0.03	0.85 ± 0.05	0.85 ± 0.03	0.70	40.9
CN14	2.07 ± 0.13	1.42 ± 0.04	1.30 ± 0.004	1.35 ± 0.01	0.65	31.4
CO14	1.84 ± 0.11	1.05 ± 0.04	1.03 ± 0.16	0.97 ± 0.02	0.79	42.9
CN15	2.63 ± 0.07	2.46 ± 0.08	N/A*	N/A*	0.17	6.5
CC15	2.64 ± 0.09	2.46 ± 0.01	N/A*	N/A*	0.18	6.8
AN14	2.87 ± 0.02	2.50 ± 0.10	2.40 ± 0.03	2.53 ± 0.07	0.37	12.9

* Experiments are still in progress, to the date of diploma thesis completion, data for 90th day of storage have become available.

5.2.2.1 Orange juices with pulp

Effect of storage

Time-dependent decrease of TEAC values of orange juices produced in 2013 under Ar modified atmosphere (CA13) during their long-term storage is depicted on *Figure 30*. As follows from the comparison of TEAC values of samples produced at different atmospheres, although the seasoning effect should be taken into consideration, TEACs of Ar modified atmosphere samples revealed the lowest values, both at the start of the storing experiment and at 90th day of experiment, 1.71 ± 0.03 mmol·l^{-1} and 1.01±0.03 mmol·l^{-1}, respectively. Thus, after 90th day of the storage, TEAC value decreased by 40.9 %.

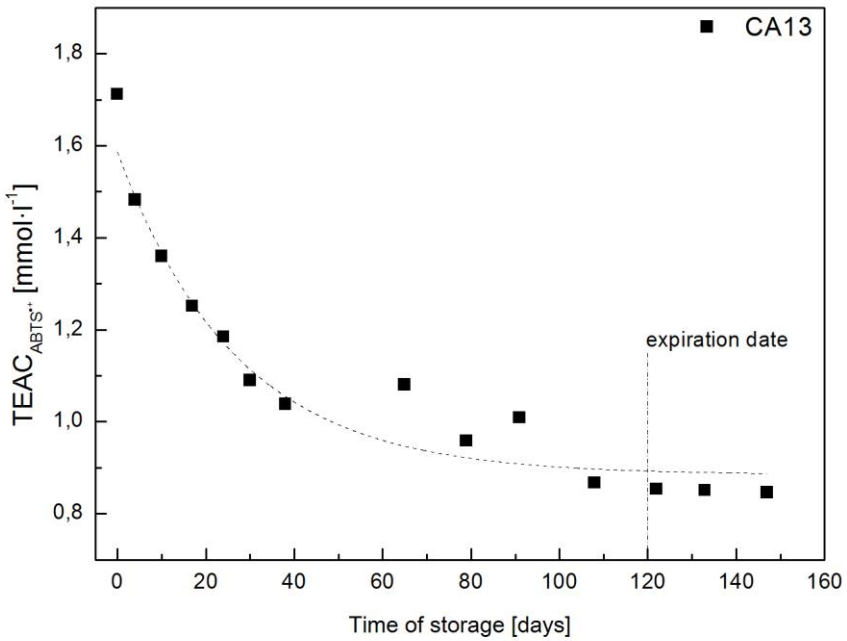

Figure 30: *Changes of $TEAC_{ABTS^{\bullet+}}$ values in orange juice sample CA13 produced under the Ar atmosphere during the storage. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

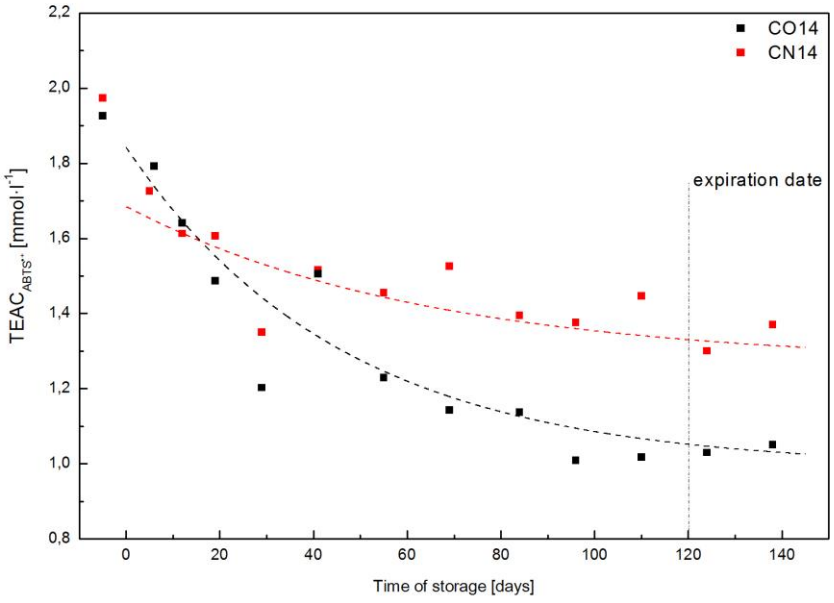

Figure 31: *Changes of TEAC values in orange juice sample CO14 and CN14 produced under the conventional air and N_2 atmosphere during the storage. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

At the end of the monitored period, additional decrease of TEAC values to 0.85 ± 0.03 mmol·l^{-1} (i.e., 49.7 % of the initial value) was found.

Orange juice produced in 2014 under the unmodified conventional atmosphere (CO14) exhibited TEAC value of 1.84 ± 0.11 mmol·l^{-1}, while the juice produced under the N_2 modified atmosphere (CN14) the TEAC value of 2.07 ± 0.13 mmol·l^{-1}. Time-dependent decrease of TEAC values during the storage of the CO14 and CN14 samples is depicted on *Figure 31*. As follows from the evaluation of the spectral data, after 90th day of storage, TEAC value of CO14 samples decreased by 42.9 % to 1.05 ± 0.04 mmol·l^{-1} and that of CN14 by 31.4 % to 1.42 ± 0.04 mmol·l^{-1}. At the end of the storage experiment, additional decrease of TEACs to 0.97 ± 0.02 mmol·l^{-1} and to 1.35 ± 0.01 mmol·l^{-1} was noticed for CO14 and CN14 samples, respectively. From results is obvious that application of N_2 modified atmosphere improves TEAC value of processed juice during processing and storage.

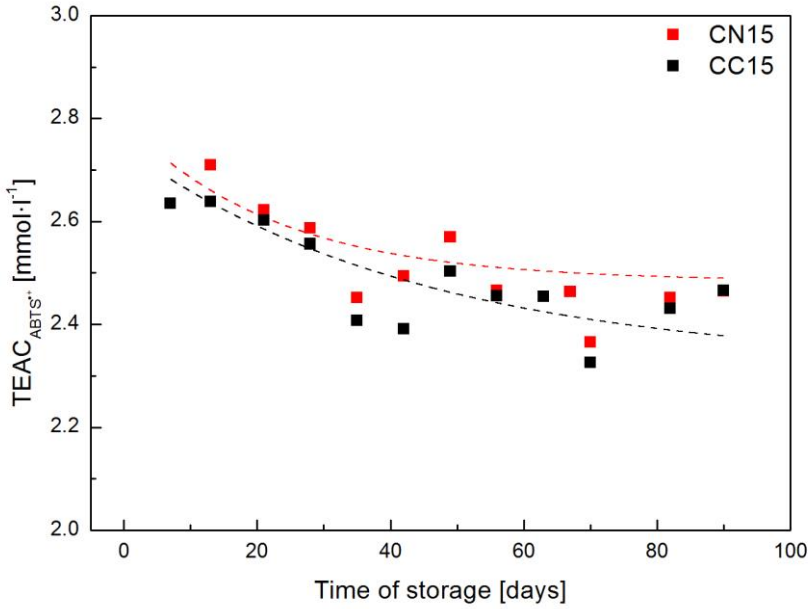

Figure 32: *Changes of TEAC values in orange juice sample CN15 and CC14 produced under the N_2 and CO_2 atmosphere during the storage. Dotted line represents the fit of experimental data to the model of 1st order kinetic equation.*

Time-dependence of TEAC values evaluated from EPR spectra recorded for orange juice samples produced under the N_2 modified atmosphere (CN15) and under the CO_2 (CC15) in 2015 are depicted on *Figure 32*. The results obtained indicate that at the start of the storage experiment, for CN15 juices TEAC value of 2.63 ± 0.07 mmol·l^{-1} and for CC15, TEAC value of 2.64 ± 0.09 mmol·l^{-1} were evaluated. After 90th day of samples storage, TEAC value of CN15 juice decreased by 6.5 % to 2.46 ± 0.08 mmol·l^{-1} and those of CC15 by 6.8 % to 2.46 ± 0.01 mmol·l^{-1}. These results are consistent to our findings described above, i.e., that in terms of efficacy, the application of CO_2 and N_2 possess practically identical protecting effect.

In the context of the results obtained for orange juices properties assessed by TEAC assay, it was proved, that:

- The application of N_2 led to general protection of samples against the oxidation, via eliminating or slowing down the deterioration of their antioxidant and radical-scavenging properties.
- Results obtained again confirmed, that the application of N_2 and CO_2 modified atmosphere had very similar impact on TEAC values during storage.

- In comparison to juices produced in 2014, those of 2015 production exhibited overall higher TEAC values, probably due to seasonal variance during the growth of oranges, as mentioned also above in other assays.

Additional remark should also be done when considering the variability in experimental data, not only TEAC. In spite of the worse results of all the parameters evaluated for juices produced under Ar atmosphere, one phenomenon should be highlighted – i.e., the fact that the Ar-produced samples exhibited the lowest variability within the measurements and between the samples, obvious from time-dependent measurements presented in the thesis. It is hardly to discuss, whether the observed lowered variability is the result of sample homogeneity, variety, season or origin. It can only be concluded on the basis of these results, that the lowered variability brings better predictability of the sample behavior at different time of storage, what could be effectively used for e.g., juice composition modulation in terms of reaching the defined values of certain nutrients/antioxidants at the desired time after the juice production. Of course, additional experiments in a wider scale performed on extended group of samples would be desirable to verify and evaluate the observed trends in a more complex way.

Effects of pasteurization

Effect of pasteurization and modified atmosphere on TEAC values of orange juices produced in 2014 is obvious from the data presented in *Table 14*.

Table 14: *Effect of modified atmosphere and pasteurization on TEAC values of juices from 2014.*

Sample label	**TEAC [mmol·l^{-1}]**
CO14 Raw	1.93 ± 0.03
CO14 Pasteurized	1.84 ± 0.11
CN14 Raw	1.97 ± 0.06
CN14 Pasteurized	2.07 ± 0.13

It is obvious, that the TEAC values evaluated for raw samples produced under both atmospheres are comparable. The same observation was done also when the effect of pasteurization was assessed – when the standard deviations of TEAC values evaluation are taken into consideration, there is practically no difference between the results. Thus, similarly like above for AAE values, it can be concluded, that the pasteurization have only negligible effect on TEAC values without respect on the production atmosphere. Or, as both TEAC and AAE are only effective sample characteristics that were not measured directly, but just calculated from the spectral data – this in fact, brings additional uncertainty to the evaluation.

5.2.2.2 Pineapple juices

Typical series of EPR spectra of $ABTS^{\bullet+}$ radical in the presence of orange juice samples processed under N_2 atmosphere (AN14) recorded at different time of their storage are presented on *Figure 33*. Whereas the signal of the reference sample (water instead of juice solution) remained stable during the experiment, the gradual decrease of the $ABTS^{\bullet+}$ EPR spectra intensity is observable after the addition of sample to $ABTS^{\bullet+}$ solution. It is apparent that ability of samples to terminate $ABTS^{\bullet+}$ radical decreases as a result of the continuous storage, analogously as described above for TEMPOL assay.

The spectral intensity decrease was again evaluated in terms of TEAC, taking into consideration the double-integrated EPR spectrum recorded at 10.5 min of the measurement, i.e., after the addition of the respective juice solution to the solution of $ABTS^{\bullet+}$ cation-radical.

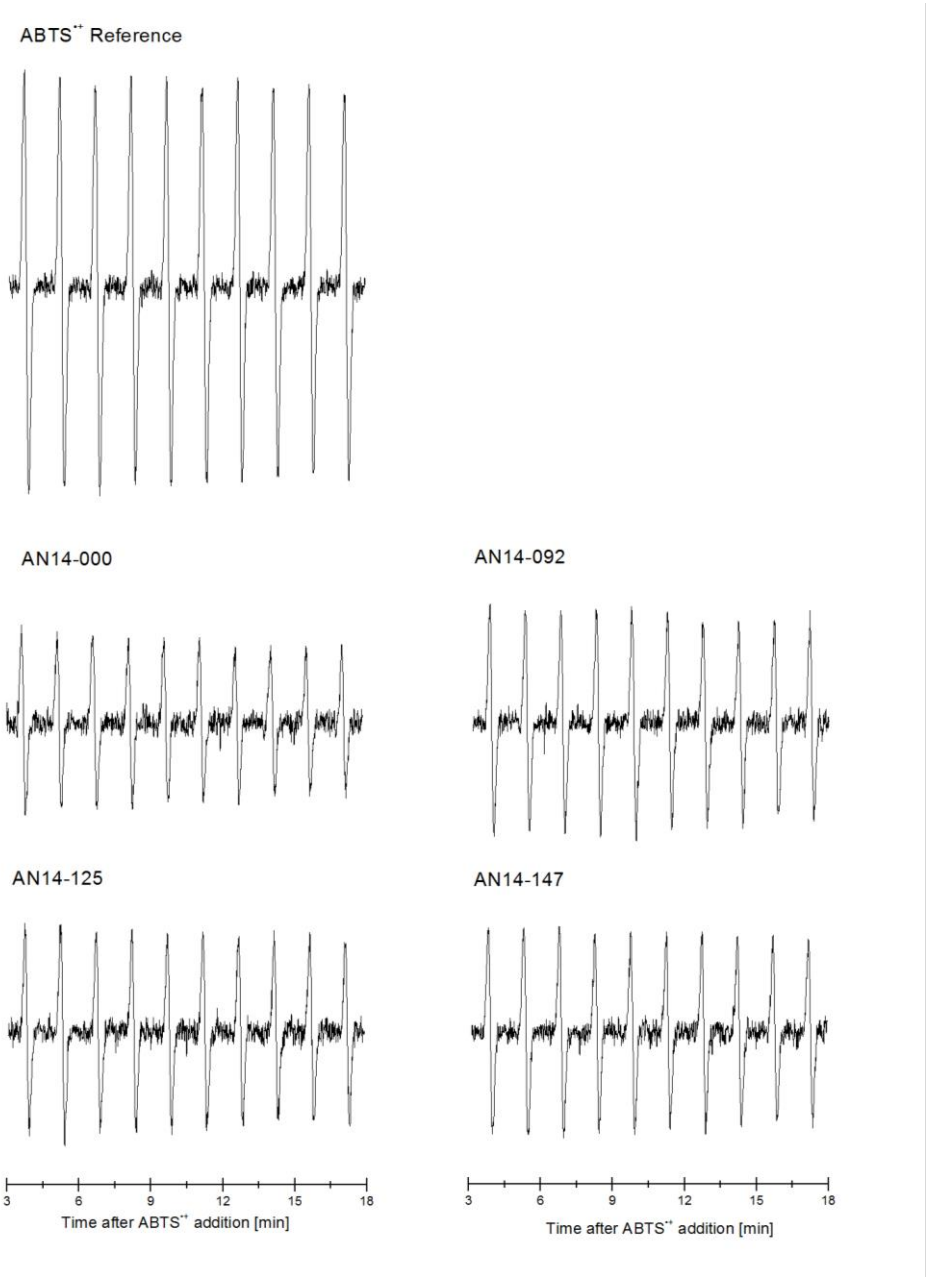

Figure 33: *Series of time evolutions of EPR spectra recorded for orange fruit juices with pulp at different time of their storage (0, 92, 125 and 147 days) and for raw orange juice sample in the presence of* $ABTS^{\bullet+}$ *free radical (*$c_{0\ ABTS^{\bullet+}} = 1.10^{-5}\ mol.l^{-1}$*). Distilled water was used instead of juice sample as the reference. Typical parameters of the measurements are defined in chapter 4.5.1.*

Time progressive decrease of TEAC values of pineapple juices produced under the N_2 atmosphere during the storage is depicted on *Figure 34*. Pineapple juice produced in 2014 under the N_2 modified atmosphere exhibited the initial TEAC value of $2.87 \pm 0.02\ mmol \cdot l^{-1}$, while after 90^{th} day of the storage, its decrease by 12.9 % to $2.50 \pm 0.10\ mmol \cdot l^{-1}$ was noticed. At the end of monitored period, TEAC value increased slightly to $2.53 \pm 0.07\ mmol \cdot l^{-1}$. However, this increase is statistically non-significant as the average values of both, TEAC at 90^{th} and 147^{th} day of storage are comparable, considering also the uncertainty of TEAC calculation.

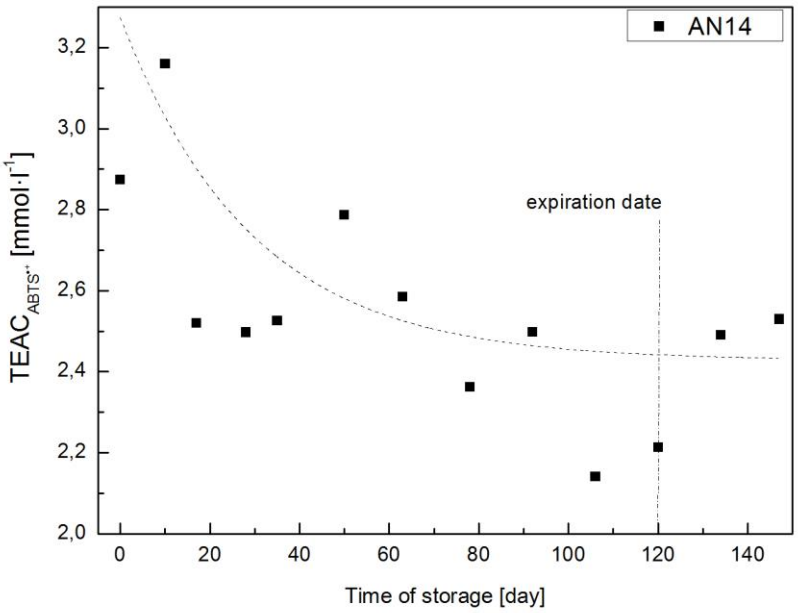

Figure 34: *Changes of the $TEAC_{ABTS^{\bullet+}}$ values in AN14 pineapple juice samples produced under N_2 atmosphere during the storage.*

Pineapple juice produced in 2014 under N_2 modified atmosphere had generally higher $TEAC_{ABTS^{\bullet+}}$ values at both the start and end of experiment in comparison to orange juices. This is probably caused by differences in fruit composition and antioxidant activity. Other effects, such as seasonal variance and country of origin play a role. Application of N_2 modified atmosphere proved to be effective in slowing and lowering decrease of $TEAC_{ABTS^{\bullet+}}$ values during storage. Results regarding $TEAC_{ABTS^{\bullet+}}$ in this thesis are in good correlation with previously published studies of the Institute [71].

5.2.2.3 Kinetic evaluation

Values of 1st order rate constant of radical/scavenging activity decrease detected in fruit juices in terms of $TEAC_{ABTS^{\bullet+}}$ values resulting from the long-term storage, together with the half-lives $t_{1/2}$ are summarized in *Table 15.*

Table 15: *Values of formal first-order kinetic parameters evaluated from the decrease of $TEAC_{ABTS^{\bullet+}}$ values in fruit juices during the storage.*

Sample label	k_{TEAC} [day^{-1}]	R^2	$t_{1/2}$
CA13	0.040 ± 0.005	0.998	3.923
CO14	0.016 ± 0.004	0.997	4.560
CN14	0.021 ± 0.006	0.994	4.848
CC15	0.022 ± 0.018	0.999	4.491
CN15	0.014 ± 0.022	0.999	4.970
AN14	0.037 ± 0.014	0.977	3.991

As follows from the values of correlation coefficients, in all the evaluated cases, high positive correlations of the fit to the experimental data were reached, proving that the optimum kinetic model was chosen. Regarding the kinetic rate constants, it is obvious that application Ar modified atmosphere "accelerated" the worsening of the radical-scavenging ability of orange juice samples which is in agreement to previously described changes of $TEAC_{ABTS^{\bullet+}}$. As the corresponding

conventionally produced counterpart for this sample is not available, this phenomenon cannot be discussed objectively.

When evaluating the effects of the modified atmosphere – CO_2 and N_2 – the problems with the variability of the experimental results affected the values of the calculated kinetic parameters, as is apparent from the data presented in *Table 15*. In fact, the values of the formal first-order rate constants are comparable, or, when considered on just absolute values basis, even contradictory. The last mentioned is particularly valid for samples produced in 2014 under the conventional air atmosphere and N_2 atmosphere, for which the reversed trend in k-values was evaluated, than would be expected. Values of rate constants evaluated for samples produced in 2015 under N_2 and CO_2 are comparable, supporting thus the conclusions presented above, i.e., that the protection effects of both these atmospheres are comparable.

In case of kinetics of TEACs values of pineapple juices decrease, in contradiction to AAE values, there is an obvious trend indicating the gradual decrease of TEAC values, expressed by the formal first order rate constant and half-live, however, due to the reasons mentioned above, any effect cannot be evaluated, as there do not exists corresponding counterpart.

5.3 HPLC experiments

HPLC experiments were performed in the laboratory of chromatography and separation techniques of the Food Research Institute, Bratislava. The changes in ascorbic acid concentration and concentration of hesperidin flavonoid, as a typical representative of flavonoids occurring in orange juice were monitored.

5.3.1 Ascorbic acid concentration

Changes in the ascorbic acid (AA) concentration in orange and pineapple juices resulting either from the modified atmosphere application during the production or from the long-term storage were evaluated by HPLC . Measurement conditions are described in chapter 4.6. Results were expressed in terms of concentration units, $mg.l^{-1}$.

For the purposes of the quantitative evaluation of the assessed effects of the storage, the identical approach was used as in the previous assays. The difference in the concentration of AA, Δ_{AA} value, representing the difference in AA content of freshly produced sample and sample stored for 90 days was calculated, as well as % change analogously as previously described. AA concentrations evaluated for orange and pineapple juice samples of different production atmosphere at the respective time of their storage are summarized in *Table 16.*

Table 16: *Ascorbic acid concentration determined by HPLC f in orange and pineapple juices at the respective time of their storage.*

Time of storage	**Ascorbic acid [mg·l^{-1}]**					
	CA13	**CN14**	**CO14**	**CN15**	**CC15**	**AN14**
Start of the experiment	193 ± 18	257 ± 19	310 ± 29	324 ± 29	353 ± 66	365 ± 16
90th day of storage	79 ± 12	162 ± 30	97 ± 7	287 ± 13	306 ± 13	274 ± 2
Expiration date	63 ± 4	155 ± 11	88 ± 21	N/A*	N/A*	261 ± 3
End of the experiment	35 ± 10	158 ± 34	71 ± 21	N/A*	N/A*	257 ± 2
Δ_{AA}	114	95	213	37	47	91
% change	59.1	37.0	68.7	11.4	13.3	24.9

* Experiments are still in progress, to the date of diploma thesis completion, data for 90th day of storage have become available.

5.3.1.1 Orange juices with pulp

Effect of storage

In accord with expectations, the highest AA concentrations were detected in freshly prepared samples in the beginning of the experiments. The lowest for samples produced in 2013 under the Ar atmosphere, followed by the samples produced in 2014 and the highest concentration of AA was noticed in both types of samples produced in 2015.

The trends in AA concentrations clearly illustrate the seasoning effect and also the variability of the experimental results, affecting the standard deviation values. In addition, it is also obvious, that when the measurement uncertainty is taken into account, the initial concentrations of AA determined in both type of samples produced in 2014 (O_2 vs. N_2) and 2015 (N_2 vs. CO_2). At the 90^{th} day of storage, the highest decrease of AA concentration was detected in CO14 samples, reaching approx. 69%, followed by CA13 samples. In fact, changes in AA concentrations determined by HPLC correlate well with changes in AAE values during the storage discussed above.

Effect of pasteurization

Impact of modified atmosphere and pasteurization on AA values of orange fruit juices with pulp produced in 2014 is summarized in *Table 17*. As follows from the evaluation, the application of N_2 modified atmosphere seemed to have negative effect on AA concentration, because the AA concentration of pasteurized samples exhibited 11.7 % decrease for samples produced under the conventional unmodified atmosphere and 27.0 % decrease for samples produced under N_2 modified atmosphere. This result is in contradiction to previously described effect of pasteurization and modified atmosphere on AAE values of juices processed in 2014, since for those samples only the minor changes of AAE were observed.

Table 17: *Effect of modified atmosphere application and pasteurization on AA concentration determined by HPLC.*

Sample label	AA concentration [mg·l^{-1}]	Δ_{AA}	% change
CO14 Raw	351 ± 27		
CO14 Pasteurized	310 ± 29	41	11.7
CN14 Raw	352 ± 40		
CN14 Pasteurized	257 ± 19	95	27.0

5.3.1.2 Pineapple juices

AA concentration of pineapple juices produced under N_2 modified atmosphere (AN14) exhibited 24.9 % decrease during the first 90 days of the storage, after which only the slight decrease was observed. In comparison to AAE values evaluated from TEMPOL assay, it is unclear if AA can be correlated to. These results are comparable to previously published studies of Food Research institute [71].

5.3.2 Hesperidin concentration

Hesperidin is one of the most abundant flavonoids usually present in orange pulp [6], released during the processing to orange juices. Thus, the changes of its concentration during the storage can be taken as a measure of flavonoids degradation and they also can be correlated to gradual decrease of total polyphenol content (TPC) of orange juices assessed by UV-VIS. For the purposes of quantitative evaluation of the individual assessed effects, again the difference in the concentration of hesperidin, Δ value, representing the difference in hesperidin content of freshly produced sample and sample stored for 90 days was calculated, as well as % change analogously as previously described for the other evaluated parameters presented in the diploma thesis.

Values of hesperidin concentration determined for orange juice samples of different production atmospheres at the respective time of their storage are summarized in *Table 18.*

Table 18: *Hesperidin concentration determined by HPLC in orange and pineapple juices at the respective time of their storage.*

	Hesperidin concentration [mg·l^{-1}]				
	CA13	**CN14**	**CO14**	**CN15**	**CC15**
Start of the experiment	296 ± 32	132 ± 1	90 ± 22	324 ± 29	353 ± 66
90th day of experiment	298 ± 197	76 ± 12	79 ± 18	287 ± 13	306 ± 13
Expiration date	134 ± 94	75 ± 11	71 ± 19	N/A*	N/A*
End of the experiment	78 ± 44	70 ± 23	74 ± 17	N/A*	N/A*
$H_{esperidin}$	2	56	11	37	47
% change	0.7	42.4	12.2	11.4	13.3

* Experiments are still in progress, to the date of diploma thesis completion, data for 90th day of storage have become available.

5.3.2.1 Orange juices with pulp

Effect of the storage

As follows from the overview of hesperidin concentrations determined in individual fruit juices summarized in *Table 18*, hesperidin content gradually decreased during the storage, what is the expected trend, in good agreement with gradual TPC decrease assessed by UV-VIS. The only exception was noticed for juices produced under the Ar modified atmosphere (CA13), where 0.7 % increase in hesperidin content was observed. However, the observed increase is within the measurement uncertainty, thus, it can be concluded, that in case of juices produced in 2013 under argon, practically steady-state of hesperidin concentration during the storage was determined.

Effect of pasteurization

Influence of the slight pasteurization on hesperidin content in orange juices produced in 2014 is summarized in *Table 19.*

Table 19: *Impact of the modified atmosphere production and pasteurization on hesperidin concentration determined by HPLC in orange juices with pulp produced in 2014.*

Sample label	**Hesperidin concentration [mg·l^{-1}]**	**$\Delta_{hesperidin}$ [mg·l^{-1}]**	**% change**
CO14-Raw	60 ± 0		
CO14-Pasteurized	90 ± 22	30	50
CN14-Raw	141 ± 23		
CN14-Pasteurized	132 ± 1	9	0.30

Unlike the TPC of the same juice samples, hesperidin content of CO14 increased by 50 % after the pasteurization. This effect is probably caused by the extraction of hesperidin from pulp or more probably, as a result of partial oxidation of polyphenols and potentially their thermally- and oxidation-induced cleavage during the pasteurization, resulting in the formation of low-molecular flavonoid and hesperidin - like structures. In addition, different pulp content in raw and pasteurized samples can also

affect the determination of the hesperidin concentration. Unfortunately, the dry matter/pulp content in these samples was not assessed.

As regards the effect of pasteurization on orange juice produced under the N_2 atmosphere, slight decrease of hesperidin concentration was observed, in good agreement with pasteurization effect on TPC values of this sample, discussed above.

5.4 Statistical evaluation of the results

As mentioned in the experimental part of the thesis, the results of all assays were processed by statistical analysis in order to evaluate the mutual relationships between the individual sample characteristics and some their properties affected by the origin, way of production and season of production. It should be noted here, that the statistical analysis was applied only on the results obtained for orange juice samples, as there is (with an exception for the routine standard deviations of means determination) practically no external factor, by which the pineapple juices should be evaluated/compared.

Beside the ANOVA Tukey HSD, also the methods of multivariate statistics, particularly the method of principal components analysis (PCA), principal component factoring (PCF) and discriminant statistics (canonical discriminant analysis, CDA, and k-th nearest neighbor classification) were involved. ANOVA (Analysis of Variance) allows performing multiple comparisons of mean values of respective parameters – it is generally based on the evaluation of the relationship between the variances of the compared samples and, in fact, gives the analysis of the effect of one selected factor on the selected dependent variable under the examination.

To evaluate the inter-relationships of multiple parameters of a larger set of samples, methods of multivariate statistics are effectively involved. They were previously successfully applied e.g., for differentiation of wines according to the production practices, milk and cheeses geographical differentiation, or as a tool to differentiate and classify the γ-treated spice samples according to the absorbed dose of γ-radiation [72-74].

PCA is effectively used to analyze the hidden relationships between the individual variables – experimental characteristics - so that a large number of potentially correlating experimental characteristics are transformed via linear/non-linear combination to the smaller (at most equal) number of non-correlating principal components. The linear components are designed so that the first component describes the maximum variability of the studied system, and any other component reflect the maximum of the remaining variability. Subsequent analysis of the statistical data brings also the information on which experimental characteristics has the highest weight (contribution) in the description of the variability of the whole system. In addition to PCA, factor analysis provides the information about the correlation of individual experimental characteristics - parameters. Using the PCF, the correlations of individual variables were analyzed, as those non-correlating could be utilized for the differentiation of samples according to the chosen criteria.

Discriminant analysis examines the patterns in the classification of the evaluated objects into groups and on the basis of the so-formulated rule, discriminant function; classification of the object in a particular group according to the selected criterion is performed. In the construction of the canonical discriminant function, all the independent – non correlating variables are considered [75].

Method of the k-th nearest neighbor classification is the simplest classification method, in which the samples are classified into groups based on comparison of properties of the classified object to these of already classified nearest objects (commonly, one to three objects – neighbors – are taken for properties consideration). It is a non-parametric test, in which the probability that an object belongs to the classified group is assessed by calculating the distance of the k-th nearest neighbor to the center of gravity of the whole group (centroid). By this approach, the effects of outliers on miss-classification is rather eliminated. The number of the neighbors taken for comparison is defined by the user, usually, $k = 1, 2$ or 3. The higher the number of the compared object (the higher k), the variability is generally higher and lower the classification score [75].

For the statistical evaluation, the TPC content, L*, a*, b*, TCD as well as AAE, TEAC, AA content and hesperidin content (altogether 9 parameters) were taken as variables, and the year of production, and the atmosphere applied were taken as factors. The effect of fruit type (pineapple vs. orange, was not considered, as these differences are obvious by nature and are not the subject of the study.

ANOVA Tukey HSD proved that all the compared experimental characteristics are significantly different in dependence on the effect of storage, the year production, the origin of sample or production atmosphere, with some exception. The test was performed on the significance level of α=0.05. These results are in good agreement with the behavior of the samples described above. Detailed insight is given by the multivariate statistical analysis. Of course, some exceptions (non-significant differences) were occasionally noticed for all the parameters and both factors, but their detailed description would be exhausting and without practical effect on the global observations.

Results of PCA analysis are depicted on *Figure 35*. For the principal components construction, all 9 experimental characteristics were used, and the analysis was performed using the year of sample production as a factor, although the factor was used just for illustration, as it has no effect in PCA on the results, just the graphical output may differ in dependence on classification factor used.

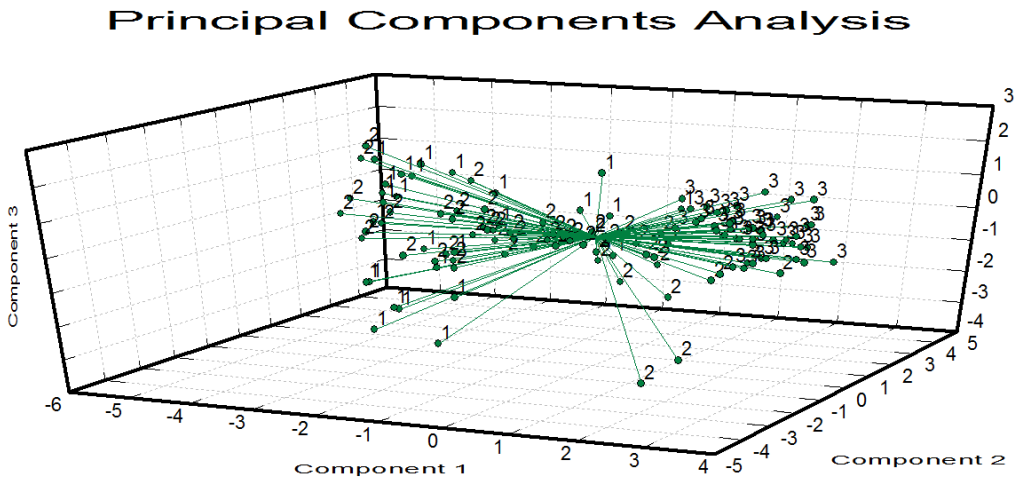

Figure 35: *Principal component analysis of orange juices with pulp produced in (1) – 2013, (2) – 2014 and (3) – 2015. All 9 experimental characteristics (TPC, L*, a*, b*, TCD, AAE, TEAC, AA and hesperidin content) were used for PCs construction.*

As is obvious from the plot of PCs, partially successful separation – differentiation – of the samples was reached, as there are apparent 3 groups of vectors, partially separated one from each other, according to the year of sample production.

Results of the analysis indicated, that first 3 PCs cumulatively explain approx. 90% of the variability of the whole dataset of experimental characteristics, what is very promising. To explain 100% of the variability, 6 PCs were needed. As follows from the values of Eigenvectors, indicating the importance of individual experimental characteristics in the construction of principal components (data not presented), it is obvious, that in the first PC, the most important role played the TEAC values, followed by L* and AA concentration assessed by HPLC. In the 2nd principal component construction, key role of TPC content as well as of hesperidin concentration and AAE values was identified, whereas in the 3rd one, the concentration of hesperidin and color characteristic b*. Thus, it can be concluded that these parameters contribute to the explanation of the variability of the results to the maximum.

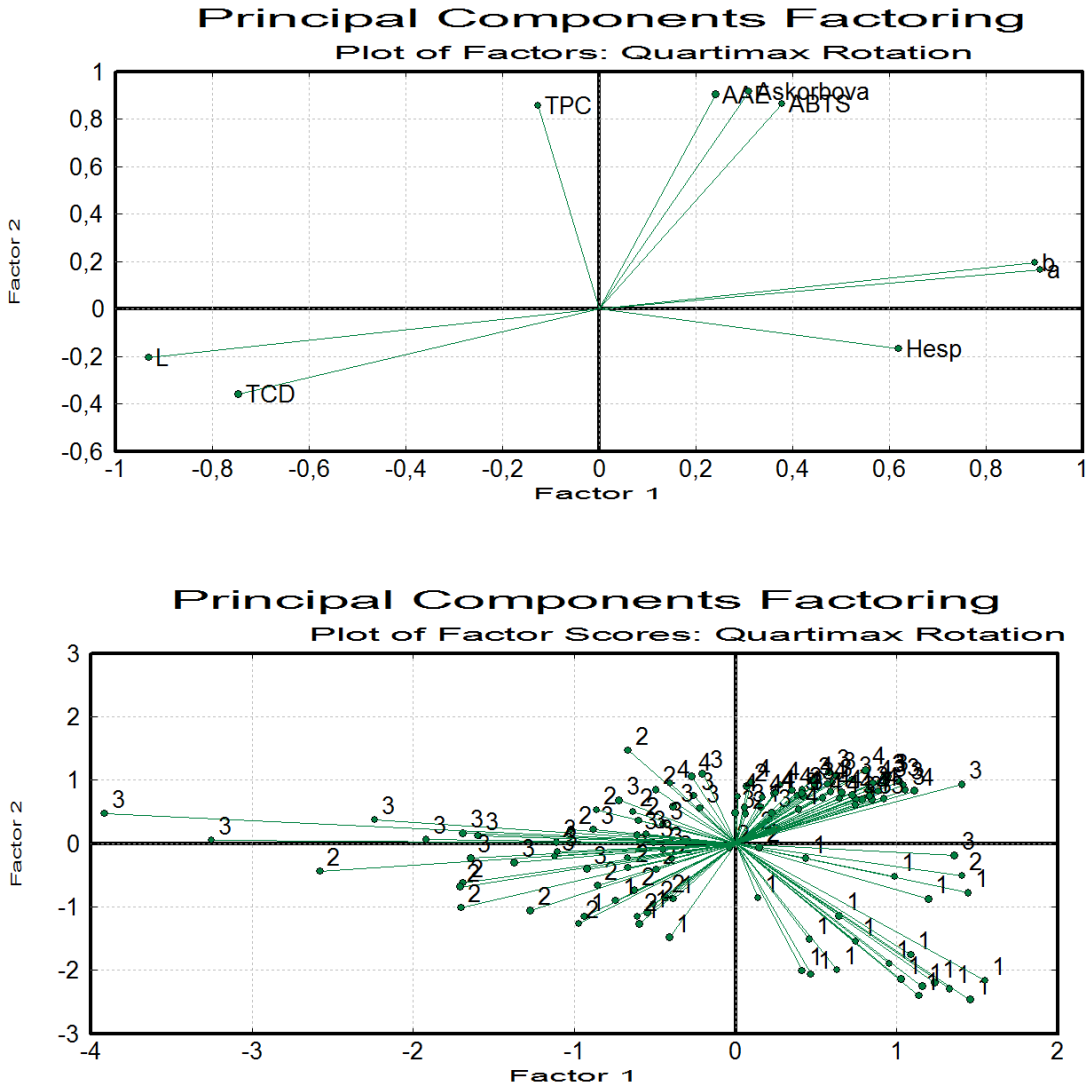

Figure 36: *Plot of factors and of factor scores in Quadrimax rotation, constructed from the whole dataset of the experimental characteristics (TPC, L*, a*, b*, TCD, AAE, TEAC, AA and hesperidin content).*

The above-described findings of PCA confirmed also the processing of the experimental dataset by the principal component factoring analysis. PCF is in fact based on similar principles like the PCA, as the PCF is usually its first step (refer to plot of factor scores depicted on *Figure 36*). However, PCF enable to determine and visualize the hidden correlations between the individual variables, as is clear from the plot of factors on *Figure 36*. The stronger the correlation, the closer is the position of individual vectors belonging to variables, and vice versa. By this approach, 5 groups of variables can be identified, for which the correlation within group is rather strong, but the between-group correlation is weak – (L*, TCD), (TPC), (HESP), (b*, a*) and (TEAC, ABTS, AA concentration).

Key role of the statistical analysis performed herein was to assess and present the effects of season and the production atmosphere on juices properties. As indicated above in the description of principles of the discriminant analysis, the correlating variables are either eliminated, or they are taken just marginally, and dominant role for the purposes of the discrimination play the non-correlating variables or groups of such variables.

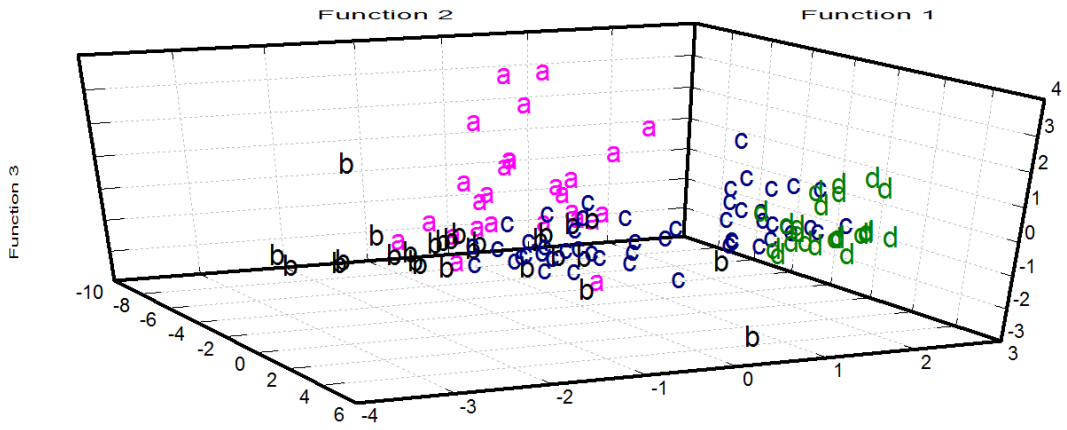

Figure 37: *Discrimination of orange juice samples by Canonical discriminant analysis, on the basis of the entire group of experimental characteristics. The production atmosphere was used as the discrimination criterion (a- Ar, b-O_2, c- N_2, d-CO_2).*

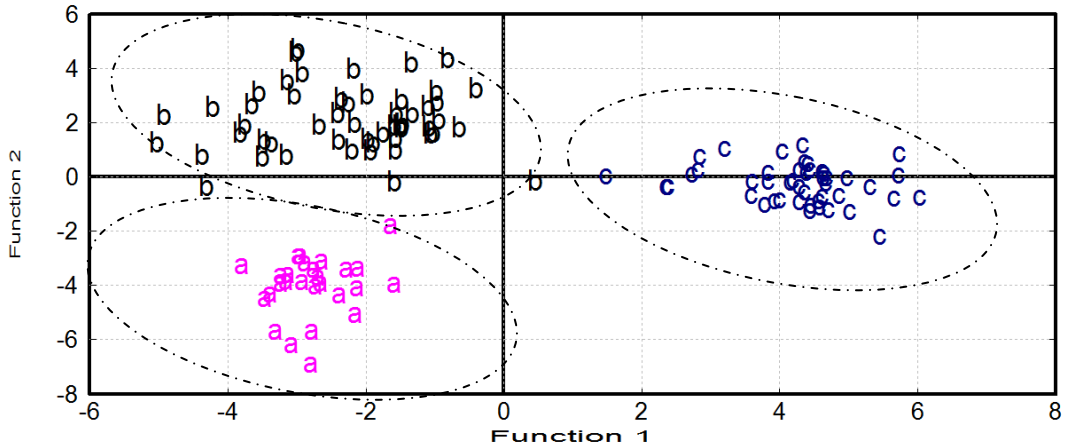

Figure 38: *Discrimination of orange juice samples by Canonical discriminant analysis, on the basis of the entire group of experimental characteristics. The year of production was used as the discrimination criterion (a- 2013, b-2014, c-2015).*

The discrimination of samples according to the chosen criteria – production atmosphere and year of production (considering thus the seasoning effect) is depicted on *Figure 37* and *Figure 38*, respectively. As is obvious, the discrimination according to the production atmosphere brought only partially successful discrimination, although high overall discrimination score of 87.4 % was reached. However, absolute discrimination from the other samples was reached for those samples produced under the Ar atmosphere and under the CO_2. Very high, 93 % correctness was reached for samples produced under the conventional air atmosphere. Problematic was particularly the discrimination of N_2 produced samples, with the highest misclassifications - the discrimination score of samples produced under N_2 reached 72 % - of totally 50 samples, 8 (16%) were misclassified as being produced under the conventional air, and 6 (12%) as being produced under the CO_2.

Most probably, the problems with samples produced under nitrogen comes from the fact, that these samples were provided 2 times – in 2014 and in 2015, thus, also the effect of season takes place. As is proved bellow, but also stressed several times in the thesis, the effect of season, i.e., year of production, affects the properties of the samples most significantly.

The seasoning effect is clearly obvious from the plot of discriminant analysis depicted on *Figure 378*. Here, 3, well detectable separate regions – clusters of points are obvious, belonging to the individual production years. The observation was confirmed also by the statistical results, as 100% overall classification score was reached.

Last way of classification was the application of the kth-nearest neighbor method. For the purposes of samples differentiation and classification, 1-3 neighbors approach was used and the classification was performed according to both, the production method and season of sample production. The results of the classification are summarized in *Table 20*.

Table 20: *Classification scores of orange juice samples in classification by kth-nearest neighbor method according to the production atmosphere and year of sample production.*

Classification parameter	**Number of neighbors, *k***	**Classification score**
Production atmosphere	*K=1*	100.00 %
	K=2	91.34 %
	K=3	92.91 %
Year of production	*K=1*	100.00 %
	K=2	100.00 %
	K=3	99.21 %

It is apparent that the kth- nearest neighbor method resulted in highly correct discrimination of the samples according to both criteria. The relative worsening of the classification scores for k = 2 and k = 3 in classification according to the production atmosphere is most probably the result of the system variability increase – and thus, with number of the neighboring objects compared, also the uncertainty of the classification increased, although the classification score is in both cases still above 91%.
Classification of samples according to the year of sample production again proved unambiguously that the properties of juice samples are affected by the seasoning effects strongly then by any other physical or even technological processing effects.

6 CONCLUSION

In this diploma thesis, the effect of modified production atmosphere application (nitrogen, argon, CO_2) on selected qualitative parameters (characteristics) of orange juices with pulp and pineapple juices, and the effect of their long-term storage (4 month) under the defined conditions were assessed. Main attention was focused on the determination of antioxidant and radical-scavenging activity and color characteristics, as well as on the concentration of ascorbic acid, total polyphenols and of selected flavonoids. For these purposes, several EPR, UV-VIS and HPLC assays were effectively involved. In addition, kinetics of changes of the individual characteristics was assessed via the kinetic rate constants and half-life of respective samples evaluation. For the purposes of the description of kinetics of changes of individual parameters, 1st order kinetics was effectively employed.

The results were also processed by means of multivariate statistical methods in order to consider the mutual relationships between the experimental characteristics as well as the effects of modified atmosphere application, samples origin and seasoning effects on quality of fruit juices.

Results obtained clearly confirmed that the pasteurization as well as the production atmosphere influence the quality of the product. Although the "slight" pasteurization was applied, in comparison to the fresh - non-pasteurized samples, slight decrease of values of practically all the monitored parameters was observed. In the samples processed under the modified atmosphere, generally the retardation of the decrease of qualitative parameters as a result of inert atmosphere application in dependence on type of production gas was noticed.

As the general conclusion on the effects of the production atmosphere and storing on TPC concentration, color characteristics, antioxidant and radical-scavenging activity as well as ascorbic acid concentration of analyzed juices, it can be stated that:

- It is obvious that the application of N_2 or CO_2 modified atmosphere had very similar impact on majority of individual characteristics during the storage.
- The applications of modified N_2 or CO_2 atmospheres positively influence the TPC and antioxidant activity in comparison to the processing without the modified atmosphere.
- The effect of the modified atmosphere production on changes in color characteristics of orange juices during their storage is influenced significantly by the sample homogeneity and/or by the presence of solid particles in the juice solution, or the turbidity of the samples caused e.g., by the presence of proteins.
- The comparison of TCD of samples produced under the respective atmospheres revealed the following positive effect (in decreasing order according to TCD values at 90th day of storage): Ar> (CO_2≈N_2).
- Application of modified atmosphere has positive effects on juices color protection during the pasteurization, recognizing the color as one of the most important parameter in attracting the potential consumer.
- The application of N_2 modified atmosphere lead to the effective protection of low-molecular organic acids present in the orange juice, slowing the deterioration of the radical-scavenging activity of the samples in comparison to those produced under the conventional air atmosphere.
- Changes in AA concentration determined by HPLC correlate well with changes in AAE values during the storage determined by EPR, without respect on the production atmosphere applied.
- Kinetic study of the changes of the monitored parameters upon the storage period indicated the retardation of their decrease as a result of inert atmosphere application in dependence on type of production gas
- Results obtained confirmed the significant seasoning effect on majority of the monitored characteristics of orange juices (in comparison to juices produced in 2014, those of 2015 production exhibited overall higher antioxidant activity).

- The application of Ar atmosphere was less effective in comparison to N_2 or CO_2 modified atmospheres.
- Pineapple juice had generally higher concentration of polyphenols and ascorbic acid than the orange juices under study, caused probably by different composition and concentrations of individual polyphenols in respective fruits.
- Application of the modified atmosphere, especially in case of Ar, resulted into better predictability of monitored parameters changes.
- In case of CO_2 application, one should expect the interaction of the gas with juice components, not just the physical but also chemical, affecting thus also the sensorial properties of the samples, although the qualitative aspects were improved and/or comparable to other inert gases.

Results of this study will help the juice producer in optimization of juice processing conditions in order to obtain the product with maximum beneficial properties kept during the expiration period and, in addition, with the prolonged shelf life.

Generally, inert gases could improve the quality of fruit juices; however the application of certain gas is the compromise of the expenses on technology modification and the effects obtained. Factors including the potential physical and\or chemical interactions of gas with juice components should also be considered carefully.

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8 LIST OF ABBREVIATIONS

2,3-DKG	2,3-diketogluconic acid
AA	Ascorbic acid
AAE	Ascorbic acid equivalent
$ABTS^{\bullet+}$	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical
AN14	Pineapple juice processed in 2014 under N_2 atmosphere
ANOVA	Analysis of variance
CA13	Orange juice processed in 2013 under Ar atmosphere
CC15	Orange juice processed in 2015 under CO_2 atmosphere
CDA	Canonical discriminant analysis
CIE	International Commission on Illumination (Commission internationale de l'éclairage)
CMY	Cyan, magneta, yellow color model
CN14	Orange juice processed in 2014 under N_2 atmosphere
CN15	Orange juice processed in 2015 under N_2 atmosphere
CO14	Orange juice processed in 2014 under conventional atmosphere
CUPRAC	Cupric reducing antioxidant capacity
DHA	Dehydroascorbic acid
$^{\bullet}$DPPH	2,2-diphenyl-1-picrylhydrazyl
DMPD	N,N-dimethyl-p-phenylenediamine dihydrochloride
DMPO	5,5-dimethyl-1-pyrolin-N-oxid
EPR	Electron Paramagnetic Resonance
F-C	Folin-Ciocalteu reagent
FRAP	Ferric reducing activity of plasma
GAE	Gallic acid equivalent
HDPE	High density polyethylene
HPLC	High-performance liquid chromatography
MAP	Modified atmosphere packaging
MDHA	Monodehydroascorbate
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PBN	α-phenyl-N-tert-butylnitrone
PCA	Principal component analysis
PCF	Principal component factoring
PE	Polyethylene
PEN	Polyethylene naphthalate
PET	Polyethylene terephthalate
POBN	α-(4-Pyridyl N-oxide)-N-tert-butylnitrone
RGB	Red, green, blue color model
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulfur species
TCD	Total color difference
TEAC	Trolox equivalent antioxidant capacity
TEMPO	2,2,6,6-tetramethyl-4-oxopiperidinyl
TEMPOL	2,2,6,6-tetramethyl-4-oxopiperidinyl-1-oxyl
TPC	Total polyphenol content
UV	Ultraviolet
UV-VIS	Ultraviolet-visible