CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
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EVALUATION OF FOOD QUALITY ACCORDING TO VOLATILE COMPOUND PROFILE

Influence of the processing in volatile compounds in apple juice

by Rita Portugal Pinho Rocha

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Influence of the processing in volatile compounds in apple juice

Training Placement Report presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Food Engineering

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ABSTRACT

Apples are one of the most extensively cultivated fruit in the world and the juice is an important product worldwide. Fruit aroma is a complex mixture of a large number of volatile compounds that contribute to the overall sensory quality of fruit. The majority of volatiles extracted from apples are esters and alcohols. Their synthesis in apples may be affected by pre, at, or post-harvest factors such as maturation process, temperature during the production process and apple's storage.

The aim of the project was to analyse the quality of apple juicesfrom different processing steps according to the volatile compounds'profile, using a solid-phase microextraction followed by gas chromatography-mass spectrometry method (SPME/GC/MS)**.** There were analyzed three different batches of apple juice production, with six samples in each batch (pulp, pulp with enzymes, juice after press, juice without aroma, concentrate and aroma).

All samples were assayed for soluble solids, formol number and acidity, ash and phosphor content as well as the profile of volatile compound, using reference methodologies.

In general, volatile compounds are lost throughout the different steps of the production process. Highest losses are reported between pulp and juice samples and the main volatile compounds lost were hexanal and E-2-hexenal. Butyl acetate were not affected by the production process. The major constituents of apple juices and aromas are E-2-hexenal, 2-hexen-1-ol and 1-hexanol. A large variability was observed between batches which may be a consequence of maturity level and cultivar type. The experiment showed that the production process influences the concentration of the volatile compounds in the apple juice.

Keywords: apple juice, volatile compounds, gas-chromatography.

RESUMO

As maçãs são uma das frutas mais cultivadas do mundo e o seu sumo é um produto relevante em todo o mundo. O aroma da fruta é uma mistura complexa de um grande número de compostos voláteis que contribuem para a qualidade sensorial geral dos frutos. A maioria dos compostos voláteis extraídos das maçãs são ésteres e álcoois. Nas maças, a sua síntese pode ser afetada por fatores antes, durante ou após a sua colheita como o estado de maturação, a temperatura durante o processo produtivo e o armazenamento.

O presente estudo teve como objetivo a análise da qualidade do sumo de maça tendo em conta o perfil dos compostos voláteis. Para isso, analisou-se através da microextração por fase sólida e cromatografia gasosa acoplada à espectrometria de massas, três linhas de produção diferentes, cada uma com seis amostras (mistura de maças homogeneizada, mistura de maças com enzimas, sumo após prensagem, sumo sem aroma, concentrado e aroma).

A parte experimental baseou-se na análise, para todas as amostras, dos sólidos solúveis, número de formol, acidez, quantidade de cinzas, conteúdo de fósforo e do perfil de compostos voláteis, através de métodos de referência.

Em geral, os compostos voláteis são perdidos ao longo dos diferentes passos do processo de produção. As maiores perdas ocorreram entre amostras de mistura de maçãs e de sumo. Os principais compostos voláteis perdidos ao longo do processo de produção foram o hexanal e o E-2-hexenal. O acetato de butilo não foi afetado pelo processo de produção. Os principais componentes de sumos e aromas de maçã são E-2-hexenal, 2-hexen-1-ol e 1-hexanol. Observou-se uma grande variabilidade entre os lotes, que pode ser consequência dos diferentes níveis de maturação e das espécies de maçãs. O presente trabalho mostrou que o processo de produção influencia a concentração dos compostos voláteis no sumo de maçã.

Palavras-chave: sumo de maça, compostos voláteis, cromatografia gasosa.

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INDEX

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAT – Alcohol acyltransferase

Acyl-CoA – Acetyl coenzyme A

ADH – Alcohol dehydrogenase

BD – Bisbee Spur Delicious

C6 – Six carbon

FJ – Fuji

GD – Golden Delicious

GRE – Golden Reinders

GRU – Gold Rush

GS – Granny Smith

HTST – High temperature, short time

LOX – Lipoxygenase

n.d. (Table 1.4.) – not detected

PL – Pink Lady

PME – Pectin methylesterase

SoC6 – Sum of Carbon 6 – aldehydes and alcohols

SPME/GC/MS – Gas chromatography-mass spectrometry method

ULO – Ultra Low Oxygen

CHAPTER 1

GENERAL INTRODUCTION

1.1. Training placement

This training placement was carried out at the University of Chemistry and Technology Prague, Faculty of Food and Biochemical Technology and, in the Food Preservation Department. During the five-month period of the training placement that took place between February and July 2017, the main objective was to carry out a research project, which main aim was to analyse the quality of apple juice according to volatile compounds profile using a solid-phase microextraction followed by gas chromatography-mass spectrometry method. The supervision was the responsibility of the doc. Ing. Helena Čížková, Ph.D. and the laboratory advisor was Ing. Vojtěch Kružík.

The University of Chemistry and Technology, Prague is the largest educational institution in Central Europe and is known for its education and research activities in almost all branches of chemistry, chemical engineering, food chemistry and technology, biochemistry, refining, water treatment, power and biological sciences and technologies, as well as environment protection, materials sciences, and other chemistry-based fields of study. The Department of Food Preservation works on many research and development activities involving: modern food packaging, food authentication, diagnostics of food defects, evaluation of shelf life and increasing of food stability, implementation of new technologies in processing of fruit and vegetables and in the meat technology.

1.2. State of the art

1.2.1. Fruit juices

Fruit juices are widely consumed in ever-increasing quantities and are very important commodities in the trade of most countries.[1] These represent a growing category in the beverage market. In 2000, juice consumption accounted for 44% of total fruit

consumption in the United States. [2] The consumption of fruit juices in Europe amounts to approximately 25 liters per capita and year. [3] In addition to orange juice, apple juice makes up a major part of it, particularly in Germany, the world's leading country in fruit juice consumption (40 liters per capita and year). [3] Fruit juice's market is centered in single flavor types, mainly orange, apple and pineapple, as well as in multi fruit juices made with a mixture of flavors. [4] Orange juice is the most popular of the juice products, accounting for 60% of all juice consumed. [2]

According to the EU directive 2012/12/EU, of 27 April 2012, fruit juice is 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. Concentrated fruit juice is 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. The fruit juice from concentrate is the product obtained by reconstituting concentrated fruit juice defined above 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. This type of fruit juice must be 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. [5]

If juice is to be sold as "not from concentrate" it is usually screened and pasteurized immediately after pressing to control the growth of spoilage micro-organisms that live on the fruit surface (mainly yeasts and moulds) and to destroy the pectolytic enzymes that occur naturally in fruit that would otherwise break down the cloudy nature of the juice. If, however, a clear juice is required (apple or raspberry), enzymes such as pectinases can be added to accelerate this natural process. An example of these type of juice is the fresh single-strength juice. These are made by squeezing fruit, subjecting it to some processing, packaging it and selling it within a cold chain distribution system. Such juice has a shelf life that varies from 1/2 weeks to 2/3 months. Juice for concentration is normally subjected to screening to remove cellular debris and then fed to a one or multi-stage evaporation process to remove most of the water and volatile

material. Evaporators are highly efficient processing units(up to nine stages are used, sometimes with thermal recompression). Increasingly, evaporators also recover the volatile aromatic substances that are partly responsible for giving fruit juices their sensory characteristics. The re-addition of such volatiles is widely practiced at the point when concentrates are reconstituted into single strength juices. The European Council Directive 2001/112/EC (the Fruit Juices Directive) states that the addition of such volatiles at reconstitution is obligatory. The UK 2003 Regulations (which are based on this Directive) state that reconstituted fruit juice is the product "obtained by replacing, in concentrated fruit juice, water extracted from that juice by concentration, and by restoring the flavors". After concentration, juices are normally held in storage until they are reconstituted. Apple concentrated juices can be held at around 10–15ºC without risk of deterioration. The degree of concentration plays an important part in determining storage conditions and, apple juice is normally concentrated to about 70ºBrix.[1]

1.2.1.1. Apple juice

Apple is the second most consumed fruit juice in the world, with total export value of single-strength and concentrated juice of US$3936 million in 2009.Thelargest exporters of apple juice concentrate are Poland, Austria, Hungary, United States, and Ukraine. Poland accounts for 28.1%, Austria for 10.5% and the rest, under 10%. The largest exporters of single-strength apple juice are China, Germany, Italy, Poland, and Austria. China accounts for 51.9%, and all others under 10% of the world exports. The largest importers of apple juice concentrate are United States, United Kingdom, Russian Federation, Japan, and Germany. The United States accounts for 36.5% of imports. The largest importers of single-strength apple juice are Germany, Netherlands, France, Austria, and United States, with Germany accounting for 33.2% of the imports.[6]

The large majority of apples are harvested for the fresh market, by hand. Small apples or not suitable apples for other processed products such as slices or sauces, are used for juice processing. However, with the increased demand for apple juice, some apples are being harvested for juice processing. [6]

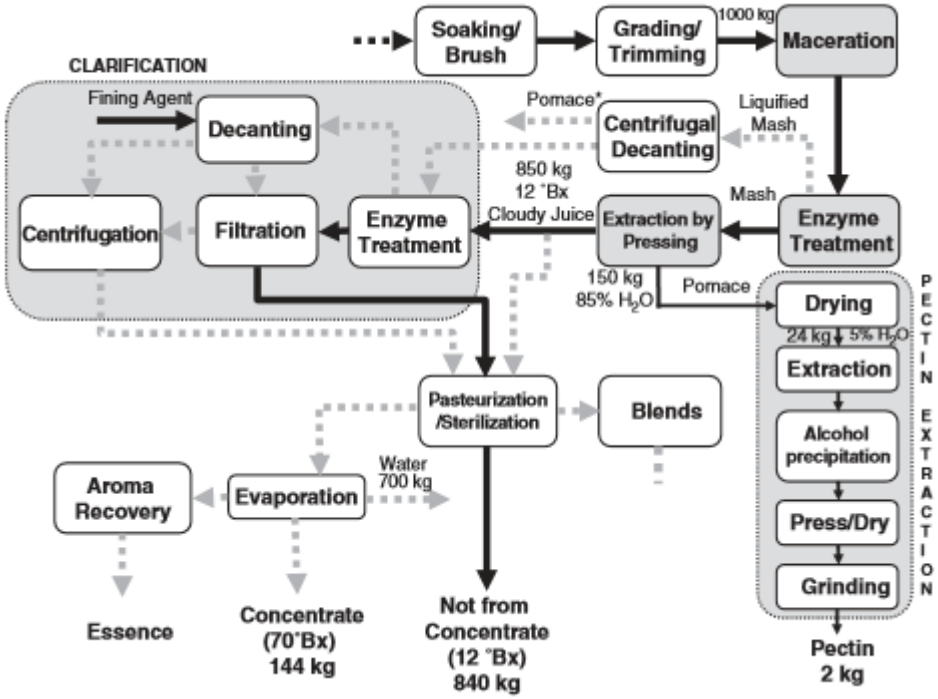

Figure 1.1. - Flow diagram of apple juice and apple by-product processing. [6]

Apple juice process is complex and figure 1.1 shows the diagram of apple juice and apple by-product processing. First of all, apples are washed to remove molds and bacteria that may spoil the juice. After, fruit with signs of spoilages are either removed or cut to remove spoiled portions. Then, all apples are macerated to produce the pulp. Enzyme treatment with cocktails of pectin methylesterase (PME), polygalacturonase, pectinlyase and cellulase are used to hydrolyze pectin and fruit cell wall, facilitating juice release during pressing and increasing yield. Enzyme treatment requires heating because it is most effective at around 50ºC and requires some reaction time. Small amounts of enzyme are required (approximately 100mL/t), making this approach cost-effective. At 50ºC some of the enzymes in the cocktail denature and lose activity. However, treatment at lower temperatures (approximately 40ºC) favors microbial growth, and even lower temperatures result in decreased enzyme activity. Therefore, enzyme cocktail formulations require a careful balance of kinetic activity and stability. A complex enzyme cocktail with increased cellulase, hemicellulase, oligomerase and other enzyme activities is used for mash liquefaction. Apple juice is extracted by pressing the mash in a belt, hydraulic, pneumatic, other type of press or a combination of presses. Typically, hydraulic presses are more efficient (3–5%) than their pneumatic counterparts. For liquefied mash, a decanting centrifuge is used. Juice yield is affected

by pressing conditions (temperature, pressure, presence, and type of pressing aid) and by the quality of the mash, which in turn depends on the quality of the apples, particle size after maceration and extent of enzyme treatment. Typical juice yields range between 70% and 95%. Cellulose or rice hulls are often added (1–2%) as press aids that help to uniformly distribute pressure across apple particles that constitute the pulp and mash. Press aids can increase extraction yields by up to 10%. Most apple juice is clarified, although the market for cloudy, "country style" apple juice appears to be growing with the demand for more fresh-like, minimally processed food products. Pectinase cocktails are currently use to clarify apple juice. Cleavage of pectin decreases juice viscosity, which facilitates filtration. Pectinase formulation is used for clarification and the conditions are also either 10–20ºC for 8–10h or 45–55ºC for 1–2h. The amount of enzyme formulation is adjusted by each processor but is in the range of 20–30mL/m^3 for liquid formulations. Unlike with citrus fruit, addition of enzymes to apple and other clarified juices does not violate the standards of identity. Fining, that is, further removal of suspended colloidal particles by decanting, is facilitated by addition of positively charged gelatin or bentonite during or after enzyme clarification. The decanted juice is further clarified by filtration and/or centrifugation. There are different types of filters, including press, vacuum, and rotary filters. Because apples of many different varieties are used for juice production, blending is commonly done to produce uniform quality product. Apple juice concentration is done mostly by evaporation, although freeze concentration can be done. Apple juice is typically concentrated to 70ºBrix in 4–5 steps. High temperature, short-time (HTST) falling film evaporators that operate under high vacuum at 90–100ºC are commonly used. Apple juice evaporators are operated in mixed configuration. Single-strength juice is fed to the second or third stage to separate volatiles at a lower temperature than if fed at the first stage. Aromas are recovered and concentrated by distillation. High temperatures are used to pasteurize the juice during evaporation to ensure sufficient microbial death and avoid concentrate spoilage. Plate evaporators and rising film evaporators have also been used for apple juice concentration.[6]

1.2.1.2. Legislation

Fruit juices are materials of commerce, to be sold direct or for use in a variety of food and drink applications. For this reason, fruit juices are subject to very detailed legislation in order to guarantee the safety and the quality of the products. The composition and quality of the products are covered by a specific European Fruit Juice Directive - Directive 2012/12/EU of European Parliament. This Directive stipulates the specific characteristics of fruit juices and fruit-based drinks to guarantee the best possible products are put on the European market. It defines the composition of the various products that can be produced, including not only fruit juices, but also dehydrated fruit juice and fruit nectars. It specifies the criteria with which the various products must comply, including which fruits can be used, their minimum content, what ingredients can or cannot be added and how these products must be designated on the label. [5]

Regulation (EC) No 1925/2006 of the European Parliament cover the requirements for the addition of nutrients. Vitamins and minerals may be added to fruit and vegetable juices and nectars to optimize their nutritional composition and make them suitable to complement the intake of certain nutrients in cases where this might not be optimal. [7]

Characteristics of enzyme preparations such as pectinases (for breakdown of pectin), proteinases (for breakdown of proteins) and amylases (for breakdown of starch) are covered by the requirements of Regulation (EC) 1332/2008 on food enzymes. This regulation cover enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. [8]

Quality can be regarded as a measure of the suitability of a fruit juice. In general, it will be the consistency in performance of the product, from batch to batch and season to season, that is the prime concern. The soluble solids content and titratable acidity are the major indicators to be taken into account when identifying the status and suitability of a juice product for use in an application. In terms of quality requirements, for juice from concentrate, the minimum relative density 20/20 must be 1.045 [9] and the minimum brix level 11,2 ºBrix. [5] Titratable acidity at pH 8.1 must give results between 2,3 and 7,5 g/l. For ash content, the results must be 1,9-3,5 g/l and for the total of phosphorus

content this must be 40-75 mg/l. The formol number results must be between 3 and 10 ml of 0,1M NaOH in 100ml of juice [9].

1.2.2. Apple varieties

In 2012/2013, apples (*Malus domestica* Brokh) were the fifth most cultivated fruit in the world and the second most produced fruit. Apples are widely cultivated in temperate climate zones and recently, apple production has been expanding into subtropical and tropical climate zones.[10]More than 60% of the apple production is sold and consumed as fresh fruit. Gala, Fuji and their clones are among the five most produced varieties due to their high productivity, high nutritive value, and good sensory quality. [6]Many of the apples that have some imperfections, such as skin blemishes or off shapes rendering them undesirable for the fresh market, are utilized by processors. [11]Around 25-35% of the total of apple production is discarded in the commercial classification process and are used as raw material in the processing of apple juice, apple wine and cider. [6]Apples contain about 85% water, 14% carbohydrate, 2,4% dietary fiber, 0,3% protein and 0,20% lipids. Some variation in these components is expected due to variety, maturity, growing location, agronomical and environmental conditions. 75% of carbohydrates in apples are sugars such as fructose, glucose and sucrose. Sorbitol is also present and the malic acid is the predominant organic acid. Variety, maturity and environmental conditions during growth and storage influence acidity of apples.[2]The processing of apple juice can include fruit at different ripening stages, which may change the composition, quality and compromise international commercialization of the products.[10]The most common apple cultivars are Red Delicious, Golden Delicious, McIntosh, Rome, Granny Smith, Fuji, and Braeburn and their characteristics are summarized in table 1.1.[1]Varietal important characteristics are size, shape, color, flavor, crisp-juicy texture and post-harvest storability. [2]

Table 1.1. - Characteristics of the most common apple cultivars. [1]

Variety	Characteristics	Use
Red Delicious	Most popular variety in the world. Red with a distinctive "typey" five pointed elongated shape. Sweet and flavorful. Good aroma apple.	For fresh eating and salads.
Golden Delicious	A chance seedling believed to be due to Golden Reinette and Grimes Golden. Pale greenish-yelow appearance. Sweet apple. Juicy yellow flesh.	Fresh eating, salads, apple sauce, baking and fresh apple cider.
McIntosh	Important commercial variety. Appearance red, striped, white juicy flesh, tender skin, medium large size.	Fresh apple cider, fresh eating and sauce making.
Rome	Large, bright red skin, sweet, juicy, white flesh. Low acidity.	Good for baking.
Granny Smith	Large size, signature green appearance. Crisp texture and white flesh.	Salads and apple juice.
Fuji	Golden hued to red appearance and round shape. Sweet and aromatic. A good storage apple.	Fresh eating, salads, pies, sauce, baking and freezing.
Braeburn	Greenish-gold with red sections. Sweet tart, spicy flavor, yellow flesh. Crisp texture and juicy.	Processing of juice, sauce and cider. For fresh eating too.

1.2.3. Volatile compounds

Flavor is an important quality parameter, together with size, color, acidity, sugar content and lack of pathological disorders. Aroma volatiles are an important group of substances that influence the flavor of apples. [12] Fruit aroma is a complex mixture of a large number of volatile compounds that contribute to the overall sensory quality of fruit specific to species and cultivar. Over 300 volatile compounds have been measured in the aroma profile of apples. These compounds include alcohols, aldehydes, carboxylic esters, ketones, and ethers. Table 1.2.shows the range of aroma thresholds of important volatiles compounds in apples. The majority of volatiles extracted from apples are esters (78-92%) and alcohols (6-16%). Esters form the largest group of volatile compounds produced by fruit. [13]

The most abundant compounds are even numbered carbon chains including combinations of acetic, butanoic, and hexanoic acids with ethyl, butyl, and hexyl alcohols. The different volatile compounds have a diverse organoleptic contribution. Some are present at very low concentrations yet contribute to the potent aroma characteristics typical of apple aroma (ethyl-2-methyl butanoate). Others contribute to aroma intensity (2-hexenal) or are related to aroma quality (ethanol). [13] In Golden

Delicious the presence of butanol and hexanol is associated with the sensation of sweetness, acetaldehyde and trans-2-hexenal with the stinging acidity, ethyl butyrate and ethyl 2-methylbutyrate with the fruity flavor and hexyl acetate with a sweet-fruity odor. Others such as propyl, butyl, pentyl acetate or butyl butyrate do not contribute with any specific character but their absence has negative effects on aroma. It is possible to typify the apple cultivars according to their specific volatile compounds. [12]

Table 1.2. - Range of aroma thresholds of important volatile compounds in apples. [15]

Compound	Cultivar	Thresold (ml/L)
Acetaldehyde	Golden delicious	0,015-0,12
Trans-2-hexenal	Golden delicious; Mclntosh; Delicious	0,00-0,017
Hexanal	Golden delicious; Mclntosh; Delicious	0,005
Butan-1-ol	Royal Gala; Golden delicious	0.5
Hexan-1-ol	Golden delicious	0.15-0.5
Trans-2-hexenol	Many varieties	
Butyl acetate	Royal gala; Cox's orange pippin; Delicious; Gala	0.066
Pentyl acetate	Cox's orange pippin; Golden delicious; Gala	0.043-0.005
Hexyl acetate	Royal gala; Cox's orange pippin; Golden delicious	0.115-0.002
2 methyl butyl acetate	Royal gala; Cox's orange pippin	0.011-0.005
Ehtyl butanoate	Golden delicious	0.001
Ethyl-2-methyl butanoate	Golden delicious; Delicious; Gala	0.0001-0.000006

The flavor of fruit juices is developed through enzymatic reactions during the maturation of the fruit and some enzymes are crucial in the production of volatile compounds, such as lipoxygenase, alcohol dehydrogenase and alcohol acyltransferase. [14] The volatile compounds are responsible for the characteristic flavor of fruits and are dependent not only on the type of fruit, but also on the cultivar. [2] These are synthesized from fatty acid, amino acid and carbohydrate metabolisms. The majority of the aldehydes derive from fatty acids catabolism. Branched chain amino acids such as isoleucine, leucine, and valine, can also form aldehydes. Alcohols are formed by the reduction of corresponding aldehydes, by the action of the enzyme alcohol dehydrogenase (ADH). Linear alcohols are derived from the catabolism of fatty acids,

whereas branched-chain alcohols are produced by the metabolism of branched amino acids. After esters, alcohols are the second most important compounds that contributes to the aroma of ripe apples. Alcohols are direct precursors of esters. Occasionally esters may be fragmented to their corresponding alcohols and fatty acids by the action of esterase enzymes, being esters and alcohols in apples a result of a balance between synthesis and hydrolysis. In the last step of volatile synthesis, the alcohol acyltransferase (AAT) enzyme transfers an acyl group from Acyl-CoA to the OH group of an alcohol to form an ester. This occurs in both fatty acid and amino acid catabolism's. In ripe apples, alcohols constitute 6-16 % of the total volatiles and esters represent, depending on the apple's variety, from 80 to 98%. Esters can have linear or branched chains, and their concentration depends on the availability of precursors and on the selectivity and activity of the enzymes involved. Fatty acids of 16 and 18 carbons are the most predominant in apples (C16:0, C18:0, C18:1, C18:2 and C18:3), which are the principal substrates for the production of volatiles because fatty acids with 20 or more carbons are major cellular structural components. The lipid content and proportion of fatty acids in pre and post-harvest climacteric apples is similar, reaching their maximum concentration in the climacteric period. [14]

1.2.3.1. Factors affecting the production of volatile compounds

The synthesis of volatile compounds in apple may be affected by several factors: pre, at or post-harvest. The volatile compounds responsible for the characteristic aroma of an apple, greatly depend on the variety. Differences in the levels of volatile compounds among different apple varieties give them a characteristic aroma pattern. For example, 2-methylbutyl acetate is the main ester in apples such as Besbee Delicious, Redchief Delicious and Fuji, whereas butyl acetate is the main ester in Golden Delicious, Royal Gala, and Mondial Gala apples. Furthermore, weather, geographic location and cultural practicescan also affect the profile of volatile compounds in apples. [14]

The concentration of volatile compounds greatly increases as apple's maturation process advances. Fruit maturity at harvest is a critical factor, which affects ripening and flavor development after harvest. Harvesting of apples before physiological maturity normally implies low volatile levels. An apple harvested in a climacteric stage will produce more

volatiles during storage. [14] Apples harvested too early are frequently very firm after storage, but small, acid-tasting or lacking in flavor. [16] At the beginning of the maturation, aldehydes are more abundant, then alcohols start to increase considerably, and finally the profile is dominated by esters. Table 1.3. shows the general behavior of some volatile compounds during maturation and ripening of McIntosh and Cortland apples on the tree. [17] There are reports of more than 25 aldehydes in apple profile and table 1.4. shows the proportion of them in the total volatiles identified in each apple variety. Aldehydes predominate in pre climacteric apples but, after ripening, the content of some becomes almost imperceptible. However, when the determination of volatile content is performed in juices, high concentrations of some aldehydes are produced, mostly hexanal. Under hypoxic conditions apples can also produce acetaldehyde, which can be converted to ethanol. Nonetheless, there are apple varieties such as Royal Gala and Golden Delicious that are very resistant to extremely low oxygen concentrations. [14]

Table 1.3. - Patterns of production of some volatiles during maturation and ripening of McIntosh and Cortland apples on the tree. [17]

Volatiles	General behavior
Hexyl acetate; Methyl butanoate	High in immature apples and decreased gradually during ripening.
E-2-Hexenal	Continuous increase during maturation and ripening.
Hexanal; Ethyl-2-methyl-1-butanoate	Produced at low level before ripening and suddenly increased after ripening.
Ethyl butanoate; Ethyl-2-methylbutanoate; Hexyl-2-methylbutanoate; Propyl butanoate.	Not detected or very low before ripening then suddenly increased after ripening.

Table 1.4. - Proportion of aldehydes in the total volatiles identified in each apple variety. [14]

	Fruit		Juice					Fruit
Volatile compounds	**GD**	**BD**	**FJ**	**GRU**	**GD**	**GS**	**PL**	**GRE**
	Before maturation		**At harvesting**					**After ULO**
Acetaldehyde	nd	nd	nd	nd	nd	nd	nd	1,13
Propanal	nd	0,60	nd	nd	nd	nd	nd	nd
Butanal	nd	3,19	0,10	0,19	0,08	0,18	0,13	0,08
Hexanal	3,66	12,2	0,34	0,25	0,32	3,08	0,34	34,37
Trans-2-hexenal	1,55	0,31	0,66	0,39	0,42	6,79	1,00	37,56
Heptanal	2,98	14,9	nd	nd	nd	nd	nd	nd
Octanal	5,76	13,24	nd	nd	nd	nd	nd	nd
Nonanal	34,54	26,04	nd	nd	nd	nd	nd	nd
Decanal	0,38	7,86	0,09	0,10	0,08	0,21	0,19	nd
Benzaldehyde	2,49	0,60	0,16	0,03	0,01	0,08	0,02	0,03

*GD – Golden Delicious; BD – Bisbee Spur Delicious; FJ – Fuji; GS – Granny Smith; GRU – Gold Rush; PL – Pink Lady; GRE – Golden Reinders; ULO – Ultra Low Oxygen.

Volatile compounds are not produced in significant amounts during apple's growth, but they increase during the climacteric period. The initiation of the climacteric rise in apples contributes to the color, texture and flavor changes associated with ripening. During this period, occur production of ethylene that contributes to the development of the enzyme systems and volatile precursors necessary for flavor formation. The production of esters is regulated by ethylene and increases quickly as soon as ethylene synthesis starts. Given that, the addition of inhibitors of this hormone in the pre-harvest stage reduces the production of volatiles in fruit. 1-methylcyclopropene and aminoethoxyvinylglycine are ethylene inhibitors that have been shown to reduce volatiles in apples.The fatty acid content of the fruit also increases with maturity. Lipid hydrolases, lipoxygenase and proteases contribute to the formation of the fatty acid and amino acid precursors of flavor formation. In the latter stages of ripening, β-oxidation of linoleic acid, lipoxygenase activity and esterification of acids with alcohols contribute to ester formation. The time of lipoxygenase activity also depends on the apple variety. For example, in Golden Delicious, the activity increases at the end of apple ripening; in Fuji, the activity remains constant and in Cox's Orange Pippin, the activity increase during fruit ripening.Since these enzyme systems and flavor precursors are not fully

developed in the immature fruit, juice processed from immature fruit often lacks the esters and other volatile compounds that contribute desirable fruity flavor characteristics. [15] [2]

Temperature during the production process can also affect the concentration of volatile compounds in apples and apple juices. Volatiles are normally recovered and concentrated for later addition of the concentrated products, resulting in the loss of volatiles if the restoration is incomplete. In apple juice, evaporation could induce a loss of around 95% of E-2-hexenal. [18]

Apple's storage is a critical point as well with regard to the loss of volatile compounds. According to the experience from Jonathan Dixon and Errol W. Hewett in 2000, ester and alcohol concentrations increased as temperature changed from -1 to 10°C, during 12 weeks in storage. Red Delicious apples have maximum ester production at 22°C, it decreased at 32°C and was inhibited at 46°C indicating that heat treatment may temporarily inhibit or inactivate enzymes responsible for producing volatiles. A heat treatment of 38°C for 4 days reduced volatile production in Golden Delicious apples compared to fruit at 22°C. Apples transferred to 20°C after low temperature storage produce greater concentrations of volatiles and reach maximum production earlier than freshly harvested apples. Maximum concentration of butyl acetate and hexyl acetate in Cox's Orange Pippin apples was reached 18 days after harvest and 27 days after harvest for butan-1-ol and hexan-1-ol. After 3,5 months at 3,3°C maximum butyl acetate concentration was reached 4 days after return to 20°C. Such increases in volatile concentration may result from accumulation of volatile precursors in fruit at low temperatures. Storage at low temperatures is a commonly used method for slowing ripening to allow transport over long distances to different markets. Exposure of apples to low temperatures for more than 3 months decreases volatile concentrations by 30-60%.Maximum concentration of Red Delicious volatiles occurred after 2-4 months at 1 °C and declined after longer storage periods. After 5 months at 1°C, Golden Delicious apples had less volatiles than fruit stored for 3 months. Total volatile concentration of 'Golden Delicious' apples after 3 months at 1°C was 50% greater than after 8 months. Butyl acetate and hexyl acetate concentrations in Golden Delicious apples increased to a maximum after 2,5 months at 4°C then decreased after 3,5 months. Ester concentrations in Law Rome and 262 Rome apples were lower after 6 months at 0,5°C than in freshly harvested fruit and in fruit stored for 3 months at 0,5°C. [15]

Apples are frequently held in controlled atmosphere storage to reduce fruit respiration, delay ripening and extend storage. However, the reduced oxygen environment alters the production of volatile compounds by the fruit resulting in decreases in the contents of butyl and hexyl esters, aldehydes, and ketones and increases in ethanol, acetaldehyde, and ethyl esters. [2]

1.2.4. Parameters for the evaluation of apple juice aroma

Due to the complexity of the apple juice aroma composition, the development of directives for the single aroma compound is not effective. Due to this, a sum parameter (sum of esters, sum of carbon-6-aldehydes and alcohols and aroma index) was developed as an alternative. These models are able to compensate specificities and particularities in the aroma composition. [19]

Esters contribute significantly to the aroma of apple juices and, due to their high volatility, they are suitable for flavoring and aroma reconstitution process. The sum of esters (SoE) includes the aroma compounds ethyl isobutyrate, methyl 2-methylbutyrate, ethyl 2-methylbutyrate, ethyl butyrate, 2-methylbutyl acetate, hexyl acetate, hexyl 2-methylbutyl acetate, ethylhexanoate, ethyl 3-methylbutyrate and 3-methylbutylacetate.Butyl acetate occurs in apple juice in high amounts and it is omitted to not dominate the parameter. The contents of these compounds are summed up to a total value which varies between species. [19] Heil et al. suggested the minimum concentration of the sum of esters of 150 $\mu g \cdot l^{-1}$. [20]

The sum of carbon-6-aldehydes and alcohols (SoC6) is a classical sum parameter as well. It includes hexanal, (E)-2hexenal, hexanol and (E)-2-hexenol. The majority of the alcohols and aldehydes has significantly higher odor threshold values than most of the esters. However, they are usually present at higher amounts and therefore do strongly contribute to the overall apple juice aroma. [19]

A disadvantage of these sum parameters is that both sum the individual aroma substances without weighting thereof. Because of this, some substances that appear at higher concentration may dominate the parameter while compounds that contribute to the aroma in even lower contents are discriminated. [20]

The aroma index is a sum parameter for which the weight is taken into account. The contents of the ten included substances are summed with consideration of standardization factors. The aroma index is calculated as the sum of the content of the aromatics multiplied by their standardization factors.[21] The standardization factors are found in table 1.5. The reference values form the lower guideline that has to be reached in order to obtain an apple juice of average aroma content. The substances ethyl 2-methylbutyrate, ethyl butyrate, 2-methylbutyl acetate, and hexyl acetate are taken into account. Additionally, the four components of the SoC6 together with butyl acetate and 2-methylbutanol are considered. These ten substances have been identified as typical apple juice aroma compounds. They contribute in view of their usual contents to apple juice. [19]

Table 1.5. – Standardization factors of the aroma index model. [19]

Aroma compound	Standardization factor
Ethyl 2-methyl butyrate	2.000
Ethyl butyrate	0.500
Hexyl acetate	0.333
2-methylbutyl acetate	0.250
Butyl acetate	0.100
Hexanal	0.100
(E)-2-hexenol	0.050
(E)-2-hexenal	0.025
Hexanol	0.017
2-methylbutanol	0.033

1.3. Objectives

The general aim of the project was to analyse the quality of apple juice according to volatile compounds profile using a solid-phase microextraction followed by gas chromatography-mass spectrometry method.

Specifically, and firstly, quality requirements as soluble solids, titratable acidity, formol number, ash and phosphorus content were analyzed and taken into consideration. Furthermore, analysis of the samples by the quantification of the nine most important volatile compounds in apples using a solid-phase microextraction followed by gas chromatography-mass spectrometry method, was performed.

CHAPTER 2

MATERIALS AND METHODS

2.1. Material

Three sets of samples from the sameapple juice process from concentrate were analyzed. Each set consists of six samples – homogenized pulp, homogenized pulp with enzymes, juice after press, juice without aroma, aroma and concentrate. Each sample was taken from different phases of the manufacturing process. The raw material used was different apples varieties harvested between August and October of 2016 and the mixture of these harvested apples corresponds to the sample “homogenized pulp”. It is expected that the stage of maturity is gradually growing but because apple juice concentrate is a mixture of apple varieties, their maturity stage and composition could be variable. Figure 2.1. shows the diagram of the process of the analyzed apple juice.

A standard sample (Relax 100% apple – distributed by Maspex Slovakia Trade s.r.o.) was used over time to check the repeatability of the method during the test period.It was purchased from a local store.

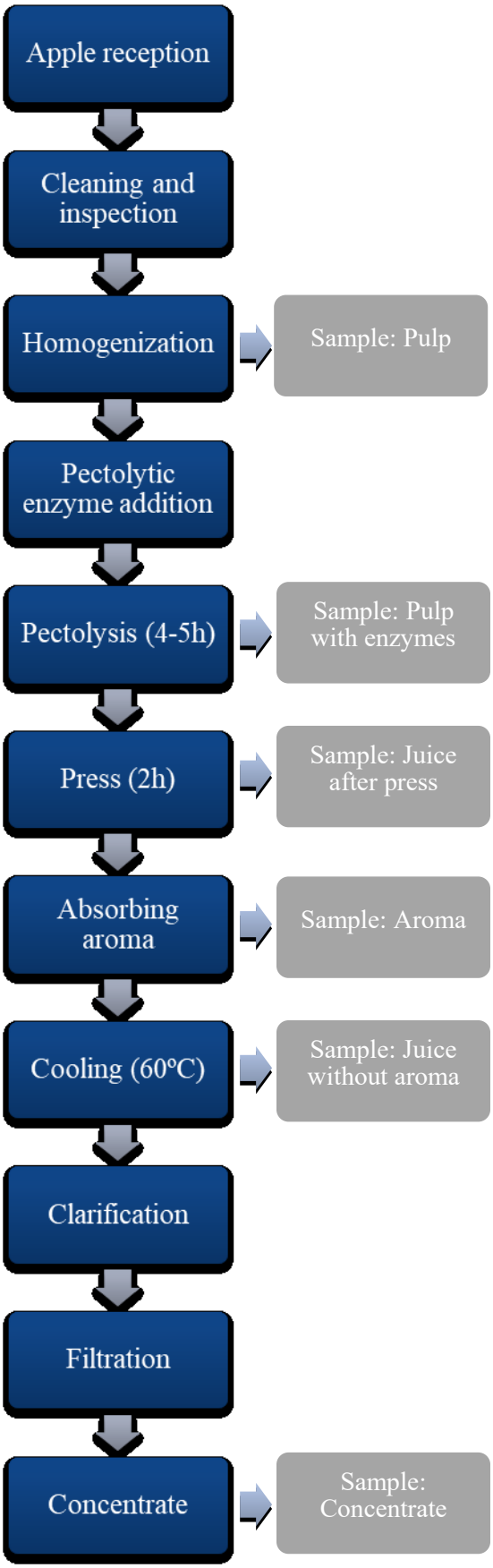

Figure 2.1. - Diagram of the apple juice production process.

2.1.1. Chemicals

Chemical standards used in this study were 2-methyl-1-butanol, ≥99% (Sigma-Aldrich); Ethyl butanoate, ≥98% (Sigma - Aldrich); Hexanal, 98%, (Sigma - Aldrich); Butyl acetate, ≥98%, (Sigma - Aldrich, GmbH); 2-hexen-1-ol, 96% (Sigma-Aldrich); Hexyl acetate, 99% (Sigma - Aldrich); 2-methylbutyl acetate, ≥94% (Sigma - Aldrich); 2-methylbutyl acetate, ≥94% (Sigma - Aldrich); E-2-hexenal, ≥95% (Sigma-Aldrich); Ethyl 2-methyl butanoate, 99%, (Sigma-Aldrich); 1-hexanol, ≥99% (Sigma - Aldrich); Potassium hydrogen phthalate, CAS: 877-24-7 (Sigma - Aldrich); Formaldehyde 36-38% (Penta); Sodium hydroxide (NaOH) (Penta); Hydrochloridric acid 35% (HCl) (Penta); Sulfuric acid 96% G.R. (H_2SO_4) (Lach:ner); Ascorbic acid ($C_6H_8O_6$) (Lach:ner); Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) (Penta); Monopotassium phosphate (KH_2PO_4).

2.1.2. Measurement material

All the material used within the experimental setup are included the list below: Analytical Balances AE 200 (Mettler); Analytical grinder A11Basic (Ika, Germany); Agilent Technologies 7890A gas chromatograph, GS system; CTC Analytics GC autosampler, Pal system; SPME fiber 50/30 μm 24 Ga DVB / CAR / PDMS (Supelco); Automatic Titrator DL 22 F&B (Mettler Toledo); Digital Refractometer DR301-95 (A.Krüss, Germany); Agilent Technologies 5975C, inert MSD with Triple-Axis Detector; Muffle furnace model L91/11/P320 (Nabertherm, Germany); Spectrophotometric Genesys 20 (Thermo Spectronic).

2.2. Experimental work procedures

2.2.1. Determination of volatile substances by the SPME/GC/MS method

Samples were analyzed on a gas chromatograph using the following conditions: Column used was a DB-5MS, 5% Phenyl Methyl Siloxane, (30 m x 25 μm x 0.25 μm); the

temperature program began at 60°C/2 min and then increased 10°C/min up to 250°C; Detection was performed using a MS detector at temperature 250°C with a mobile phase of helium, flow rate 1.2 ml/min.
For SPME extraction the conditions used included SPME fiber 50/30 µm 24 Ga DVB / CAR / PDMS, with an extraction time of 1800s, at 30ºC, and desorption time of 120s.

Samples preparation

Samples were prepared in 10 ml SPME vials. The content of distilled water, sodium chloride and the analyzed sample is shown in Table 2.1. A sample of apple juice made from concentrate was also analyzed. All samples were measured with SPME /GC/MS.

Table 2.1. - Quantity of distilled water, sodium chloride and samples quantity in vials.

Sample name	Sample quantity	NaCl (g)	Distilled water (ml)
Pulp	1 g	1,5	3
Pulp with enzyme	1 g	1,5	3
Juice after press	4 ml	1,5	-
Juice without aroma	4 ml	1,5	-
Aroma	0,040 ml	1,5	3,96
Concentrate	4 ml	1,5	-

Preparation of aromatic standards

A mixture of nine different aromatic standards, with a specific volume, was pipetted with an automatic pipette to a 1L volumetric flask. The final concentration of the individual standards in the solution was 50 mg/l. Specifically: Ethyl butanoate 57 µl, Hexanal 61 µl, Butyl acetate 57 µl, Ethyl 2-methyl butanoate 58 µl, E-2-Hexenal 59 µl, 2-hexen-1-ol 59 µl, 1-hexanol 61 µl, 2-methylbutyl acetate 57 µl, Hexyl acetate 57 µl, was added.

The standard mixture solution was filled up to the mark with distilled water and mixed. Subsequently, almost 100 ml of distilled water was poured into 100 ml volumetric flasks and then pipetted with 2, 10, 100, 700, 1500, 2000 µl of the standard solution and filled to the mark with distilled water. This was made to obtain a calibration series of

standards with 1, 5, 50, 350, 750 and 1000 μg/l concentration. 4 ml of the standards was pipetted into vials, previously weighed with 1.5 g of NaCl. All standards were repeatedly measured in duplicate using SPME / GC / MS.

2.2.2. Total titratable acidity and formol number determination

Determination of titratable acidity was made by potentiometric titration with a standard solution of NaOH (0,1 eq/l) to pH 8,1. The formol number measures the total amino acid concentration in the sample. Using two successive endpoints titrations, it is possible to determine total titratable acidity and formol number.

The determination of formol number takes place in three steps:

1) Neutralization of titratable acidity by means of an endpoint titration at pH 8.1 with NaOH 0.1 eq/l.

2) Addition of an excess of formaldehyde (HCHO) to the solution. This operation locks the NH2 groups of amino acids due to the pH decrease and allows titration of the COOH groups of amino acids with an endpoint titration at pH 8.1.

3) Second endpoint titration at pH 8.1 to determine total amino acid content. The result is then expressed as milliequivalents/100 ml or milliequivalent/l.

Sample preparation

An aliquot of 10g of sample was weighed into the titration cup and 40ml of distilled water was added. The sample was titrated with 0.25M NaOH to pH 8.1. After, 10 ml of formaldehyde at pH 8.1 (previously treated with 0.25 M NaOH) was added with stirring and the solution allowed to stand for exactly one minute. Then, 0.25 M NaOH was titrated to pH 8.1.Titratable acidity was determined by titration according to ČSNEN

12147:1998. Formol number was determined by titration under pH 8.1 according to ČSNEN 1133:1996.

2.2.3. Determination of soluble solids

Soluble dry matter was determined refractometrically (based on light refractive index) using a digital refractometer. The instrument was calibrated using distilled water, the sample was measured and the observed value was expressed in ºBrix.Soluble solids content was determined according to the standard ČSNEN 12143:1998.

2.2.4. Ash and phosphorus content determination

Ash was determined by gravimetric methods according to EN 1135:1995. The samples were weighed (1g of homogenized sample and 5g of the others) into ceramic cups and then placed in a muffle furnace with a temperature of 525ºC +- 25ºC, during 24 hours. Afterwards, the cups were weighted and the ash content determined. The obtained ash was dissolved in 3 ml of hydrochloridric acid and the solution was placed in a 50 ml graduated flask and added to the mark. Then, for the spectrophotometric analysis, 5 ml of this solution was placed in a 100 ml graduated flask with approximately 50 ml of distilled water, 20 ml of sulfuric acid, 4 ml of ammonium molybdate tetrahydrate solution and 2 ml of ascorbic acid solution. After 15 minutes in a water bath, the samples were cooled down and adjusted the volume to 100ml. Then, the samples were analyzed in a spectrophotometer adjustable to 720 nm.

2.2.5. Aroma index determination

The aroma index was calculated as the sum of the content of the volatile compounds multiplied by their standardization factors. The standardization factors used are in chapter 1.2.4., table 1.5.

2.2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Software with analysis of variance and tuckey test. The experimental tests were done in duplicate for each sample and the values are reported in appendix II and III.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Calibration curve

Calibration of the GC/MS machine was made as explained in chapter 2.2. Figure 3.1. shows an example of a calibration curve for 1-Hexanol. In appendix I is a table that presents the equations used to calculate the concentration of each volatile compound with the respective coefficient of correlation. In sequence of a coelution of two compounds (hexanal and ethyl butanoate), an extration of ions was made – 44 and 56 for hexanal and 88 and 60 for ethyl butanoate. The ion 56 is specific for hexanal and the 88 for ethyl butanoate.

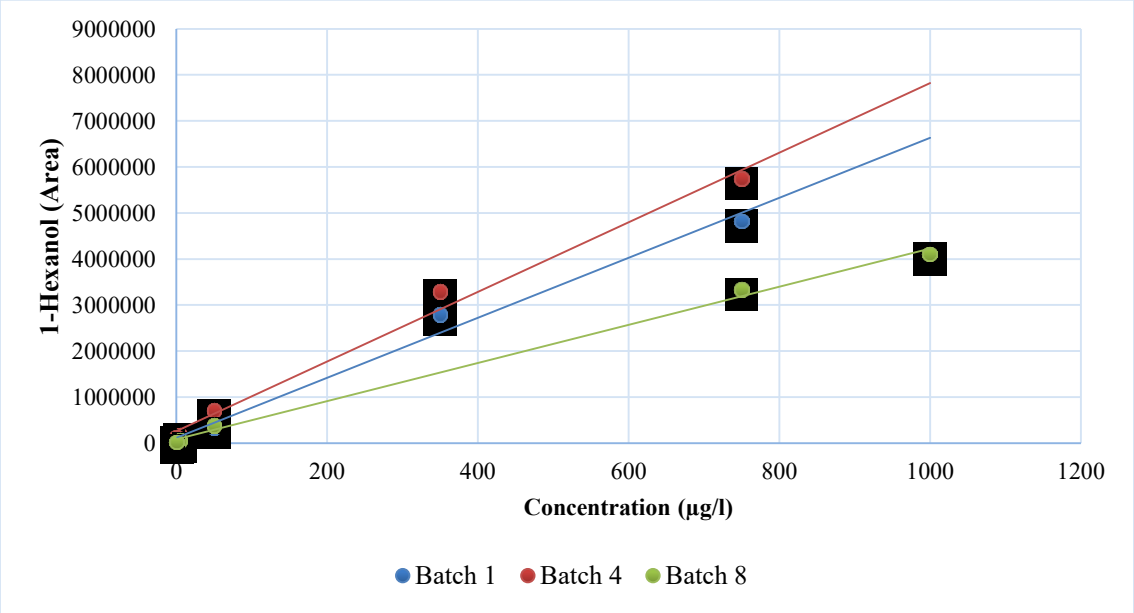

Figure 3.1. - Example of calibration curve's compilation for 1-hexanol. (Appendix I)

3.2. Soluble solids

The soluble solids content relates directly to both the sugars and organic acids, as these are the main contributors. Figure 3.2. shows the variations of soluble solids for each batch measured.The values for the first four samples (pulp, pulp with enzymes, juice after press and juice without aroma) are in agreement with those found in the literature (chapter 1.2.1.2.). It can be observed that the samples have similar and constant values for brix degree between them, as was expected.The statistical analysisconfirmed that there are no significant differences between the samples in all batches analyzed. Thus it can be concluded that the production process didn't influence the soluble solids content.For the aroma sample,thesoluble solids content is negligible and, according to the sample's character, since the majority of its composition is volatile compounds, there are no carbohydrates to quantify. The value of the sample "concentrate" is in agreement with values found in literature (chapter 1.2.1.2.).

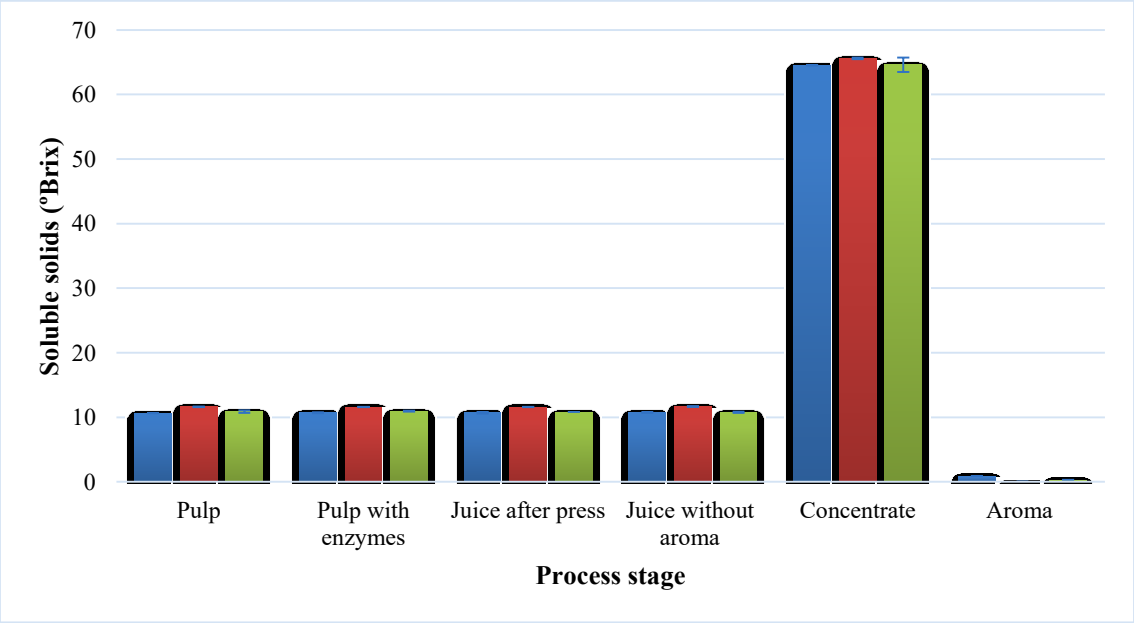

Figure 3.2. - Changes in soluble solids content at different stages - pulp, pulp with enzymes, juice after press, juice without aroma, concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

3.3. Formol number and titratable acidity

According to chapter 1.2.1.2, the formol number results must be between 3 and 10 ml of 0,1M NaOH in 100ml of juice. After a careful analysis of figure3.3., it's possible to conclude that the samples, except concentrate and aroma, showed values in the range of the literature limits. Also, there is an observed decrease in each batch, from the sample "pulp" to the "juice without aroma". Analysis of variance showed that there are significant differences between, at least, two of the samples in each batch.

Values in batch 4 are almost two times higher than those reported for batches 1 and 8. As the apple juice from concentrate is made from a mixture of apple varieties, their maturity stage and composition may vary. For example, the apple mixture used for batch 4 may contain apples that were at the beginning of the harvest season, characterized by a higher concentration of amino acids.

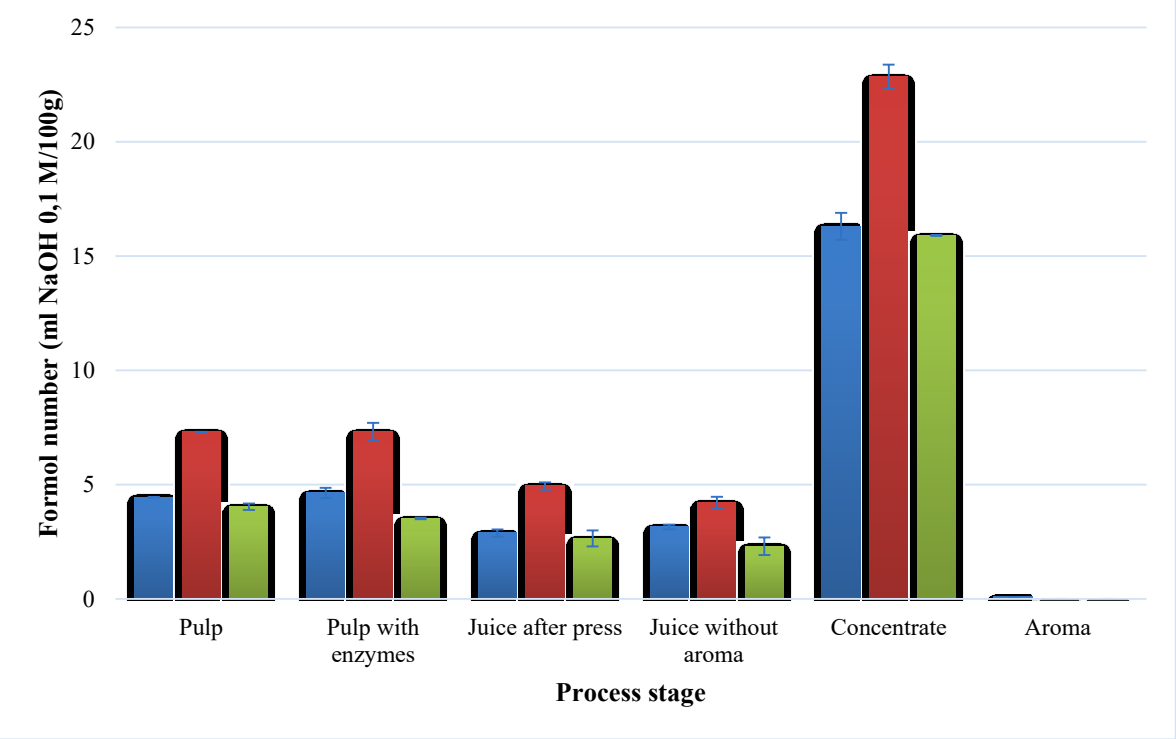

Figure 3.3. - Evolution in the formol number at different stages – pulp, pulp with enzymes, juice after press, juice without aroma, concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

The major organic acids in apple juice are malic and citric acids, although malic acid predominates.Generally, the acidity of juices will decrease with increasing maturity of the fruit source, or with increasing levels of sugars in the resulting juice. [1] According to this and, assuming that the stage of maturity is gradually growing from batch 1 to 8, the values reported in figure 3.4. for titratable acidity in the different samples are normal, especially in the sample "concentrate".

Analysis of variance of the first four sample types, showed that, there are significant differences between, at least, two of them, for the three batches.

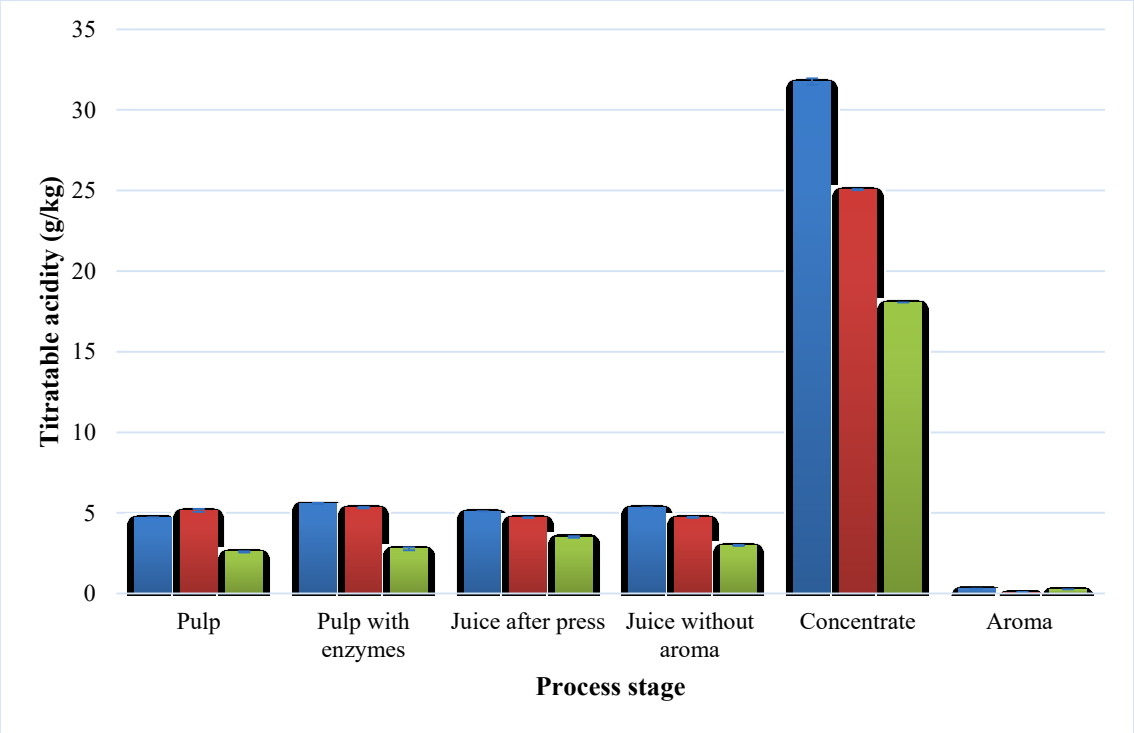

Figure 3.4. - Evolution of titratable acidity at different stages – pulp, pulp with enzymes, juice after press, juice without aroma, concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

3.4. Ash and phosphorus content

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. As mentioned in chapter 1.2.1.2. and according to literature, for ash content, the results must be between 1,9 and 3,5 g/l.

From figure 3.5., it can be concluded that the values of ash content in the samples are in agreement with literature values since the relative density is approximately 1. Analysis of variance showed that, for batches 1 and 4, there are significant differences between, at least, two of the samples. For batch 8, no significant differences were detected. It can then be concluded that the production process did not influence the ash content in batch 8.

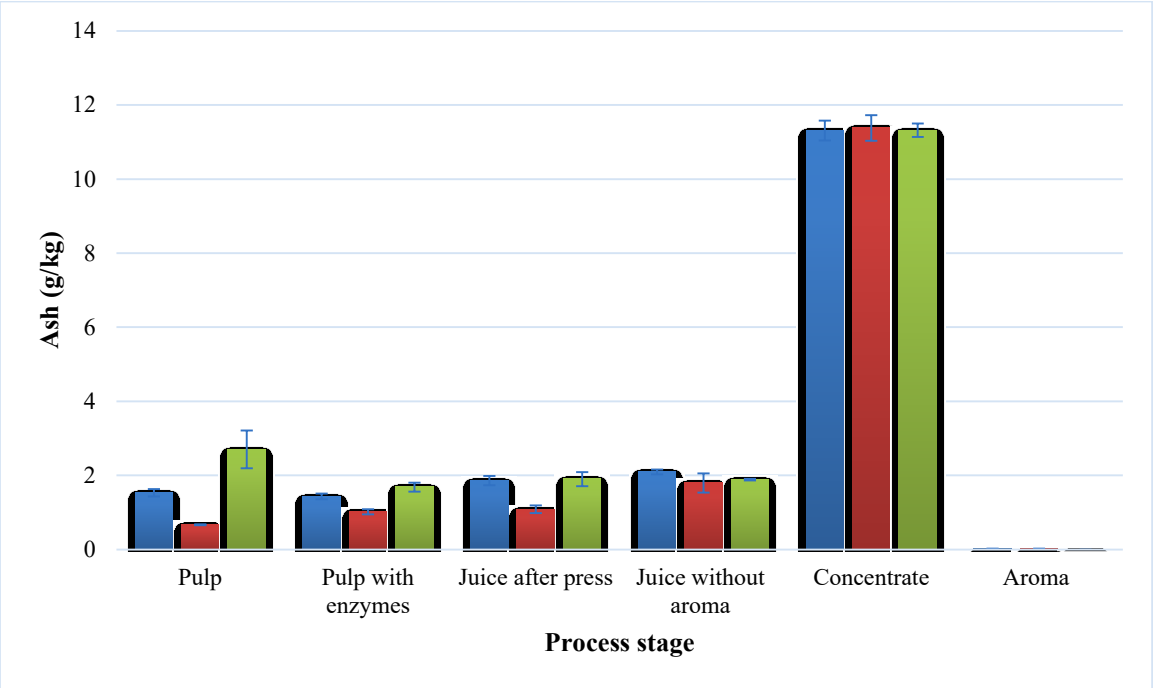

Figure 3.5. - Changes in the ash content in pulp, apple juice, concentrate and aroma samples, for batches 1 (o), 4 (o) and 8 (o).

Figure 3.6. shows the values obtained for phosphorus analysis. It can be observed that, as the production process develops, the phosphorus content in the sample decrease. According to literature (chapter 1.2.1.2.), the phosphorus content in apple juice must be between 40 to 75 mg/l. The values obtained in this experiment for the phosphorus content in the juice samples (juice after press and juice without aroma) are similar to literature values for all batches analyzed. Analysis of variance showed that, for batch 1, there are no significant differences between samples. It can be concluded that the process does not influence the concentration of phosphorus in the samples. However, for batches 4 and 8, the analysis of variance showed that there are significant

differences between, at least two of the samples. For these batches, the process influenced the concentration of phosphorus.

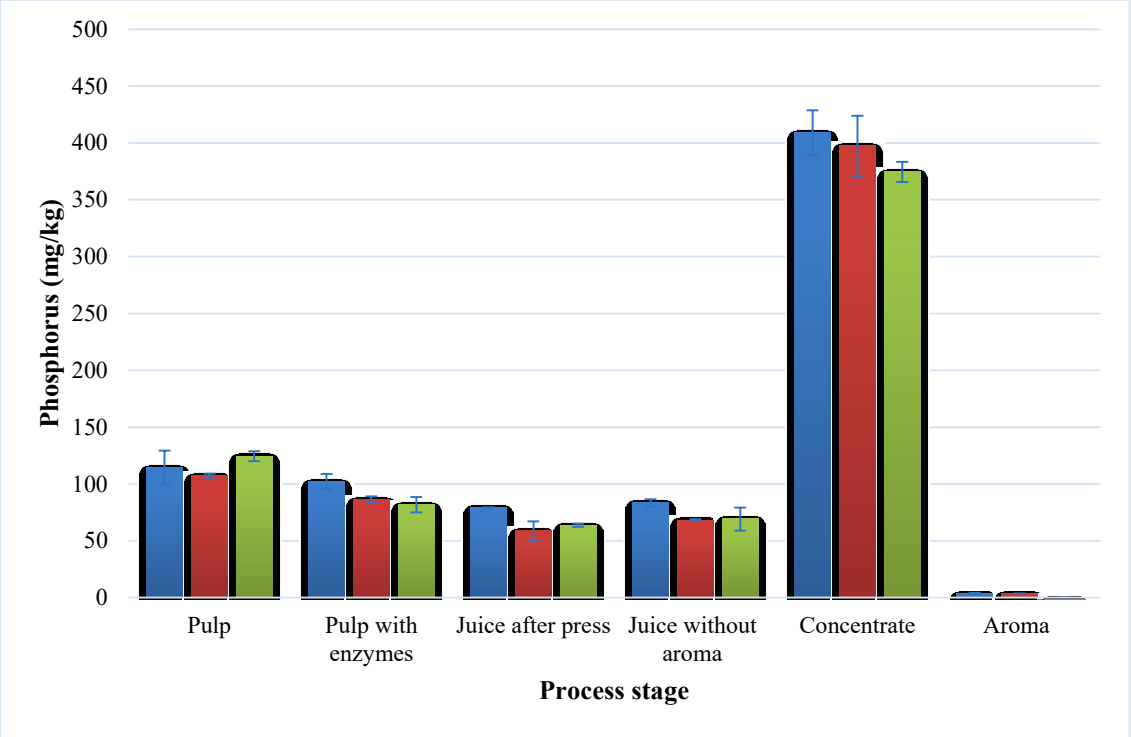

Figure 3.6.–Evolution of phosphorus content at different stages – pulp, pulp with enzymes, juice after press, juice without aroma, concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

3.5. Volatile compounds

Figure 3.7. shows the total concentration of volatile compounds in each batch analyzed. By analyzing figure 3.7., it can be concluded that, for all batches, the concentration of volatile compounds in the sample "pulp with enzymes" is higher than in the sample"pulp". Some aldehydes are produced after disruption of the cell structure during processing. [22] In the apple juice process, the disruption of the cell structure is made by addition of enzymes, so the higher concentration of volatile compounds in sample "pulp with enzymes" was expected. As mentioned in chapter 1.2.3.1., the concentration of volatile compounds greatly increases as the apples' maturation process advances. Batches 1,4 and 8 were harvested from August to October and it is expected that the stage of maturity is gradually increasing. However, the raw material used in the batches

was a mixture of different apple species and the harvest time varies within different species. [16] In fact, it can be observed in figure 3.7. that the concentration of aromatic volatile compounds is increasing from batches 1 to 8 and, in a first analysis, it can be concluded that the maturity process is growing and that batch 8 is the one with the ripest apples.

Statistical analysis of variance, performed for each batch for all volatile compounds measured, showed that there are significant differences between at least two of the samples: pulp, pulp with enzymes, juice after press and juice without aroma. The results express that the production process influences the concentration of volatile compounds in samples. Data used to calculate statistical analysis and the respective resultsare summarized in appendix II.

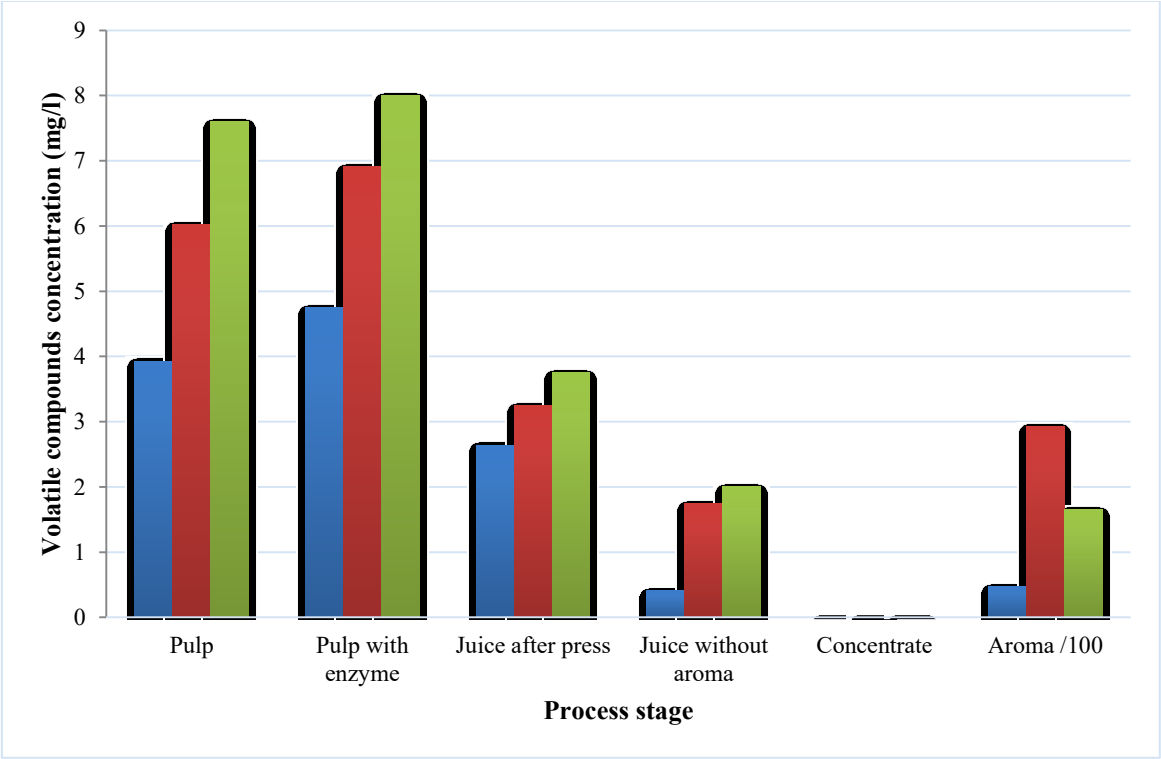

Figure 3.7. - Total concentration of the nine volatile compounds measured for batches 1 (o), 4 (o) and 8 (o), throughout the apple juice processing. (Appendix II)

According to Wolter et al., the major constituents of apple juices and aromas are E-2-hexenal, 2-hexen-1-ol and 1-hexanol. [19] The range of normal concentration for these compounds are summarized in table 3.1. As it turns out, the values obtained in the present experiment showed that these three compounds were the ones that appeared at higher concentrations. Therefore, these three compounds were the ones that influenced the total concentration of volatile compounds present in figure 3.7.the most.

Table 3.1. - Range of typical concentrations in juices and aromas for each volatile compound. [23]

Compound	Concentration in juices (mg/l)	Concentration in aromas (mg/l)
E-2-hexenal	0 – 3,0	0 – 470
1-Hexanol	0,06 – 5,9	47 – 685
2-Hexen-1-ol	0,01 – 3,4	12 – 300
Butyl acetate	0 – 1,7	0 – 165

E-2-hexenal and hexanal are aldehydes that contribute to aroma intensity in apples. Aldehydes are more abundant at the beginning of maturation and in pre climacteric apples. [15] Figures 3.8.and 3.9.show that the concentration of E-2-hexenal and hexanal inthe two types of pulp samples are highest in batch 4. Since batches 1 and 4 were harvested before batch 8, it can be said that, based on the figure, batch 8 is the ripest. It's also visible in figures 3.8. and 3.9.that concentration of both pulp samples is higher than the juice samples. In addition, the results from figure 3.8. are according to literature (table 3.1.). As was mentioned before, there are higher values of aldehydes in the "pulp with enzymes" sample than in the "pulp" sample, as expected and according to literature.

A statistical analysis of variance and a tuckey test were performed for the two compounds separately and the conclusion was that there are significant differences between samples for batches 1, 4 and 8. For E-2-hexenal, figure 3.8. shows the results for the different batches. For hexanal, the results were the same for all batches. The pulp-type samples are significantly different from the juice-type samples. The results are shown in figure 3.9.

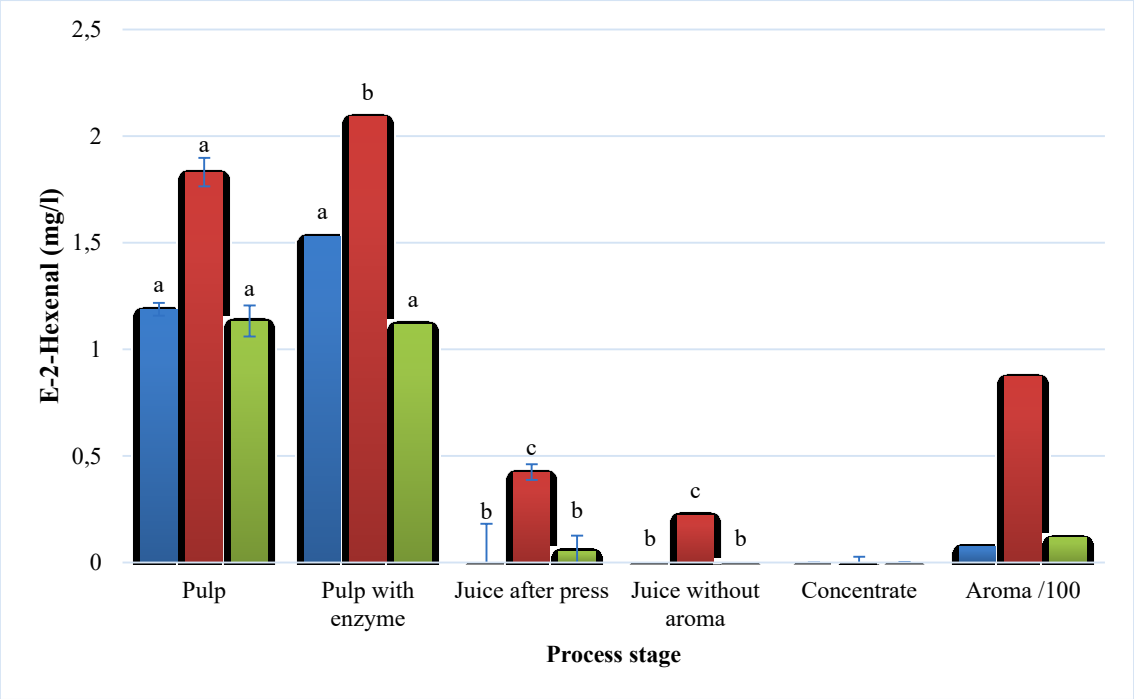

Figure 3.8. - Total concentration of E-2-hexenal at different stages - pulp, pulp with enzymes, juice after press, juice without aroma concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

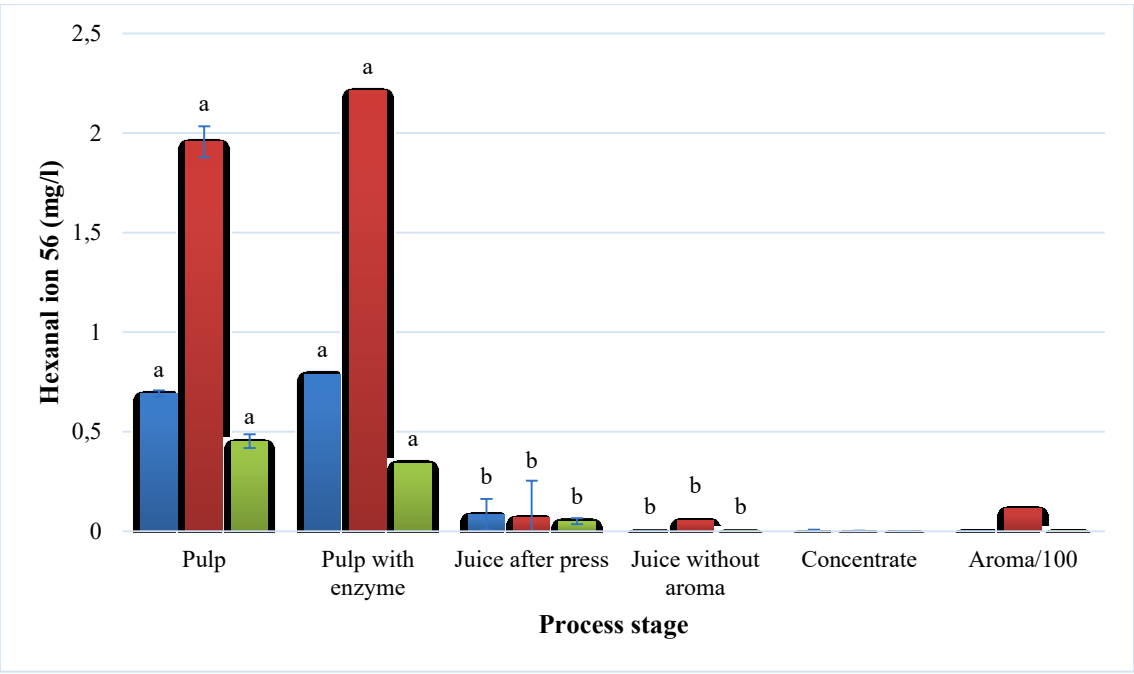

Figure 3.9. - Total concentration of hexanal at different stages - pulp, pulp with enzymes, juice after press, juice without aroma concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

For 1-hexanol (figure 3.10.) the values are also in agreement with those listed in table 3.1. By observation of figure 3.10., concentration of 1-hexanol is highest in batch 8. As mentioned in chapter 1.2.3., alcohols are the second most important group of compounds that contribute to the aroma of ripe apples and are present in a range of 6 to 16%. This information suggests, once more, that the ripest batch is batch 8.

A statistical analysis of variance and a tuckey test were performed, and the conclusion (present in figure 3.10.) was that there are significant differences between samples for all batches.

Figure 3.11. shows the total concentration of 2-hexen-1-ol during the production process for the three batches analyzed. 2-hexen-1-ol is an alcohol and, as was mentioned before, the concentration of this compound is usually higher in ripe apples. In figure 3.11., batches 4 and 8 have higher concentrations of this alcohol than batch 1. E-2-hexen-1-ol is formed immediately after destruction of the cell [13], so the higher concentration found inthe sample “pulp with enzymes”is in agreement with that found in literature. The values for juice and aroma concentration are in accordance with table 3.1.

A statistical analysis of variance and a tuckey test were performed and the conclusion was that there are significant differences between samples for all batches. Differences found in different batches are shown in figure 3.11.

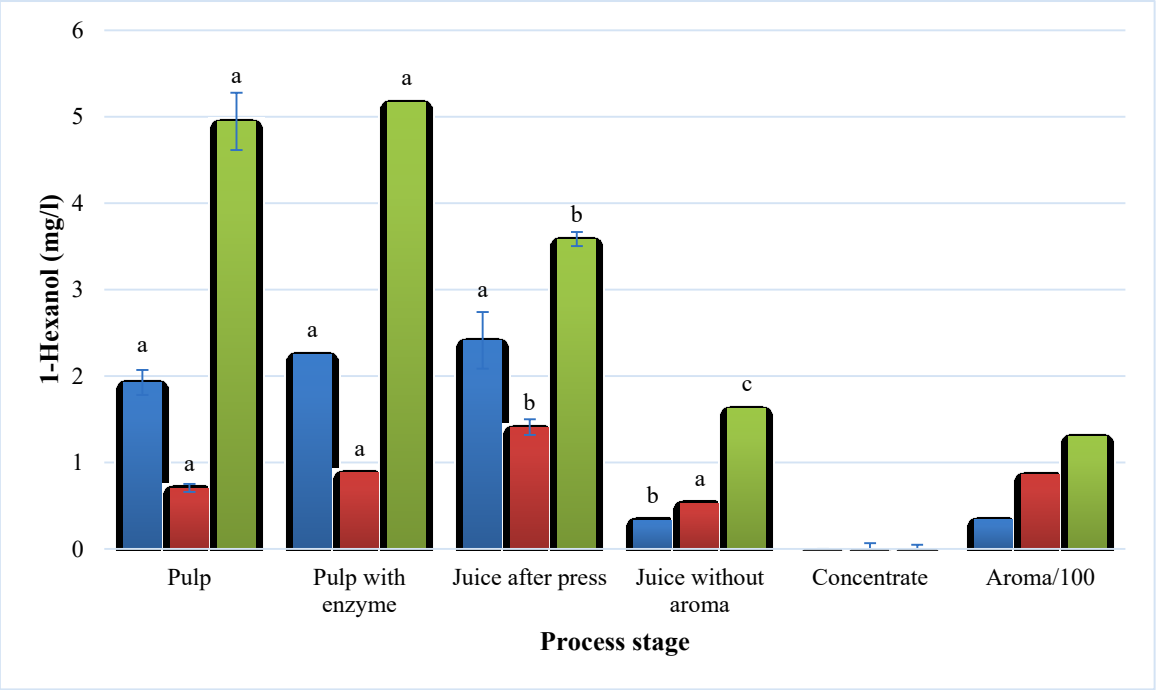

Figure 3.10. - Total concentration of 1-hexanol at different stages - pulp, pulp with enzymes, juice after press, juice without aroma concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

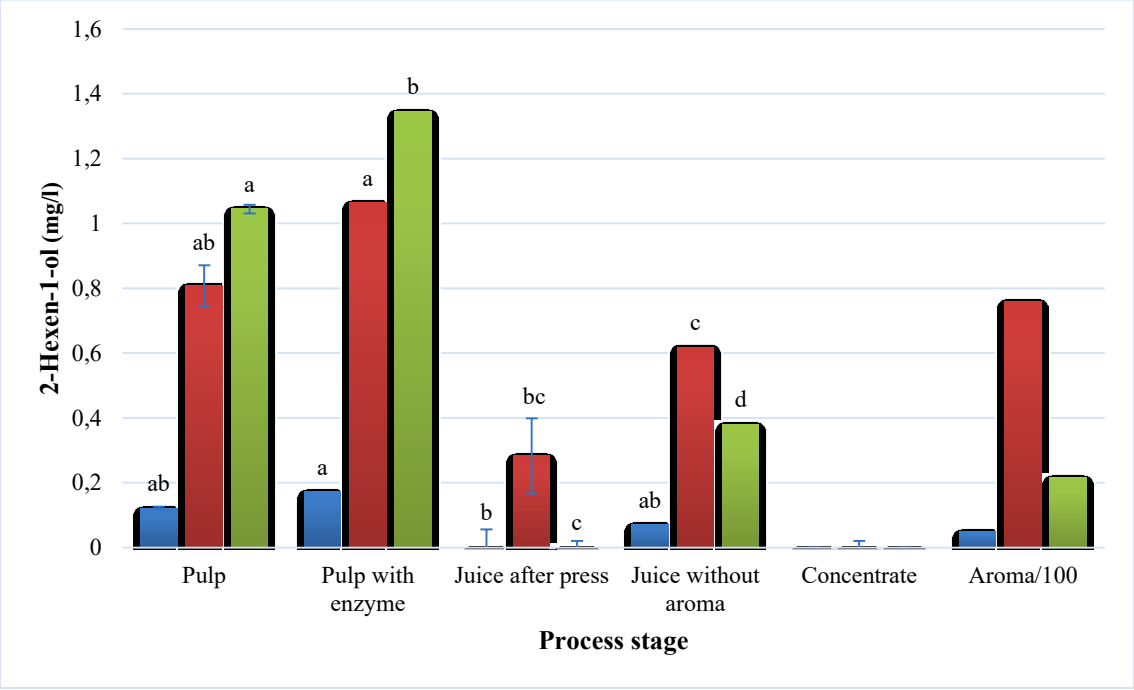

Figure 3.11. - Total concentration of E-2-hexen-1-ol at different stages - pulp, pulp with enzymes, juice after press, juice without aroma concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

Figures 3.12. and 3.13. show the total concentration of two esters that are important volatile compounds for the aroma profile characterization of apples. The results have come to suggest that batch 8 is the most mature. By analyzing these two figures (3.12. and 3.13.), it can be concluded that esters are predominant in batch 4 and, according to literature (see chapter 1.2.3.), in ripe apples, esters represent, depending on the apple's variety, 80% to 98% of the total volatile compounds.
Another important fact that has to be analyzed is the fact that alcohols are direct precursors of esters (see chapter 1.2.3.). Figures 3.10. and 3.11. show that the alcohols (2-hexen-1-ol and 1-hexanol) have a significant, and similar concentration (varies between 0,70 and 1,06 mg/l in the samples "pulp" and "pulp with enzymes"), in batch 4. The concentration of esters in batch 4 varies between 0,15 and 0,86 mg/l. These results were expected and in agreement with literature. For butyl acetate, the values are also in agreement with table 3.1.

A statistical analysis of variance and a tuckey test were performed for the two compounds separately. In the case of compound 2-methyl-1-butyl acetate, the result was that there are significant differences between at least two samples, for all batches (results in figure 3.12.). The butyl acetate showed different results in the three batches. For batches 1 and 8, the statistical analysis of variance showed that there are significant differences between at least two samples. However, for batch 4, there are no evidences of significant differences between samples. In batch 1, the sample "juice after press" is different from all the others. In batch 8, all pulp samples are different from the juice samples. The results are present in figure 3.13.

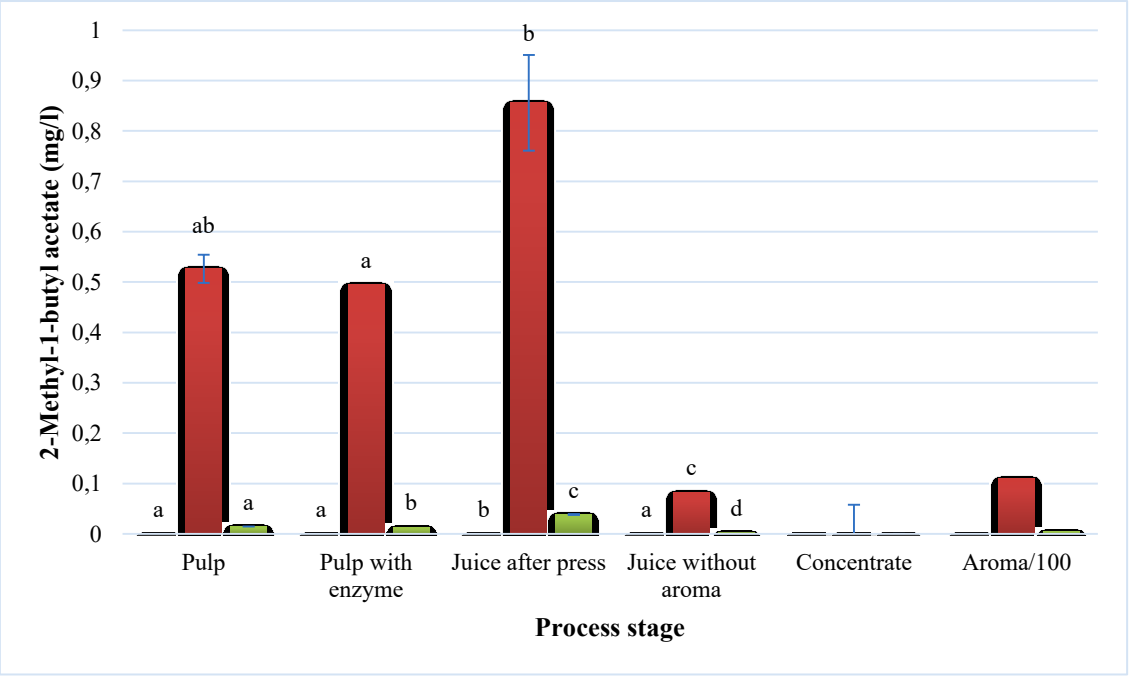

Figure 3.12. - Total concentration of 2-methyl-1-butyl acetate at different stages - pulp, pulp with enzymes, juice after press, juice without aroma concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

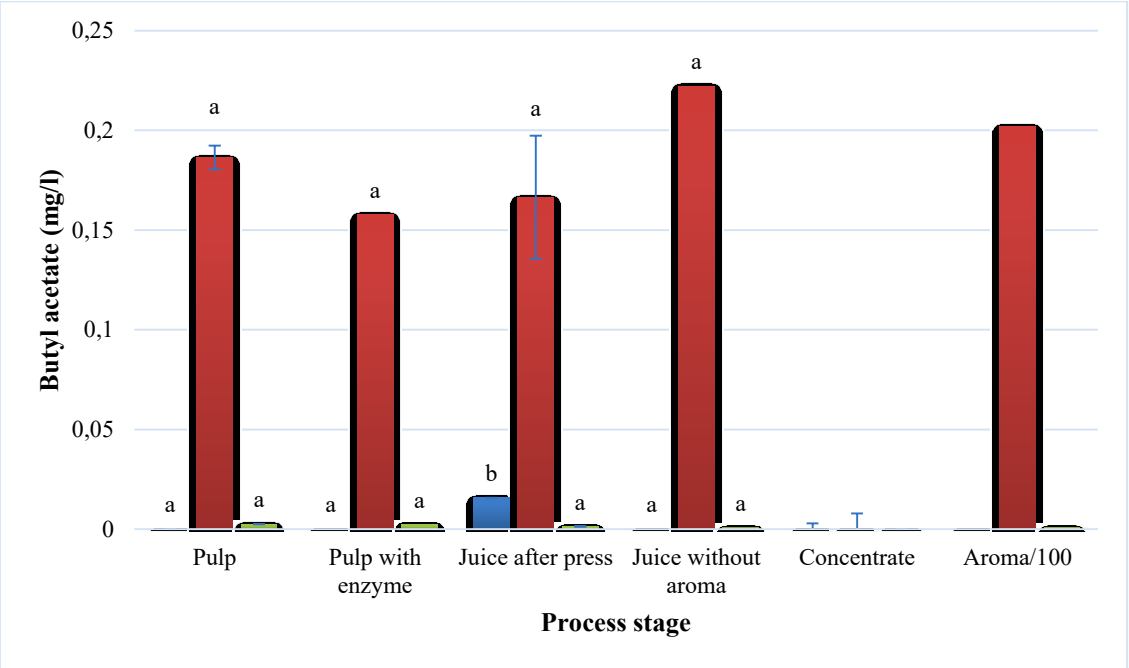

Figure 3.13. - Total concentration of butyl acetate at different stages - pulp, pulp with enzymes, juice after press, juice without aroma concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o).

3.6. Standard samples

Standard samples were used over time to check the repeatability of the method during the test period. GC/MS was also usedto find the nine most important volatile compounds of apple juice in the different samples. The values vary between 8,70 to 8,90 mg/l, maintaining the repeatability of the method.

3.7. Aroma index

The aroma index is a weighted sum parameter that reflects the influence of each individual volatile compound on the final product. Changes in the aroma index of individual samples for each batch are summarized in figure 3.14. To calculate it, it was used the standardization factors showed in table 1.5. in chapter 1.2.4.

Figure 3.14.shows that the aroma index concentration for batch 4 is much higher than in batches 1 and 8. As was seen in figures 3.12. and 3.13., esters concentration is higher in batch 4 and almost imperceptible in the other batches (1 and 8). Furthermore, it can be seen in table 1.5. (chapter 1.2.4.) that the standardization factors are higher for esters. In this way, it can be concluded that the higher concentration for the aroma index in batch 4 comes from the esters concentration.

Several important odor active volatiles are produced via processes initiated by cellular disruption. [19] Six carbon (C6) volatiles including aldehydes and their corresponding alcohols, are produced from action of the lipoxygenase (LOX) pathway on substrates released by tissue disruption. Contreras et al. showed that, for crushed fruit tissue, the synthesis of LOX-derived C6-aldehydes E-2-hexenal and hexanal was, at first, extremely high such that the concentration of the aldehydes in the sample cup was several hundred times higher than their odor thresholds. [24] Thus, the higher concentration in the sample “pulp with enzymes” than in the sample “pulp” are according to literature.

A statistical analysis of variance and a tuckey test were performed and the general conclusion was that there are significant differences between at least two samples, for

all batches. In batches 1 and 8, the sample "juice without aroma" is significantly different from the others. In batch 4, all pulp samples are different from sample "juice without aroma". The sample "juice after press" is equal to all samples. The statistical results are in accordance with what was expected, since the sample "juice without aroma" was analyzed after the aroma was removed. The results prove that the production process influences the aroma index concentration. All the data used in statistics calculations is present in appendix III.

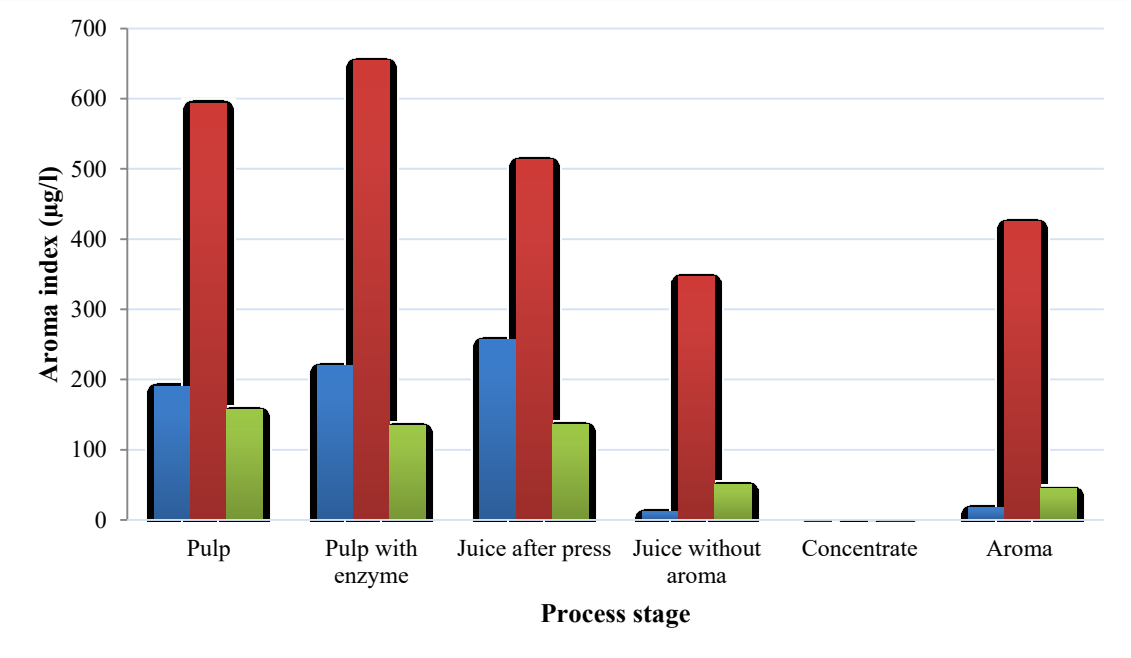

Figure 3.14. - Aroma index at different stages - pulp, pulp with enzymes, juice after press, juice without aroma concentrate and aroma – of the apple juice processing for batches 1 (o), 4 (o) and 8 (o). (Appendix III)

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

The aim of this experiment was to analyse the quality of apple juice according to volatile compound profile, drawing conclusions about the influence thatthe apple juice production process has on the volatile compounds profile. The overall objectives were achieved, despite several limitations.

Sample preparation is often the most time and effort consuming step of an analytical process. Furthermore, it's the most susceptible step to commit errors. Errors encountered during sample preparation that were due to human factors or to the sample's nature were taken into account.

From the quality requirements analysis, it was concluded that soluble solids' content of the different apple pulp and juice samples is not affected by the production process. However, the formol number varies during the process, and the differences between batches are related with the variation of the pulp's composition, since apples at the beginning of harvesting imply higher concentration of amino acids. As far as their titratable acidity values are concerned, statistical analysis proved that the apple juice production process influences the acidity. In addition, the values obtained showed that acidity decreases with the increase of apple maturation. For ash and phosphorus content, the values reported were in agreement with literature. Statistical analyses revealed great variability between batches, which may be explained by geographical factors, apple physiological factors or even instrumental variability in the analyses.

In relation to the volatile compounds, it can be concluded that the concentration of volatiles in the samples pulp with enzymes was higher than in samples pulp,since enzymes induce the production of some volatile compounds due to the release of different precursor compounds. Another important fact that can be concluded from the experimental values reported is that, as evidenced in the sample "pulp", with the overall concentration of volatile compounds, batch 8 is the one with the ripest apples.

Statistical analysis showed that there are significant differences between at least two of the samples and this result proves that the production process influences the concentration of volatile compounds in apple juices.

The separate analysis of each volatile compound allowed the confirmation of the pattern of aldehydes, alcohols and esters in the apple juice. Analysis of results, enabled to

confirm that the major constituents of apple juices and aromas are E-2-hexenal, 2-hexen-1-ol and 1-hexanol. For aldehydes, it was concluded that they are present at higher concentrations at the beginning of harvesting and in pre climacteric apples. Of the aldehydes analyzed, the pulp-type samples are significantly different from juice-type samples, and the concentration in the pulp-type samples is higher than in the juice-type samples. Furthermore, it was possible to observe and statistically confirm that aportion of aldehydes is lost during the production process of apple juice. For alcohols, 1-hexanol is present at higher concentration in batch 8 and 2-hexen-1-ol in batches 4 and 8. It can be concluded that alcohols are related to ripe apples and that the production process affects the concentration of alcohols ($p < 0,05$). Esters are present in batch 4 and are imperceptible in batches 1 and 8. Statistical analysis showed that 2-methyl-1-butyl acetate's concentration is affected by the production process and it is significantly lower in the juice samples. On the other hand, statistical analysis for butyl acetate allowed to conclude that the production process does not influence its concentration.

Aroma index was also affected by the production process of apple juice, as was expected. This parameter takes into account the influence of each volatile in the final product and it can be concluded that batch 4 was the one with highest content of apples' volatile compounds.

This experiment also showed that the reconstitution of the aroma of apple juices produced from the concentrate is an important step in the process,since all the volatile compoundsare lost in the concentration step, and need to be reincorporated.

To sum up, the present study serves to show that the production process of apple juices influences the profile of volatile compounds.

In the future, it is important to start by repeating the experiment and by using three replicas for each sample, to obtain more trustworthy and statistically significant results. Afterwards, it is recommended that the technology used in each step of the production process be analyzed, to better know where the volatile compounds are lost. Lastly, it will be important tostudy in depth the reason for losing the compounds, in order to propose improvements to the apple juice production process.

CHAPTER 5

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APPENDIX

Appendix I - Calibration curve

Table I - Equations and correlation coefficients of the calibration curve used to analyzed each compound.

Compound	Batch 1		Batch 4		Batch 8	
	Equation	**R^2**	**Equation**	**R^2**	**Equation**	**R^2**
Ethyl 2 methyl butanoate	y=12556x + 325686	0,9887	Y=116926x + 3000000	0,9993	Y=15483x+281605	0,9984
Trans-2-Hexen-1-al	Y=4837,9x+68308	0,994	Y=45441x+341031	0,9995	Y=4862,8x+106685	0,9962
Butyl acetate	Y=10177x+284706	0,985	Y=66711x+2000000	0,9975	Y=7993,6x+273204	0,9926
1-Hexanol	Y=6512,2x+119539	0,9885	Y=7559,7x+262927	0,9907	Y=4141,3x+8511	0,9968
2-Hexen-1-ol	Y=2792,3x+12289	0,9945	Y=27837x+811829	0,9904	Y=2332,7x+49128	0,9997
2-methyl –1-butyl acetate	Y=12371x+1000000	0,9873	Y=93653x+2000000	0,9948	Y=12529x+210933	0,9984
Hexyl acetate	Y=13829x+986055	0,9925	Y=118517x+3000000	0,9922	Y=9574x+375754	0,992
Hexanal 44	Y=1046,4x+62537	0,9857	Y=8508,1x+184350	0,9908	Y=962,1x+29520	0,9951
Hexanal 56	Y=998,76x+42947	0,9858	Y=8400,3x+119800	0,992	Y=1015,7x+26188	0,9948
Ethyl butanoate 60	Y=410,46x+6134,7	0,9872	Y=1128,4x+53,815	0,9949	Y=449,7x+13868	0,9877
Ethyl butanoate 88	Y=1240,8x+14086	0,9888	Y=1090,8x+25062	0,9968	Y=1632,7x+48476	0,9884

Appendix II – Volatile compounds

Table II – Medium concentration and Tuckey test results of each compound in samples of batch 1.

Compounds	Pulp (mg/l)	Pulp with enzymes (mg/l)	Juice after press (mg/l)	Juice without aroma (mg/l)	Concentrate (mg/l)	Aroma (mg/l)	Sum (mg/l)
Hexanal - ion 56	0,6914 [a]	0,7892 [a]	0,0843 [b]	0,0021[b]	0,0000	0,4302	1,9973
Butyl acetate	0,000 [a]	0,000 [a]	0,0157 [b]	0,0000 [a]	0,0000	0,0000	0,0157
Ethyl-2-methylbutanoate	0,0005 [a]	0,0008 [a]	0,0357 [b]	0,0000 [a]	0,0000	0,0386	0,0757
E-2-hexenal	1,1871[a]	1,5306 [a]	0,0000 [b]	0,0000 [b]	0,0000	7,4524	8,6395
2-Hexen-1-ol	0,1213 [ab]	0,1720 [a]	0,0000 [b]	0,0705[ab]	0,0000	4,9812	5,3450
1-Hexanol	1,9242 [a]	2,2532 [a]	2,4112 [a]	0,3342 [b]	0,0000	33,6663	40,5891
2-methyl-1-butyl acetate	0,0000 [a]	0,0000 [a]	0,0084 [b]	0,0000 [a]	0,0000	0,3504	0,3589
hexyl acetate	0,0000 [a]	0,0000 [a]	0,0001 [b]	0,0000 [a]	0,0000	0,0579	0,0580
Ethyl butanoate - ion 88	0,0015 [a]	0,0028 [a]	0,050 [b]	0,0024 [a]	0,0000	0,3031	0,3947

Table III - Medium concentration and Tuckey test results of each compound in samples of batch 4.

Compounds	Pulp (mg/l)	Pulp with enzymes (mg/l)	Juice after press (mg/l)	Juice without aroma (mg/l)	Concentrate (mg/l)	Aroma (mg/l)	Sum (mg/l)
Hexanal - ion 56	1,9556[a]	2,2113[a]	0,0698 [b]	0,0549 [b]	0,0000	11,2031	15,4947
Butyl acetate	0,1864[a]	0,1578[a]	0,1665[a]	0,2222[a]	0,0000	20,2025	20,9353
Ethyl-2-methylbutanoate	0,000 [a]	0,000 [a]	0,000 [a]	0,000 [a]	0,0000	0,0000	0,0000
E-2-hexenal	1,8305[a]	2,0908[b]	0,4243[c]	0,2223 [c]	0,0012	87,0618	91,4241
2-Hexen-1-ol	0,8080 [ab]	1,0643 [a]	0,2826[bc]	0,6184 [c]	0,0000	75,9453	78,7187
1-Hexanol	0,7048[a]	0,8814 [a]	1,4066 [b]	0,5301 [a]	0,0000	85,5673	89,0901
2-Methyl-1-butyl acetate	0,5263 [ab]	0,4946 [a]	0,8558 [b]	0,0827 [c]	0,0000	10,9712	12,9306
Hexyl acetate	0,0070 [ab]	0,0044[ab]	0,0345 [a]	0,0023 [b]	0,0000	0,3830	0,4312
Ethyl butanoate - ion 88	0,0019 [a]	0,0009 [a]	0,0068 [b]	0,0053[b]	0,0000	1,4909	1,9692

Table IV - Medium concentration and Tuckey test results of each compound in samples of batch 8.

Compounds	Pulp (mg/l)	Pulp with enzymes (mg/l)	Juice after press (mg/l)	Juice without aroma (mg/l)	Concentrate (mg/l)	Aroma (mg/l)	Sum (mg/l)
Hexanal - ion 56	0,4513[a]	0,3420[a]	0,0507 [b]	0,0020 [b]	0,0000	0,3753	1,2213
Butyl acetate	0,0025[a]	0,0022[a]	0,001[b]	0,0007 [b]	0,0000	0,0170	0,0237
Ethyl-2-methylbutanoate	0,0000[a]	0,0000 [a]	0,0000 [a]	0,0000 [a]	0,0000	0,0000	0,0000
E-2-hexenal	1,1322[a]	1,1183 [a]	0,0540 [b]	0,0000 [b]	0,0000	11,5612	13,8658
2-Hexen-1-ol	1,0444[a]	1,3451 [b]	0,0000 [c]	0,3803[d]	0,0000	21,5594	24,3292
1-Hexanol	4,9441[a]	5,1688 [a]	3,5830 [b]	1,6233[c]	0,0000	130,2316	145,5508
2-Methyl-1-butyl acetate	0,0144[a]	0,0131 [b]	0,0383c	0,0023[d]	0,0000	0,5590	0,6272
Hexyl acetate	0,0000 [a]	0,0000 [a]	0,0068 [b]	0,0000 [a]	0,0000	0,3093	0,3161
Ethyl butanoate - ion 88	0,0105 [a]	0,0116 [a]	0,0184[b]	0,0011[c]	0,0000	0,8713	0,9410

Appendix III – Aroma index

Table V - Aroma index for batch 1.

Compounds	Pulp (ug/l)	Pulp with enzymes (ug/l)	Juice after press (ug/l)	Juice without aroma (ug/l)
Hexanal - ion 56	176,5649	177,9847	92,0021	2,0246
	169,1487	216,6216	76,6501	2,2111
Butyl acetate	0,0000	0,0000	12,8525	0,0000
	0,0000	0,0000	18,5549	0,0000
Ethyl 2-methylbutanoate	0,2994	0,3532	86,4212	0,0000
	0,2465	0,4530	56,4508	0,0000
E-2-Hexanal	7,6086	8,4350	0,0000	0,0000
	7,2306	10,6975	0,0000	0,0000
2-Hexen-1-ol	1,5715	1,4506	0,0000	3,1299
	1,4616	2,8502	0,0000	3,9164
1-Hexanol	8,7977	8,1873	38,8853	5,3008
	7,5578	10,9650	43,0958	6,0630
2-methyl-1-butyl acetate	0,0000	0,0000	2,2499	0,0000
	0,0000	0,0000	1,9575	0,0000
Hexyl acetate	0,0000	0,0000	0,0483	0,0000
	0,0000	0,0000	0,0273	0,0000
Ethyl butanoate ion 88	0,1864	0,3165	46,2863	1,1673
	0,1950	0,3854	38,6755	1,2085
Sum 1	$195,0286^{ab}$	$196,7273^{a}$	$278,7457^{b}$	$11,62266^{c}$
Sum 2	$185,8403^{ab}$	$241,9726^{a}$	$235,4118^{b}$	$13,39904^{c}$

Table VI - Aroma index for batch 4.

Compounds	Pulp (ug/l)	Pulp with enzymes (ug/l)	Juice after press (ug/l)	Juice without aroma (ug/l)
Hexanal - ion 56	469,4928	598,8463	71,9985	59,6357
	508,3031	506,8104	67,6941	50,1351
Butyl acetate	45,1002	47,1469	174,3965	248,0185
	48,0786	31,7329	158,5903	196,3073
Ethyl 2-methylbutanoate	0,0000	0,0000	0,0000	0,0000
	0,0000	0,0000	0,0000	0,0000
E-2-Hexanal	11,0255	13,2961	9,9422	5,4802
	11,8563	12,8393	11,2720	5,6346
2-Hexen-1-ol	9,3212	11,8477	13,0965	30,3746
	10,8789	14,7602	15,1663	31,4634
1-Hexanol	2,7954	3,3597	22,7902	8,6204
	3,1950	4,1325	25,0333	9,4020
2-methyl-1-butyl acetate	31,1468	36,8596	228,3247	25,5216
	34,6467	24,9698	199,5503	15,8403
Hexyl acetate	0,4998	0,4350	15,0367	1,0069
	0,6629	0,2913	7,9484	0,5296
Ethyl butanoate ion 88	0,2242	0,1454	3,6279	2,9393
	0,2410	0,0848	3,1714	2,4051
Sum 1	569,6060[a]	711,9365[a]	539,2131[b]	381,5972[c]
Sum 2	617,8625[a]	595,6210[a]	488,4261[b]	311,7173[c]

Table VII - Aroma index for batch 8.

Compounds	Pulp (ug/l)	Pulp with enzymes (ug/l)	Juice after press (ug/l)	Juice without aroma (ug/l)
Hexanal - ion 56	121,6355	81,7517	51,7212	2,0062
	104,1635	89,3872	49,7350	1,9661
Butyl acetate	0,6387	0,5192	1,3446	0,5172
	0,5883	0,5674	1,3114	0,81146
Ethyl 2-methylbutanoate	0,0000	0,0000	0,0000	0,0000
	0,0000	0,0000	0,0000	0,0000
E-2-Hexanal	7,5311	6,5422	1,3254	0,0000
	6,6220	7,4369	1,3765	0,0000
2-Hexen-1-ol	12,8895	17,0740	0,0000	18,7297
	13,2198	16,5528	0,0000	19,3002
1-Hexanol	22,4214	21,6173	60,1361	27,3001
	19,6037	22,3177	61,6852	27,8925
2-methyl-1-butyl acetate	0,9105	0,8071	9,5431	0,6290
	0,8926	0,8310	9,6254	0,5439
Hexyl acetate	0,0000	0,0000	2,3873	0,0000
	0,0000	0,0000	2,1403	0,0000
Ethyl butanoate ion 88	1,4967	1,5959	9,2553	0,6058
	1,1350	1,3111	9,1073	0,4827
Sum 1	167,5233[a]	129,9076[a]	135,7130[b]	49,7879[c]
Sum 2	146,2248[a]	138,4041[a]	134,9810[b]	50,9968[c]