Chemical and biological analysis of *Tramazeira*

Mhadhbi Chaimaa

Dissertation submitted to **Escola Superior Agrária de Bragança – Instituto Politécnico de Bragança** to obtain the Degree of Master in **Food Quality and Safety.**

Supervised by

Prof. Letícia Estevinho (IPB)
Prof. Luís Guimarães Dias (IPB)

Bragança, Portugal

July 2021

Abstract

Rowanberries also called as *Sorbus aucuparia L*. fruits (Tramazeira fruits in Portugal), an ornamental tree, have been traditionally used in various processed foods due to their high relevance to human health. Scientific research demonstrates the nutritional effects of *Sorbus L*. fruits are determined by their unique composition of chemically and biologically active compounds. The extraction of phenolic compounds from *Tramazeira* fruits is of interest because they can be used as natural food additives but, extractive processes must be optimized to ensure that the relevant levels of all phenolic compounds groups are extracted from fruit pulp (lyophilized).

A central composite design with two factors (percentage of Ethanol and pH values) was applied, with ethanol % in the range of 24.6 to 95.4% and pH between 0.9 and 5.1. From the response surface methodology (RSM) data treatment, the selected hydroethanolic extract solution had pH value of 3 and ethanol % of 85%, which allowed to have high amounts of the three main groups of phenolic compounds: flavonoids, hydroxybenzoic acids and hydroxycinnamic acids.

Extracts of Tramazeira (*Sorbus L.)* fruit were applied as an additive in orange fresh juice and showed antimicrobial activity against gram positive and gram-negative bacteria, being more effective against gram-positive bacteria. The time effect on orange juice with the different concentrations of the Tramazeira fruit extract showed influence on the microbiological quality of the orange juice. However, the results suggest that the juices obtained with the highest concentration are more stable from a microbiological point of view.

Keywords: Food additive; Phenolic compounds; Central composite design; Response surface methodology.

Resumo

A fruta Tramazeira também chamada de *Sorbus aucuparia L.*, uma árvore ornamental, tem sido tradicionalmente usada em vários alimentos processados devido à sua grande relevância para a saúde humana. Trabalhos científicas demonstram que os efeitos nutricionais das frutas *Sorbus L.* são determinados por sua composição única de compostos química e biologicamente ativos. A extração de compostos fenólicos dos frutos da Tramazeira é interessante porque podem ser usados como aditivos alimentares naturais, mas os processos extrativos devem ser otimizados para garantir que os níveis relevantes de todos os grupos de compostos fenólicos sejam extraídos da polpa do fruto (liofilizada).

Foi aplicado um desenho compósito central com dois fatores (percentagem de etanol e valores de pH), com % de etanol na faixa de 24,6 a 95,4% e pH entre 0,9 e 5,1. A partir do tratamento dos dados com a metodologia de superfície de resposta, a solução extractora hidroetanólica selecionada apresentava valor de pH de 3 e % de etanol de 85%, o que permitiu ter quantidades elevadas dos três grupos principais de compostos fenólicos: flavonóides, ácidos hidroxibenzóico e ácidos hidroxicinâmicos .

Extratos da fruta Tramazeira (*Sorbus L.*) foram aplicados como aditivo em sumo fresco de laranja e apresentaram atividade antimicrobiana contra bactérias gram-positivas e gram-negativas, sendo mais eficazes contra bactérias gram-positivas. O efeito do tempo nos sumos de laranja com as concentrações do extrato da fruta Tramazeira mostrou influência na qualidade microbiológica do sumo de laranja. No entanto, os resultados sugerem que os sumos obtidos com as maiores concentrações são mais estáveis do ponto de vista microbiológico.

Palavras-chave: Aditivo alimentar; Compostos fenólicos; Desenho compósito central; Metodologia de superfície de resposta.

Acknowledgements

To say that this thesis was completed as a personal achievement would be an understatement. The completion of this work was done so as a collaborative effort by the incredible people who surround me. I would first of all like to thank my two advisers and supervisors, Dr. Letícia Estevinho and Dr. Luís G. Dias. I will never be to be able to express how grateful I am for all the advice and trust you have placed in me. The skills and knowledge I have gained under your guidance are invaluable and I will be forever grateful to have been one of your graduate students.

I also want to thank Vanessa B. Paula for your support and advice, for being so generous, listening and, overall, for all the efforts, you have provided throughout this project, without you I could not have finished this thesis at the right time.

Finally, I want to thank my family. To my parents, Kamel and Amel, forever support me in all my efforts. I attribute all my success to your continued support.

To my Brothers Mohamed Aziz, you are a constant source of inspiration for me and you always manage to make me smile. Finally, to my fiancé, Wael. Thank you for being by my side during my several years of study. You have continually encouraged me to pursue my dreams, no matter what the cost, and I can never really thank you enough. This thesis is dedicated to all of you.

Index

Index of figures

Index of tables

General Introduction

In our days, many societies suffer from having many unhealthy dietary habits. The insufficient consumption of healthy foods causes a major dietary imbalance, which triggers chronic diseases such as obesity, hypertension, diabetes, cardiovascular disease, and several types of cancer. However, over the past few decades, the diet of consumers has undergone a remarkable evolution in the consumption of fruits and vegetables, which has caused a decrease in these diseases.

Rowanberries also called the rowans (mountain-ashes) belong to the genus *Sorbus L.* and are traditionally used in the nutrition of Northern Europeans in different processes as accompanied, jelly and drinks that have high nutritional and health potential advertising (Poyrazoglu, 2004; Kylli et al., 2010). Scientific studies have shown anti-inflammatory (Yu et al., 2011), antioxidant (Olszewska et al., 2009), ant diabetic effects (Grussu et al., 2011), attributed to their unique composition of biologically active compounds, such as, carotenoids, ascorbic acid, as well as organic acids, sugars (Berna et al., 2011) and phenolic compounds, as flavonoids, and particularly phenolic acids (hydroxybenzoic and hydroxycinnamic acids). They contribute significantly to antioxidant activity (Tunde et al., 2014; Mikulic-Petkovsek et al., 2017). Therefore, the study of phenolic compounds in Tramazeira fruits has been of increasing interest in recent years.

Natural antioxidants also occupy importance in different fields such as cosmetics, pharmaceuticals and, particularly, in food industries, as they can also be used to maintain the flavor and color of food which prevents the destruction of vitamins (Moure et al., 2001). The growing interest in the antioxidant properties of the phenolic compounds from different plant sources also derives from their strong activity and low toxicity compared with those of synthetic phenolic antioxidants, such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), which are commonly used as antioxidants in processed foods (Nakatani, 1996). Polyphenols are compounds naturally synthesized by the secondary metabolism of plants, which have potential therapeutic effects on health. These bioactive compounds delay or inhibit the autoxidation of lipids by acting as radical scavengers, consequently, they protect against the propagation of the oxidative chain (Navarro et al., 2006).

The main objective of the present work was to study the best hydroethanolic composition (mixture of ethanol and hydrochloric acid) that allows to extract higher levels of phenolic

compounds (hydroxybenzoic acids hydroxycinnamic acids, and flavonoids) in fruit pulp. Also, to apply the pulp fruit extract as a natural food additive for contributing to positively influence the shelf life and safety of a food product, which in this work was orange juice.

1. Tramazeira tree botanical description

Tramazeira which is the Portuguese name also known as rowanberrie and mountain-ash *Sorbus aucuparia L.*.This tree belongs to the *Rosaceae* family which can reach up to 15-20 meter long, normaly less than 15m, with a shape of an oval crown tree (Phipps et al., 1990). The ripening of the fruits takes place during the autumn period (September to October), this tree is characterized by its great resistance since it is indifferent to the pH but, better in siliceous soil, requires a moderate humidity, supports low temperatures, tolerates urban pollution and is very strong to sea winds. The tree with many flowers (usually around 250 flowers during the flowering period, in the spring), and woolly pubescent when flowering, a fruit with two- to five-celled, berry-like pome, each cell containing one or two small brown seeds (Raspe et al., 2000). Flowers have storm receptacle and white petals, until 8 to 10 mm in diameter. The leaves length have 2.5 to 6 cm, with 9-15 leaflets of sawn margin, green in the superior part (upper part) and white tomentosa in the lower or inferior part.

Figure 1. Tramazeira tree and their fruits.

1.1. Geographical distribution

The genus *Sorbus L*. includes a wide variety of species widely distributed in the temperate zones of Northern hemisphere. However, the distribution of some species extends to northern latitudes, as in Greenland and Siberia, and even to the tropics of Asia (Malaysie). The main centers of diversity are in Europe, *Sorbus* species are mainly concentrated in Czechoslovakia, Hungary, Great Britain, and Germany. There are 91 species in Europe, and there are at least 111 species in China, Vietnam, Burma and the Himalaya (Phipps et al., 1990).

It is thought that the genus *Sorbus* could have originated in East Asia, and migrated to Europe and North America (Jankun 1993). Probably, a high level of hybridization is the cause of the high number of species present in European (70 out of 91 species), according to Phipps et al. (1990).

In Europe and North Africa, Tramazeira is represented by five different sexually reproducing species: *S. aria L. Crantz, S. aucuparia L., S. chamaemespilus L. Crantz, S. torminalis L. Crantz* and *S. domestica L.* (Aas et al., 1994).

And more precisely in Portugal, Tramazeira or *Sorbus aucuparia L.* is present in the mountain ranges of the central and northern regions.

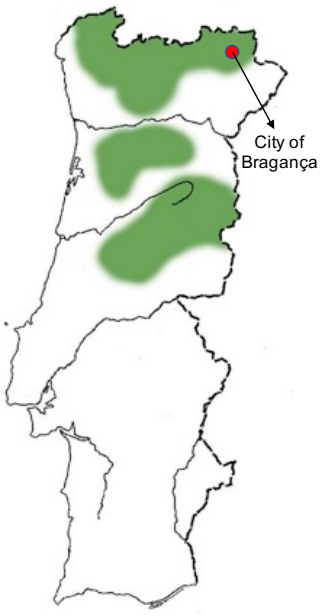


Figure 2. Zones most suitable for planting Tramazeira in Portugal.

1.2. Ethno pharmacological and ethno botanical importance

Several *Sorbus L.* species are known for their biological activity and therapeutic properties. Fruits, inflorescences, leaves and bark have been traditionally used for a wide variety of ethno medical properties, such as bronchitis, gastritis, asthma, diabetes, inflammation, antidiarrheal, vasoprotective and vasorelaxant activities, as well as vitamin and antioxidant agents (Yu et al., 2011; Hukkanen et al., 2006; Kahkonen et al., 2001; Termentzi et al., 2006).

Four species typical of Europe have found their way into traditional medical material, being the *S. aucuparia* a very popular natural remedy against various diseases recognized in Europe and Asia. In Lithuania (the Baltic region of Europe), mountain ash preparations were used for constipation and cough, while a bark decoction was used to wash wounds (Pranskuniene et al., 2019). On the other hand, in Estonia (in Northern Europe) a tea prepared from the bark of *S. aucuparia* has been used to treat cancerous diseases (Sak et al., 2014). They have also been used in gastrointestinal problems or prostatitis (Neves et al., 2009). Finally, the inflorescences have been used as diuretic and anti-inflammatory agents (Olszewska, 2011).

In Asia the *Sorbus L*. is used in folk medicine to treat asthma, dyspnea, ventricular myocytes and gastritis (Sarv et al., 2020). The peel of the fruit is applied in bronchitis, asthma or cough (Sohn et al., 2005), used as expectorant agent, and as an anti-atherosclerotic (Yin et al., 2005). The fruits are used for throat infections or as a laxative (Lee et al., 2017) and as remedy for scurvy (Khan et al., 2015). In addition, in South Korea, the leaves, stem and fruits of Tramazeira are traditionally used as a decoction or herbal tea in liver disorders (Kim et al., 2013).

Sorbus L. is also a species native in North America and it is used to relieve symptoms related to diabetes by the First Nations of Quebec, Canada (Guerrero-Analco et al., 2010). In addition, it is also administered as a traditional remedy in the boreal regions of Canada, where it is recommended in the treatment of diabetes and its complications (McCune et al., 2007).

1.3. Nutritional and chemical characterization

The berries obtained from different species of *Sorbus*, besides their medicinal use, are known for their nutritional value. In fact, *Sorbus (S. aucuparia)* are very popular edible products,

especially in Europe, often consumed raw or processed into confectionery, syrups, liquors, etc (Tardio et al., 2006; Łuczaj et al., 2013; Pranskuniene et al., 2019).

Indeed, Rowanberries are rich in organic acids, carotenoids, microelements, ascorbic acid, and phenolic compounds (Raudonis et al., 2014). In addition, the chemical composition of the genus *Sorbus* has been studied since the 1960s to the present data.

However, various studies (Olszewka, 2008; Mrkonjic et al., 2017; Isaikina et al., 2018) of *Sorbus* species have yielded twenty structurally new compounds, such as triterpenes, phenols, coumarins, fatty acids and other compounds. Some species have been analyzed for the presence of fatty acids. And more precisely, at the level of the seeds of *S. aucuparia* which contains 27 carboxylic acids, of which 17 were fatty acids and 2 were aromatic. The dominant acids (mg/kg) were linoleic (18mg/kg.), oleic (841.0 mg/kg), palmitic (11 mg/kg), malic (210.0 mg/kg) and citric (5149.7 mg/kg). The presence of fatty acids has also been confirmed in leaves of the rowan berries, being the dominant acids (mg/kg) the palmitic (3793.5 mg/kg), citric (1929.5 mg/kg), malic (1825.5 mg/kg), and oxalic acids (1288.0 mg/kg) (Krivoruchko et al., 2013).

Rowanberries are exceptionally rich sources of sugars, the most abundant were sorbitol, glucose and fructose, and they contribute to their texture, flavor and nutrition. In fruits of *S. aucuparia*, sorbitol and glucose levels reached 134.1 g/kg fresh weigth (fw) and 52.9 g/kg fw, respectively (Mikilic-Petkovsek et al., 2012).

1.4. Biological characterization

Several *Sorbus* species are known for their biological activity and therapeutic properties. The majority of biological activity studies have focused on determining their antioxidant potential due to the high number of phenolic compounds that have been detected in various species of *Sorbus.*

1.4.1. Antioxidant activity

The antioxidant activity of rowanberries has been compared to the activity of many valuable berries or even superior to them, eg. blueberries (*Aronia mitschurinii*), blueberries (*Vaccinium myrtillus*) and camarines (*Empetrum nigrum*) (Kahkonen et al., 2001). Indeed, the antioxidant

activity of several species of *Sorbus* such as *S. aucuparia* was tested using a human plasma model. Using the water-methanol flower extract of *S. aucuparia* and its fractions, they protected human plasma exposed to oxidative/nitrative stress (Olszewska et al., 2019). A few studies have looked at methanol extracts from *Sorbus* fruits with different stages of ripeness and found that unripe yellow fruits and fruit pulp were the most effective (Termentzi et al., 2006).

1.4.2. Antimicrobial activity

Problems related to the risk of the presence of multidrug-resistant strains of bacteria in the food chain, may cause the growth of foodborne illnesses. Therefore, there has been a growing demand for natural preservatives of plant origin and antimicrobial compounds (Tamkute et al., 2019). According to Lacombe et al. (2017), berries and their constituents such as phenolic compounds and organic acids can inhibit growth of bacterial pathogens. Fruit extracts of *S. aucuparia*, obtained using 50% of ethanol, inhibited the growth of *Bacillus cereus* MSCL 330, *Staphylococcus aureus* MSCL 334 and *Pseudomonas aeruginosa* MSCL 331 (Liepina et al., 2013).

1.4.3. Cytotoxic activity

Several studies have been performed to evaluate the cytotoxicity of *Sorbus* to confirm the cell safety or the potential anticancer activity of the analyses studied. A screening test was carried out for the evaluation of the cytotoxicity of various agents towards HepG2, Caco-2, A549, HMEC-1 and 3T3 cells (Boncler et al., 2017). Indeed, cell membrane integrity, mitochondrial membrane potential and nuclear size were measured. The fruit extract of *S. aucuparia* exerted a relatively high toxicity, in particular with regard to the nuclear zone. In addition, the use of the methylene chloride fraction from the ethanol extract of *S. aucuparia* showed a decrease in the viability of HeLa cells (Bozkurt-Guzel et al., 2018). Further studies examined the *in vivo* antitumor activity of extracts acidified with 95% ethanol of *S. aucuparia* fruits. This extract was administered to female mice with B-16 melanoma cells, noticing a decrease in tumour growth (Razina et al., 2016).

1.4.4. Other activities

As an example of neuroprotective activity, it was shown that some species of *Sorbus* have the ability to inhibit AChE and or BuChE (butyrylcholinesterase). In this connection, methanol extracts (75%) of leaves of several species of *Sorbus* and in particular *S. aucuparia*, were examined for possible inhibitory activities of the latter (Ekin et al., 2016).

Another example showed that the triterpenoid fraction obtained from the fruits of *Sorbus* was tested for a potential hepatoprotective effect. Levels of some selective cytokines (TNF-α, IL-1β, IL-6), MDA (lipid peroxidation malondialdehyde), SOD (superoxide dismutase), GSH (glutathione), CAT (catalase), aminotransferases (AST, ALT), were normalized after treatment, and, on the other hand, necrosis of hepatic tissues, haemorrhage and infiltration of inflammatory cells were inhibited (Yongxia et al., 2019).

Also, flavonoid fraction of *Sorbus* has shown a protective effect against cardiotoxicity in male mice. Indeed, apoptosis and oxidative stress were eliminated. On the other hand, changes in cardiac tissue have been observed such as myofibrillar loss, cytoplasmic vacuolation and cardiomyocyte necrosis. Hence, pre-treatment with *Sorbus* flavonoids (20 mg/kg, intraperitoneal injection) alleviated these abnormalities (Xiaojin et al., 2017).

1.5. Phenolic compounds in Tramazeira fruit

It's well known that (Fraser et al., 2007, Klensporf-Pawlik et al., 2015) plant phenolic are a very important class of antioxidants. Within endogenous antioxidant systems, they play a significant role in optimal protection from oxidative stress caused by an increase in the levels of damaging reactive oxygen species in the human body. The growing interest in the antioxidant properties of phenolic compounds from different plant sources also stems from their high activity and low toxicity compared to those of synthetic phenolic antioxidants, such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), which are commonly used as antioxidants in processed foods (Nakatani, 1996).The antioxidant activity of some *Sorbus* fruits has been recently related to the presence of their polyphenolic compounds (Hukkanen, 2006; Termentzi et al., 2006). According to some studies, the total phenolic content of wild blackberries was 18.7 mg/g of dry weight (Kahkonen et al., 1999).

1.5.1. Chemical structures

The phenolic compounds are heterogeneous groups of secondary metabolites that biosynthesize the pentose phosphate, shikimate, and phenylpropanoid pathways. They also have an effect on the often-accepted quality properties of fruit, such as bitterness, colour and taste. According to the number of phenolic rings and the structural elements that bind the rings together, such compounds are divided and classified into two main groups: flavonoids and non-flavonoids (Vujanović et al., 2019).

1.5.1.1. Flavonoids

Flavonoids are phenolic compounds that are found in fruits in different forms and can be divided into six groups or families showed in Figure 3. They represent a large class of at least 6000 phenolic compounds found in fruits, vegetables, and other food products, with great bioactive properties (Manach et al., 2004).

Flavones are present in some fruits, especially melon and watermelon, and vegetables (Vujanović et al., 2019). Flavonols are some of the best antioxidant flavonoids due to their hydroxylation pattern and they are also the most common flavonoids in fruits and vegetables. Flavan-3-ols are present in foods, especially high in tea, but also in chocolate, red wine, nuts, and some fruits like grape, strawberry, blackberry, peach, nectarine, apple, and fruit juices. Anthocyanidins are abundant in fruits such as peaches, nectarines, plums, and apples, and vegetables such as beans. Also, proanthocyanidins are common in the peel and seeds of grape, being apple, almond and blueberry other sources.

1.5.1.2. Non-flavonoids

The group of non-flavonoids contains phenolic compounds with very different chemical structures, most of them smaller and simpler than flavonoids, but there are also compounds with complex structures and high molecular weights. The non-flavonoids group can be classified into various subgroups, the main group of non-flavonoids in fruits are phenolic acids, which

contain a single phenyl group substituted by a carboxyl group and one or more OH groups. Phenolic acids can be subdivided into hydroxybenzoic acids and hydroxycinnamic acids.

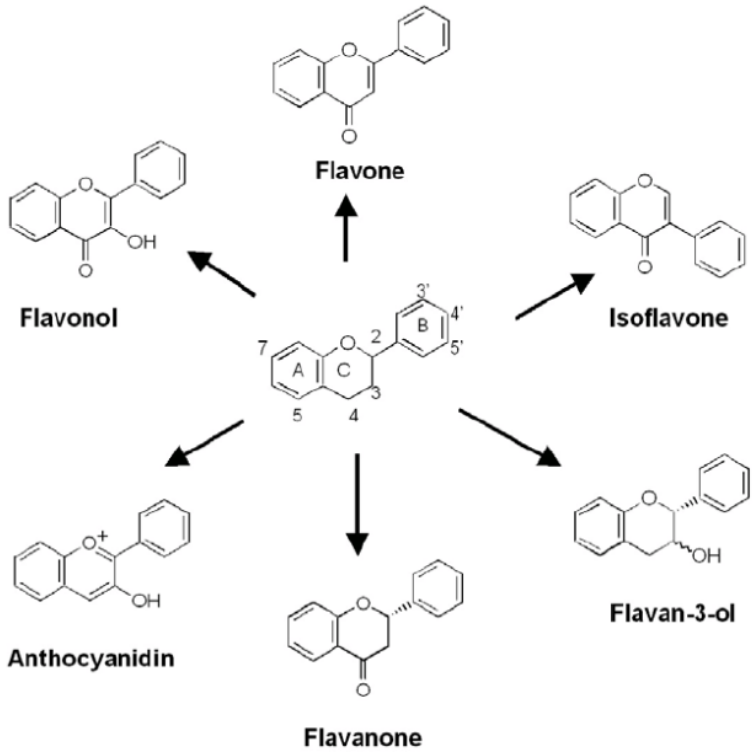


Figure 3. Flavonoids group structures (Nishiumi et al., 2011)

The most common hydroxybenzoic acids can be found in many fruits, vegetables, and other edible products (for instance, nuts, tea, chicory and some spices). They are rarely found in their free form, they often appear glycosylated, bound to small organic acids (quinine, malein or tartar) or bound to structural particles of plant cells (cellulose, protein or lignin). Hydroxycinnamic acids are usually bound to small or large molecules. The most abundant is an ester of caffeic and quinic acids, named chlorogenic acid, and its isomers are also found in many fruits such as plum, berries, nectarine, peach, apple, and pear. Lignans are non-flavonoids that form two phenylpropanoid units, they are widespread, but they are few in fruits.

Hydrolysable tannins, which can be divided into ellagic acid, and punicalagin is an ellagitannin that is abundant in pomegranate peels and is also contained in its juice. They are also found in berries, mangoes, and nuts.

1.5.2. Properties in human health

Due to their powerful antioxidant properties, phenolic compounds have been shown to be scientifically proven to prevent various diseases related to oxidative stress and chronic, such as cancer, cardiovascular and neurodegenerative diseases (Loef et al., 2012; Smith-Warner et al., 2001). Figure 4 presents a schematic of the biological properties attributed to phenolic compounds, which shows the general focus of the research line in the study of fruits, in exploiting the potential of natural antioxidants and determining their relationship to human health (Dai et al., 2010). Flavonoids and phenolic acids make up the two main classes of dietary phenolic compounds (Tapiero et al., 2002). Both categories have been extensively studied, but phenolic acids have recently received much attention due to studies linking their presence in food products, such as fruits, especially berries, and whole grains, with good human health benefits (Costa et al., 2014).

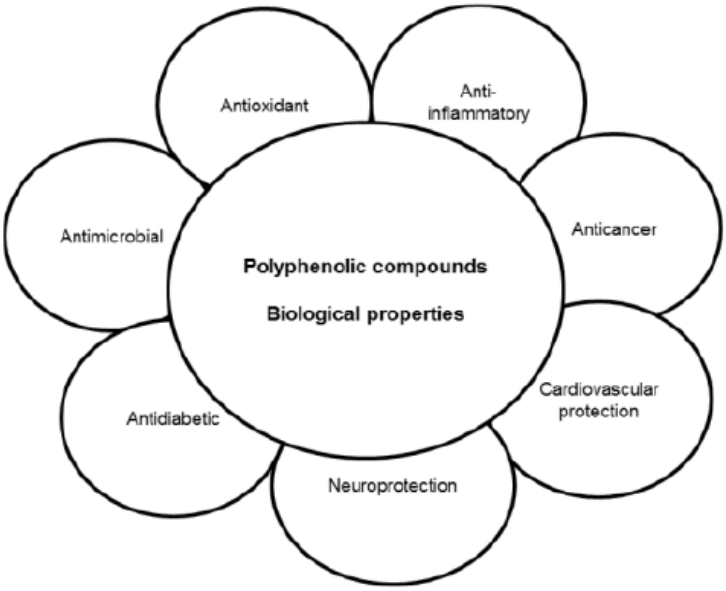


Figure 4. Main biological proprieties of phenolic compounds (Loef et al., 2012; Smith-Warmer et al., 2001)

1.5.3. Solvents most used as extractors

Various solvents have been mentioned in the literature to extract phenolic antioxidant compounds from fruits, such as hexane, ethyl acetate, methanol, ethanol, acetone and water

(Ross et al., 2009). The extraction should be done with the most suitable solvent and under analytical conditions of ideally predetermined temperature and pH. Furthermore, it is necessary to consider the polyphenol structure, as these compounds may have several hydroxyl groups which can be conjugated to sugars, alkyl groups or acids. Thus, the polarities of phenolic compounds vary considerably and it is difficult to develop a solvent for optimal extraction of all phenolic compounds. Solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, and their compounds have also been used for the extraction of phenols, often with different proportions of water. For example, phenolic compounds can be efficiently extracted from vegetables through an ethanol/water system (70:30, v/v) (Prati et al., 2007). Table 1 shows examples of works where the solvents used in the extraction of phenolic compounds in various food samples and the number of identified polyphenol compounds are highlighted.

Anthocyanins are usually extracted from plant material with an acidic organic solvent, often methanol. This solvent system destroys cell membranes, simultaneously dissolves anthocyanins and stabilizes them. However, the acid can cause changes in the original form of anthocyanins by breaking down their complexes with metals and co-pigments (Naczk et al., 2006).

Table 1. Various solvents used for fruits extraction (Liquid-Liquid Extraction)

Sample	Solvent	Nº polyphenols identified	Reference
Bayberry	Ethyl acetate	10	Zhongxiang et al., 2007; Garcia-Salas et al., 2010
Quince	Methanol (100%)	18	Magalhãeset al., 2009; Urbanaviciut, 2020
Naranjilla	Acetone 70%	2	Mertz et al., 2009; Garcia-Salas et al., 2010
Tree tomato	Acetone 70%	8	Mertz et al., 2009; Chávez-González et al., 2020
Papaya	Methanol (100%)	12	Simirgiotis et al., 2009; Addai et al., 2013

Also, as described by Ross et al. (2009), aglycon forms of glycosidic flavonoids are obtained by acid hydrolysis of bean extracts using a methanol/water system (85:15, v/v). These works show that in the extraction of phenolic compounds different solvents are used, but, in most cases, without any optimization study. It is in this context that the present work will be developed, in the optimization of the extraction of phenolic compounds with the solvent binary

system, percentage of ethanol and aqueous HCl solution (establishing different pHs), through the method of surface optimization according to an experimental design.

1.6. Response Surface Methodology

Response surface methodology (RSM) is a set of mathematical and statistical techniques useful for the modelling and analysis of problems. The objective is to optimize a response (output variable), which is influenced by several variables (input variables) (Montgomery, 2005). For example, the extraction of a substance from a plant is affected by a certain amount of ethanol (which acts as the solvent; factor x1) and an acidic aqueous solution (hydrochloric acid solution to adjust the pH; factor x2). Extraction of phenolic compounds from a plant can be carried out with any levels of both factors (x1 and x2) and, therefore, the phenol extract composition can vary continuously. When treatments are from a continuous range of values, then a RSM is useful for developing, improving, and optimizing the response variable. In this case, the concentration of total phenolic extract can be the response variable, since it is a function of pH and ethanol percentage.

The RSM model corresponds to a mathematical fit between the response variable (dependent) and the experimental variables (independent) and if the response can be defined by a linear function, then the approximating function is a first-order model. But if there is a curvature in the response surface, then a higher degree polynomial should be used, which is the case in this work. The approximating function with 2 variables is called a second-order model and it can be expressed as:

$$Y = \beta_0 + \beta_1 \cdot x_1 + \beta_2 \cdot x_2 + \beta_{11} \cdot x^2_{11} + \beta_{22} \cdot x^2_{22} + \beta_{12} \cdot x_1 \cdot x_2 + \varepsilon,$$

where β_1 is the coefficient of factor 1 (x1), β_2 is the coefficient of factor 2, $x_1 \cdot x_2$ is the interaction term and ε is the error of the mathematical adjustment.

In order to get the most efficient result of the RSM technique, an appropriate experimental design must be used to collect data. The most popular design for fitting a second-order model is Central Composite Design (CCD) due to the following three properties:

- A CCD can be run sequentially (incomplete blocks). A block is a set of relatively homogeneous experimental conditions so that an experimenter divides the observations into groups that are run in each block.
- CCDs are very efficient, providing much information on experiment variable effects and overall experimental error in a minimum number of required runs.
- CCDs are very flexible. The availability of several varieties of CCDs enables their use under different experimental regions of interest and operability.

2. Materials and methods

2.1. Sampling

The plant material used in this study was the Tramazeira fruit, which is yellow to orange depending on the ripeness, more specifically the fruit pulp. The Tramazeira (*Sorbus aucuparia L.*) fruits were harvested from five different trees in November, coming from Polis Bragança, Portugal. The geographical origin of the samples is in Figure 5, which shows the precise location in the Bragança city, Trás-os-Montes, Portugal (Google maps).

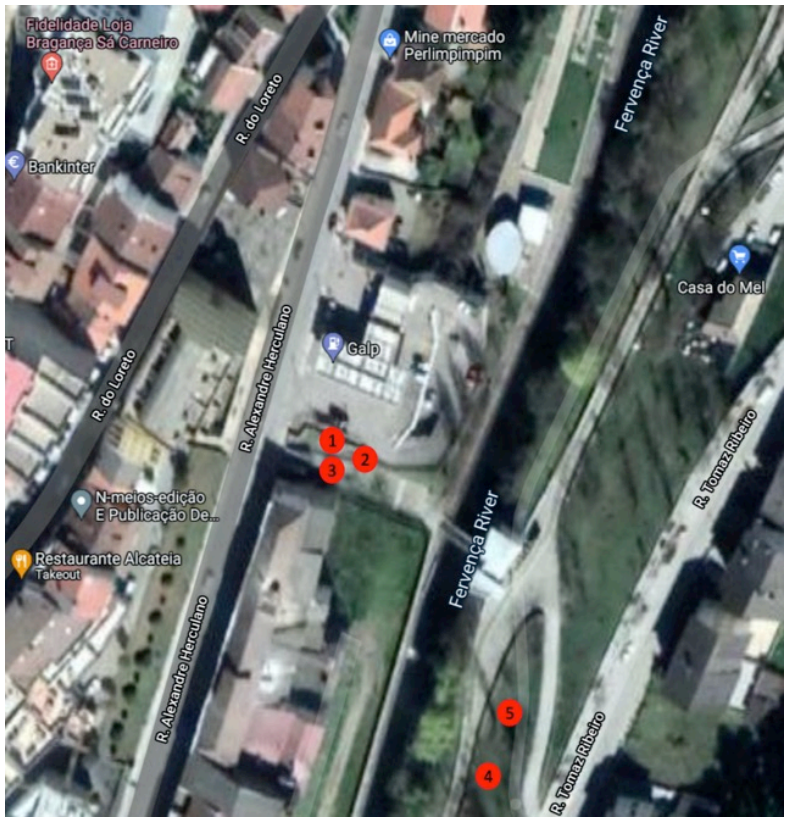


Figure 5. Tramazeira trees localization in Bragança's city, by Google maps.

2.1.1. Pre-treatment analyses

Branches, leaves, and damaged fruits were removed (considered as waste) and only the fruits in good conditions were selected. As 2nd step, the fruits were washed several times with distilled water, filtered, dried and pressed using a 1 mm sieve to get the pomace. This step was done manually in order not to damage the seeds that were also removed.

Figure 6. Pomace of Tramazeira fruits.

And, finally the obtained samples were freeze-dried (Beijer Electronics, TelStar LyoQuest HT 40) at -57, 4°C, 0.419 mBar, for 5 days, to obtained a dried Tramazeira pulp fruit, which has been stored cold (at -25 ºC) until used.

2.2. HPLC analysis

2.2.1. Equipment and experimental conditions

An HPLC Varian ProStar equipped with a Varian 220 pump, a 7725i Rheodyne manual injector, provided with a loop 20 µl, a 7981 Jones Chromatography column oven, an ultraviolet (UV) detector (Varian, model 9050) coupled to a refractive index (RI) detector (Varian. model RI-4) was used to simultaneously separate and quantify sugars. A flow rate of 0.6 mL/min was applied in an Aminex HPX-87H column (30 cm × 7.8 mm id), thermostated at 30 °C. An isocratic elution with a mobile phase consisted of a 0.005 mol/L of sulphuric acid aqueous solution was used. Star Chromatography Workstation software (version 6.4., Varian Inc.) was used for data acquisition and peak integration. Organic acids were detected with the UV detector at 210 nm, while sugars were detected with RI detector.

Chromatographic peaks of sugars and organic acids were identified by comparing retention times of pure compounds with those recorded for standard mixtures containing all the analysed compounds or sample solutions. Peaks were quantified with external standard calibration based on areas. The HPLC performance was evaluated considering linearity parameters, instrument and method precision (including repeatability and intermediate precision assays) and accuracy. For HPLC calibration and performance evaluation, a serial dilution of the sugars and organic acids used was made by dissolving the required amount of standard in deionized water. Before HPLC analysis, all standards were filtered through a 0.2 µm nylon filter (Whatman).

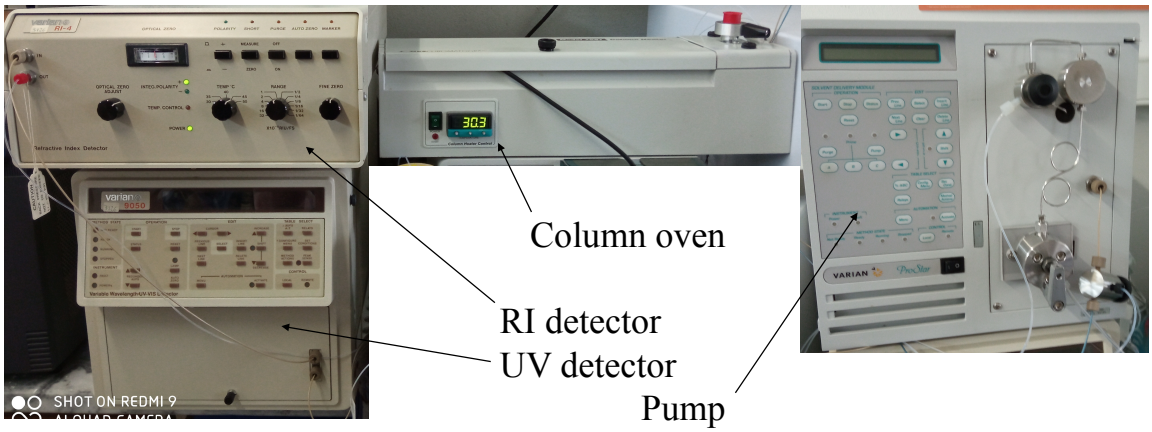


Figure 7. HPLC system used.

2.2.2. Standards solutions of calibration

Different standard concentrations were used with a range from 0.02 to 0.5 g/L for glucose, fructose, malic acids and sorbitol and were analysed in a single run. The sample solutions were prepared by 0.1 g of each sample into a 100 mL volumetric flask, whose volume was adjusted with deionized water. All samples were filtered through a 0.2 µm nylon filter (Whatman) and stored at -5°C until analysis. Analysis were carried out with repetitions until two or more concordant values were obtained. The eluent was degassed during 5 min in an ultrasonic bath (Elma Transsonic 460/H) before filtration.

2.3. Phenolic compounds extraction optimization

The study of the extraction procedure optimization involved two works. The first consisted in establishing calibrations through UV spectrum of mixtures of phenolic compounds for the measurements of the total concentrations of the groups of compounds of hydroxybenzoic acids, hydroxycinnamic acids and flavonoids, as well as the concentration of total phenolic compounds. The second, the application of the calibrations obtained using the spectra obtained in the tests defined by CCD to determine the responses (concentrations of the 4 parameters analysed) and establish the respective dependence with the two factors studied in the optimization of the extraction process of phenolic compounds (pH and ethanol %) from the pulp fruit of Tramazeira.

2.3.1. UV spectra of mixed standard solutions of phenolic compounds

A fractional factorial design was used for mixing the compounds gallic acid (hydroxybenzoic acid), ferulic acid (hydroxycinnamic acid) and quercetin (flavonoid), in order to guarantee the orthogonality between five concentration levels. The mixed calibration standard solutions were prepared as shown in Table 3. The UV spectra will allow to establish the calibrations for measuring the total hydroxybenzoic acids (THBA), total hydroxycinnamic acids (THCA), total flavonoid (TF) and total phenolic (TP) contents, as described in the work of Paula et al. (2017). To measure the UV spectrum, each mixed calibration standard solution was prepared using the Obied et al. (2005) methodology modified by Singleton and Rossi (1965). A volume of 1 mL of each mixed calibration standard was mixed with 1 mL of ethanol at 95%, containing 0.1% hydrochloric acid, and adding aqueous 2% hydrochloric acid solution until the final volume is adjusted to 10 mL (spectrum solution). The UV spectra was obtained using the spectrophotometer (VWR UV-3100PC), in the wavelength range between 200 and 450 nm.

2.3.2. CCD assays for extraction optimization with RSM

In order to optimize the phenolic compounds extraction from the *Sorbus aucuparia L.*, a RSM was used to evaluate the effect of the two factors (Ethanol % and pH, controlled by adding a HCl aqueous solution). A CCD was used with two blocks (cube and star) in order to estimate a second-degree polynomial model for the response variable considering those two factorial

variables (Lenth, 2009). The following table shows the tests performed by the experimental design (Lenth, 2009).

Table 2. Multilevel fractional factorial design for five-level mixed calibration standard solutions (Wu et al., 2011).

Assay number	Gallic acid mg/L	Ferulic acid mg/L	Quercetin mg/L
1	40	40	35
2	40	10	20
3	10	20	10
4	20	10	60
5	10	80	60
6	80	80	35
7	80	40	20
8	40	20	60
9	20	80	20
10	80	20	50
11	20	60	50
12	60	60	35
13	60	40	60
14	40	80	50
15	80	60	60
16	60	80	10
17	80	10	10
18	10	10	35
19	10	40	50
20	40	60	10
21	60	10	50
22	10	60	20
23	60	20	20
24	20	20	35
25	20	40	10

In this study, a mass of 0.1 g of a lyophilized Tramazeira fruit pulp was dissolved with 25 ml of hydroethanolic solution, a mixture of ethanol and HCl aqueous solution (concentrations related to each pH value) as defined by the experimental design. Each solution was stirred in vortex and homogenate for 24 hours (this process was repeated 3 times). Finally, a centrifugation was carried out for 10 minutes to be able to sediment the solid component. The solutions obtained were treated in order to obtain the respective UV spectrum using the Obied et al. (2005) methodology, as described in the previous section.

Table 3. CCD to optimize the extraction of phenolic compounds in Tramazeira fruits.

		Real values		Coded values	
Assay Number	Block	pH	Ethanol %	pH	Ethanol %
1	1	3,00	60	0	0
2	1	3,00	60	0	0
3	1	4,50	85	+1	+1
4	1	3,00	60	0	0
5	1	1,50	35	-1	-1
6	1	4,50	35	+1	-1
7	1	1,50	85	-1	+1
8	2	3,00	60	0	0
9	2	3,00	24,6	0	-1.4
10	2	3,00	95,4	0	+1.4
11	2	0,88	60	-1.4	0
12	2	5,12	60	+1.4	0
13	2	3,00	60	0	0
14	2	3,00	60	0	0

The UV spectrum will allow to determine the concentrations of THBA, THCA, TF and TP contents (four responses) in these solutions, by applying the calibrations that will be defined from the solutions prepared in the previous section. The optimal extractor solution will be obtained using the RSM statistical analysis.

2.4. Studies of Tramazeira fruits extracts

2.4.1. Phenolic compounds analysis in extracts by UV spectrum

Each dry crushed sample (5 g) was weighed in a beaker and 100 ml of ethanol at 85% with pH 3 was added (the optimal solution). The solutions were shaken overnight and filtered. The solution was put in rotary evaporator (IKA RV 8; Figure 8) for 1h at 50°C. The UV spectrum of these extracts were obtained using the Obied et al. (2005) methodology, as described in the previous section (Figure 9), for analysis of THBA, THCA, TF and TP contents. All experiments were carried out in triplicate.

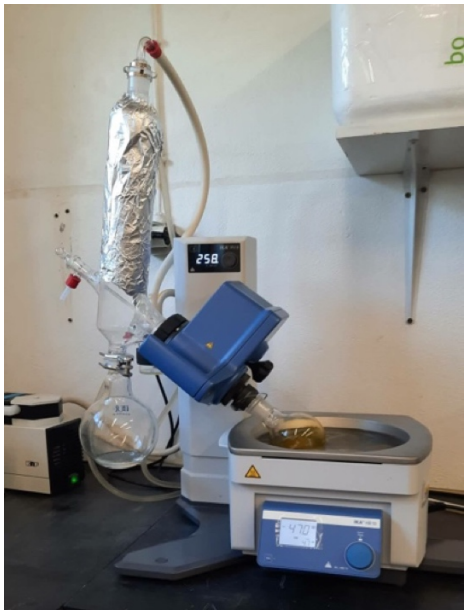


Figure 8. Rotary evaporator (IKA RV 8).

Figure 9. Obied assays using the optimal solvent for the 5 extracts.

These solutions were also analysed in their total phenols and flavonoids contents by the Folin-Ciocalteu and $AlCl_3$ methods, respectively. Other chemical tests were used to evaluate the antioxidant activity of theses extracts as, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, which evaluate antioxidant power from the inhibition of free radicals, and the ferric reducing antioxidant power (FRAP) assay, in which the antioxidant property is evaluated from a redox reaction occurring between the substrate and Fe^{3+} ions, producing Fe^{2+} ions. These tests were applied, in triplicate, on the five samples, which were extracted using the optimal solvent solution (85% of ethanol and 3 pH).

2.4.2. Total phenolic compounds by Folin-Ciocalteu method

The quantitative determination of total phenols was determined by the Folin-Ciocalteu method (Singleton et al., 1999). The volume of 0.5 ml of the sample was diluted with 2.5 ml of Folin-Ciocalteu (10% v/v) reagent, and after, 2 ml of sodium carbonate (Na_2CO_3, 75 g/L) was added (Figure 10). The absorbance was measured at a wavelength of 760 nm, after incubation for 2 hours in the dark, using a spectrophotometer (VWR UV-3100PC). The calibration curve was prepared using standard solutions of galic acid (AG) with concentrations between 15 and 105 mg/L.

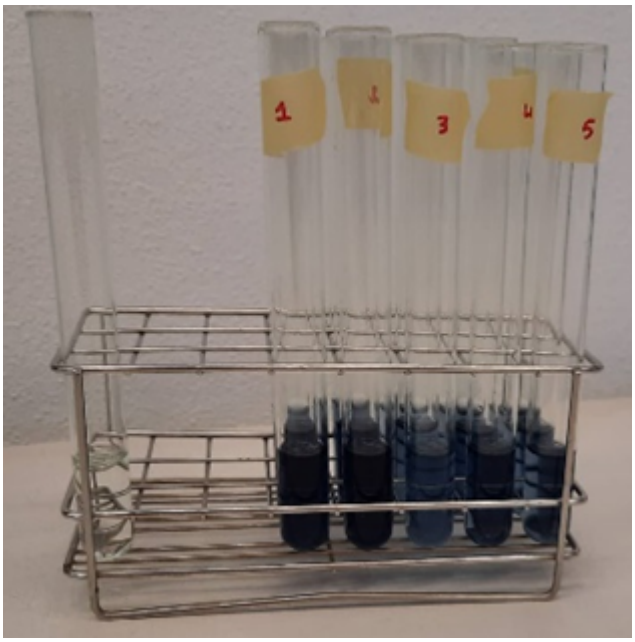


Figure 10. Total phenolic compounds assays.

2.4.3. Total flavonoids content by $AlCl_3$ method

The flavonoids contained in the phenolic extracts were estimated by the method of $AlCl_3$ (Woisky et al., 1998). Samples were prepared by mixing 2.5 ml of the aqueous extract with 2.5 ml of ethanol solution of $AlCl_3$ (2%). After 1 hour of incubation, in the dark at room temperature, the absorbance of the mixture (Figure 11) was read at a wavelength of 420 nm. To express the flavonoid content, quercetin was used as standard. The calibration curve was prepared using standard solutions of quercetin with concentrations between 0.48 and 20 mg/L.

Figure 11. Total flavonoids assays.

2.4.4. Radical scavenging activity

The free radical-scavenging activities were measured using DPPH as described by Hatano et al. (1988). To 0.3 mL of each sample, 2.7 mL of DPPH solution (2.0×10^{-4} M, 85% of ethanol) was added. After 60 min incubation period, at room temperature, the absorbance of the solutions was read against a blank at 517 nm in a spectrophotometer (VWR UV-3100PC). Inhibition of the free radical, DPPH, in percent (I %) was calculated according to the formula:

$$I\ (\%) = (A0 - As)/A0\times100,$$

Where A0 is the absorbance of the control (containing all reagents except the sample) and As is the absorbance of the tested sample.

The actual decrease in absorbance induced by the tested sample was visualized by the change of colour from deep-violet to light yellow), as shown in Figure 12. Reference standard used was Trolox with concentrations varying between 12.8 to 128 mg/L.

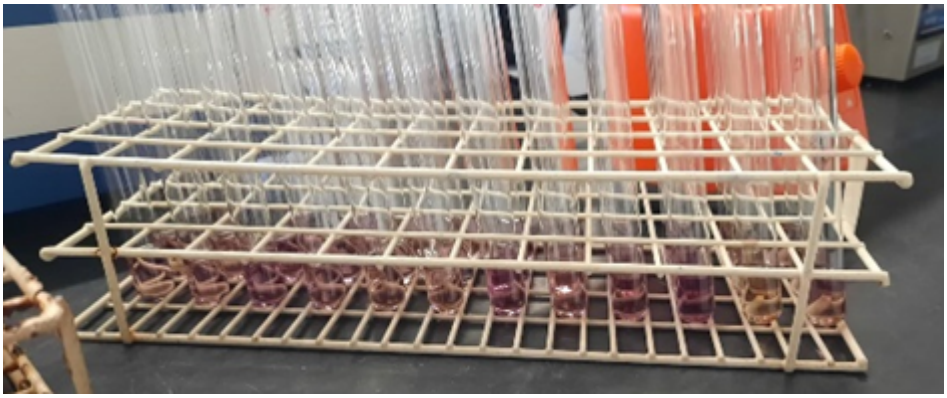


Figure 12. DPPH assays.

2.4.5. Ferric reducing antioxidant power

The FRAP assay was conducted in accordance with Berker et al., (2007). The FRAP reagent was prepared by mixing 0.3 M CH3COOH/CH3COONa buffer solution at pH 3.6, 1.0×10^{-2} M of TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) and 2.0×10^{-2} M of $FeCl_3\cdot6H_2O$ in the ratio 10:1:1. For a volume of 0.1 ml of the extract, 3 ml of the FRAP reagent was added and the mixture was left at rest in the dark for 6 min at ambient temperature. The absorbance was measured in comparison to a blank at a wavelength of 595 nm. The calibration curve was prepared using standard solutions of the compound ammonium iron (II) sulphate hexahydrate ($(NH_4)_2Fe(SO_4)_2\cdot6H_2O$) with concentrations between 25 and 350 mg/L.

2.4.6. Antimicrobial activity

Antibacterial capacity was assessed according to the microplate method described by Molla et al. (2016). One strain of Gram-negative bacteria (*Escherichia coli*) and a strain of Gram-positive bacteria (*Staphylococcus aureus*) were used.

Initially, the bacterial strains were inoculated on a sterile nutrient agar plate and incubated at 37 ºC, for 24 h. Subsequently, prior to the assay, bacterial strains were sub cultured into "overnight" nutrient broth.

Tramazeira extract (2.58 g/mL in 25% of ETOH) was diluted using the serial microdilution method in nutrient broth (100 µL). To determine the minimum inhibitory concentration (MIC), 30 µL of 0.01% of rezasurin was used. Then, 20 µL of bacterium, MacFarland scale (1×10^8

cfu/ml) was inoculated. The experiments were carried out in triplicate. Ethanol was used as a negative control. The plate was incubated at 37 ºC, for 24 h (Figure 13).

To evaluate the minimum bactericidal concentration (MBC), 20 µL of bacterial growth negative wells were seeded by plating on nutrient agar containing plates incubated at 37 ºC, for 24 h. The result with growth below 10 colonies was considered bactericidal and, more than 10 colonies were considered bacteriostatic.

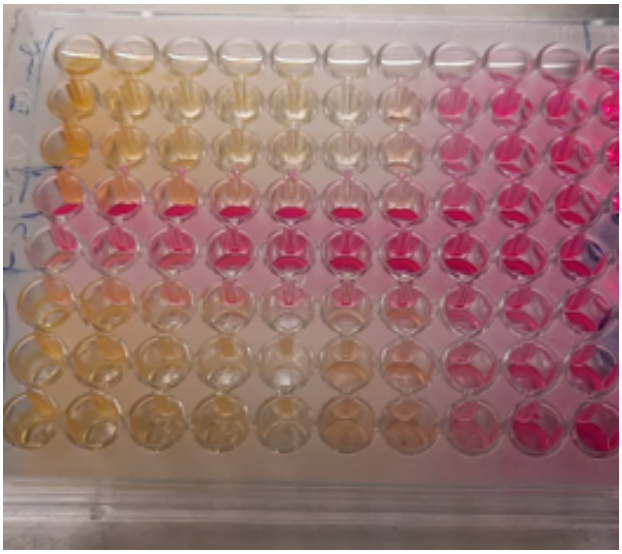


Figure 13. MicroPlate assays for the Tramazeira extract.

2.5. Addition of Tramazeira extracts to the orange juice

Several food industries are looking for natural preservatives and antioxidants and antimicrobial agents due to the increasing increase in drug-resistant multi-organisms.

For evaluated the capacity of the extract of Tramazeira as a preservative, two concentrations of extract were used and the shelf life of juices with and without addition of extract was evaluated. The extract obtained for sample number 2, for being the one with the highest content of bioactive properties, was used as a natural additive, being introduced in fresh orange juice at concentrations of 0.04 g/mL and 0.08 g/mL in order to test its preservative power through microbial growth (Figure 14).

Figure 14. Orange juice assays without (J1) and with additive (J2 and J3).

2.5.1. Sample preparation and microbiological analysis

Three different orange juice were prepared, one without Tramazeira extract, one with 0.04 g/mL of extract and other, with 0.08 g/mL. Aliquots of 10 mL of each orange juice were transferred into sterile bags, containing 90 mL of buffer peptone water (Himedia), and the mixture was homogenized. All analyses were performed in duplicate.

Yeast and fungi: The enumeration of yeasts and fungi was carried out on the Rose Bengal agar with chloramphenicol (Biolife) (ISO 21527-1:2008). Aliquots of 100 µl of the decimal dilutions were inoculated by surface spreading and incubated at 25ºC, 48 h for yeasts until 5 days for filamentous fungi. The results can be seen in Figure 15 and they were expressed in colony-forming units per milliliter (CFU/mL).

Total Mesophilic bacteria: The enumeration of total mesophilic bacteria was carried out on Plate Count Agar (AppliChem Panreac) (NP 4405:2002), which were inoculated by incorporation, with 1 ml of each decimal dilution. The plates were incubated at 30°C for 48 to 72 h. Figure 16 shows the expected results, which are expressed in colony-forming units per milliliter of sample (CFU/mL).

Staphylococcus aureus: Baird-Parker Agar Base (VWR chemicals) was supplemented with egg yolk tellurite (Himedia) and 100 µl of serial decimal dilution was spread on the surface of

the medium and incubated at 37 ºC, for 24 to 48 h. Subsequently, confirmation of 5 characteristic (clear halo around the colony) and non-characteristic colonies was done in test tubes 5ml of Brain Heart Infusion (Applichem) incubate at 24 hours in 37°C. The coagulase tube test with rabbit plasma and examination of tubes after incubation for 6 hours is the standard test for routine identification of S. aureus. *S aureus* coagulase positive was considered when a clot formed at the bottom of the tube (NP 4400-1: 2002). The visual aspect can be seen in Figure 17. The results were expressed as colony-forming units per milliliter of sample (CFU/mL).

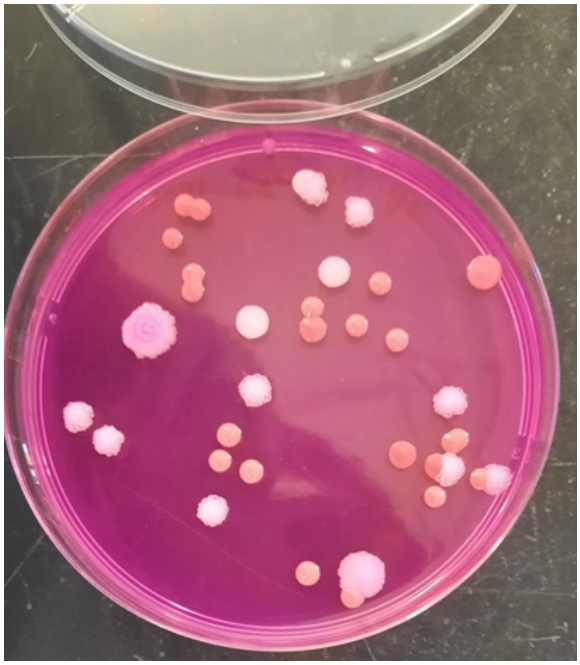


Figure 15. Yeast and fungi detection using the Rose Bengal as medium.

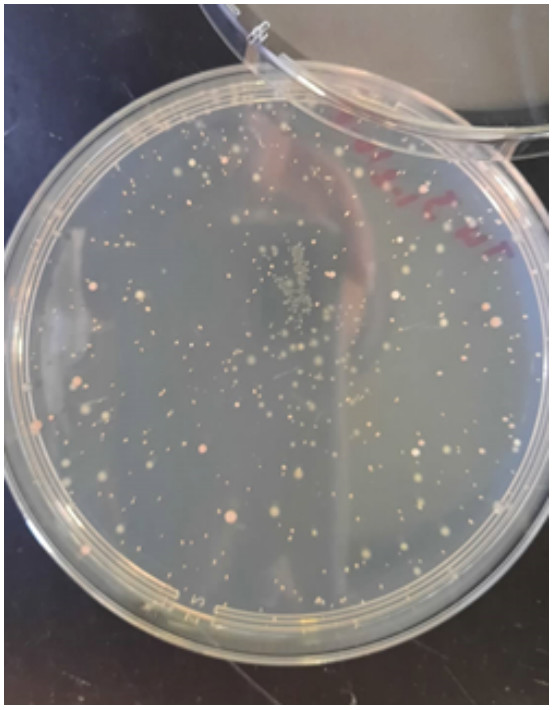


Figure 16. Total Mesophilic bacteria detection using the Plate Count Agar (PCA) as medium.

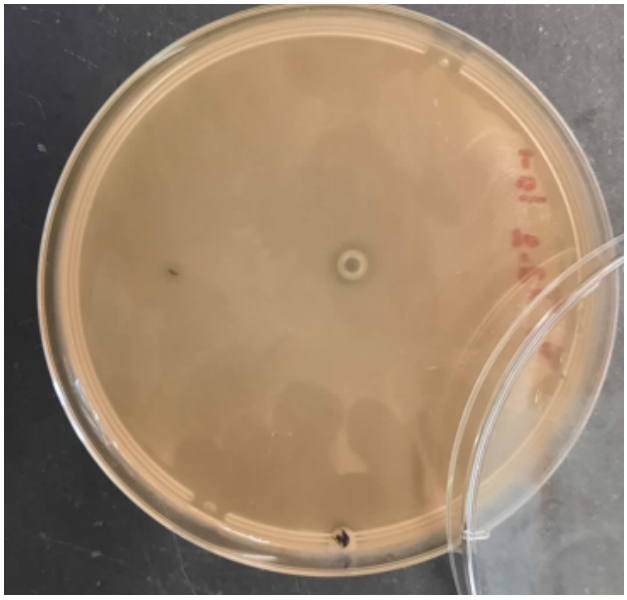


Figure 17. *Staphylococcus aureus* detection using the Baird Parker (BP) as medium.

Total Enterobacteriaceae: Total Enterobacteriaceae populations were enumerated using the simpPlate kit (Biocontrol®, AOAC, 2012) method, which is used for the detection and quantification of aerobics and facultative anaerobes microorganism. Into the rehydrated medium with 9 mL of sterile distilled water, 1 mL of the suspension was inoculated, following

the manufacturer instructions. The content was poured into the center of simPlate and gently swirled to distribute the sample into all the wells. Finally, the simPlate was incubated for 24 hours, at 37 °C. The results (Figure 18) were related according to the table provided by the manufacturer (AOAC, 2012).

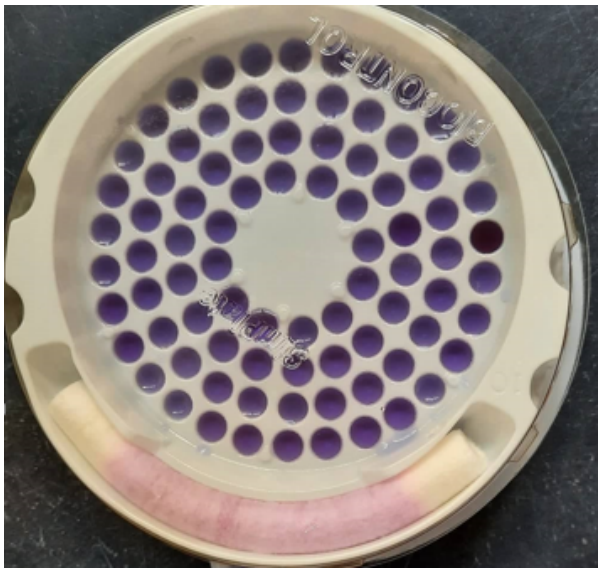


Figure 18. SimPlate assays for the detection of Enterobacteriaceae.

Determination of sulphite reducing clostridium spores: For sulphite-reducing clostridia counting (ISO 15213:2003), 1 mL of the decimal dilutions were added to an empty tube, thermally treated at 80 ºC for 10 min and covered with Iron Sulphite Agar (Liofilchem), being incubated at 37 ºC, for 5 days (Figure 19). At the end, it was counted the black colonies. Results were expressed in colony forming units per milliliter of sample (CFU/mL).

Figure 19. Clostridium sulphite reducing spores detection using Iron sulphite agar as medium.

2.6. Statistical analysis

In the study of the optimization conditions for the extraction of phenolic compounds from Tramazeira fruits, a response surface methodology (RSM) was applied. The R package rsm (allowed to generate a standard design with two factors: pH and ethanol %) was used to fit a second-degree polynomial model and also to test the meaning of their interaction.

Models validation (Maroco, 2007) was performed by checking:

- randomness and normality of the residuals;

- Cook's distance (values greater than 1 are indicative that these are excessively influential in the model);

- leverage values (values below 0.2 are acceptable, values between 0.2 and 0.5 are risky and values higher than 0.5 indicate the presence of an influential value or outlier);

- model's p-value (to evaluate the significance of the model obtained using the significance level of 0.05);

- determination coefficient value (R^2, to verify the amount of variance explained by the model);

- relative standard error (RSE, to confirm the magnitude of the model errors).

To evaluate the predictive capacity of a model, a simple linear regression model was established between the concentrations predicted by the model and the experimental values. The results are considered satisfactory if the linear regression parameters are close to the theoretical values (Roig et al., 2003): “zero” (0) for root square error (RSE) and intercept; “one” (1) for slope and the determination coefficient (R^2).

3. Results and discussions

3.1. HPLC results

3.1.1. Chromatograms

Figure 20 shows examples of HPLC chromatograms obtained for the Tramazeira fruit sample (number 4) and for two mixed standard calibration solutions visualized in the UV and RI detectors, with different concentrations of glucose, fructose, malic acid and sorbitol (the one with the lowest concentration corresponds to the 10-fold dilution of the most concentrated).

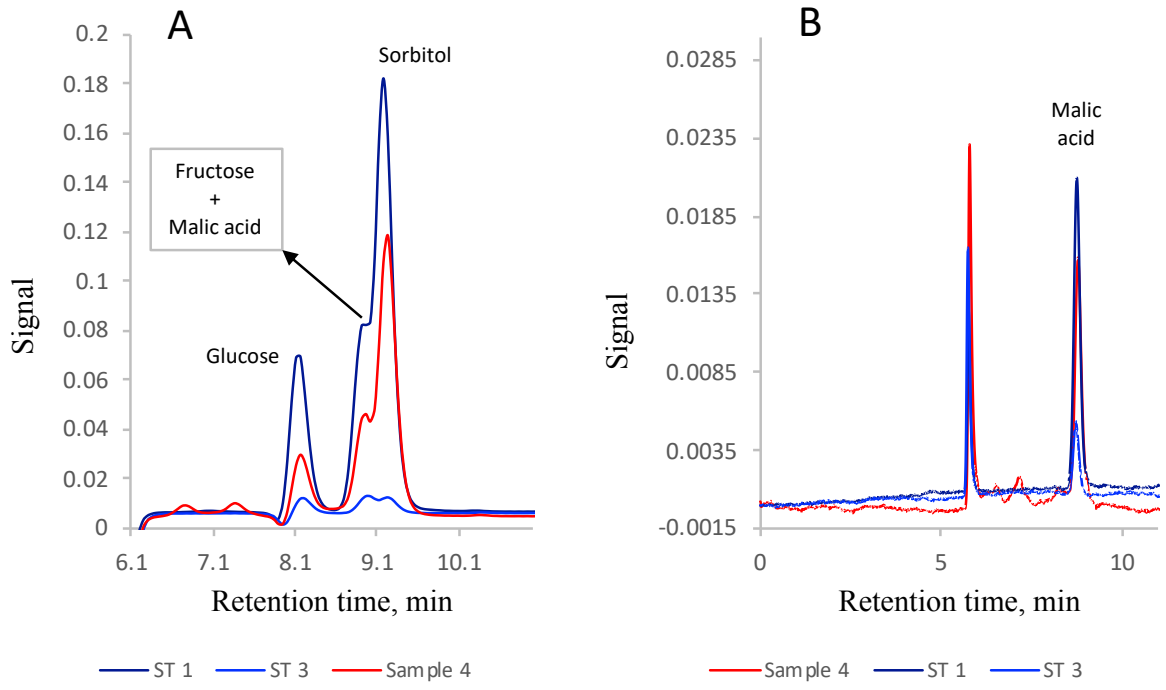


Figure 20. Chromatograph of the sample 4 with the two standard (ST1 and ST3) showing in the UV (A) and RI (B) detectors. ST1 had 0.50 g of glucose, 0.26 g of fructose, 0.50 g of malic acid and 0.42 g of sorbitol. ST3 had 0.050 g of glucose, 0.026 g of fructose, 0.050 g of malic acid and 0.042 g of sorbitol.

The sample's chromatogram presents 3 different peaks in the UV detector, being the second peak an overlap of two compounds (fructose and malic acid). In the RI detector, the first peak was attributed to the solvent and the second, to malic acid.

3.1.2. Linearity

Linearity was demonstrated by the linear relation between peaks areas and the respective concentrations (Figure 21) for the compounds of glucose and sorbitol, measured by the RI detector, and malic acid, measured by UV detector. The concentrations used in calibration were in the range of: 0.025 g/L to 0.401 g/L for malic acid; 0.025 g/L to 0.60 g/L for glucose; 0.025 g/L to 1.50 g/L for sorbitol; 0.035 g/L to 0.35 g/L for fructose.

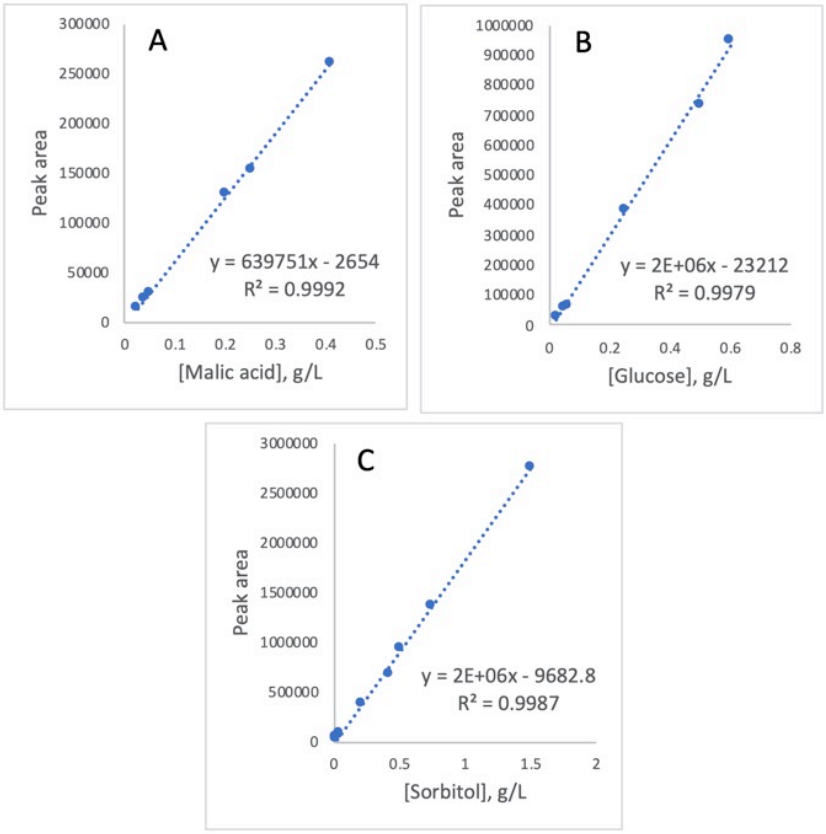


Figure 21. HPLC calibration of (A) malic acid, (B) glucose and (C) sorbitol.

The calibration results are presented in the Table 4, as well the limits of detection and quantification, which were calculated with the linear regression parameters. All models were significant (p-value < 0.001) and presented correlation coefficients higher than 0.998.

Table 4. HPLC calibration results of malic acid, glucose and sorbitol analysis. *

Compounds	Detector	Slope	Intercept	R	LD, mg/L	LQ, mg/L
Malic acid	RI	639751 (±9192)	-2654 (±1970)	0.9996	0.010	0.031
Glucose	UV	1593679 (±32992)	-23212 (±12734)	0.9989	0.026	0.080
Sorbitol	UV	1825639 (±24947)	-9683 (±15106)	0.9993	0.027	0.083

*) R – correlation coefficient; LD – limit of detection; LQ – limit of quantification; UV – ultra-violet; RI – refractive index.

For the analysis of fructose, it is necessary to consider the peak area of fructose with the additive influence of malic acid, which is analysed by the UV detector, using the following equation:

$$\text{Peak area} = 1218655\ (\pm 41882)*[\text{malic acid}] + 1532143\ (\pm 33852)*[\text{fructose}]$$

Rearranging the equation, the fructose concentration was calculated by the equation:

$$[\text{Fructose}] = (\text{Peak area} - 1218655*[\text{malic acid}])/1532143,$$

where the malic acid concentration was determined using its calibration in the RI detector. This model allowed to explain 99,92% of the data variability (R=0.9996), being a significant model (p-value < 0.001). These results are in accordance to the ones obtained in the work of Paula et al. (2017).

3.1.3. Sample analysis

Table 5 shows the measured concentrations results of the 4 compounds, analysed by HPLC, in the five collected samples, as well the ANOVA results that was used verify which sample mean values where statistically different (same letters in each column represent means that were not different statistically at significant levels of 0.05). Overall, malic acid determinations showed percentage relative standard deviations (RSD %) between 0.5 to 6.9%; glucose, between 0.9 to 6.5%; sorbitol, ranged between 0.4 and 11.8%; and, fructose between 4.4 and 14.9%. These last

two high RSD % values can be attributed to the overlap between the fructose + malic acid and sorbitol peaks, introducing greater analytical errors. Figure 22 presents the results from HPLC analysis for the five samples, which are ordered by the increasing concentration of glucose.

Overall, sample 5 is the least matured due to its low levels of sugars (by tasting, matured fruit showed to be sweeter; Carmichael, 2011), followed by sample 1. The three other samples (2, 3 and 4) are the most matured since they present similar (at significant level 0.05) high content of sugars (glucose and fructose), which is also repeated with malic acid and sorbitol concentrations. Sample 1 showed similar concentration of malic acid as the sample 5 and different concentration of glucose and sorbitol in all the samples, but similar concentration of fructose as the samples 2, 3, and 4. The Figure 22 shows that the values for sugars and malic acids had the same behaviour. However, the sorbitol results showed a not expected behaviour between the sample 5 and 1 (respectively, 112 and 61 g/100g of sample) since there was not increase or decrease tendency.

Table 5. The concentration of the four compounds in the five samples.

Sample	Malic Acid (g/100g)	Glucose (g/100g)	Sorbitol (g/100g)	Fructose (g/100g)
1	8,73 (±0.05) b	17,7 (±0.8) b	61,5 (±0.2) c	11,0 (±0.7) a
2	17,1 (±0.8) a	22,5 (±0.4) a	81,6 (±9.6) b	13,6 (±2.0) a
3	15,2 (±0.8) a	22,2 (±0.2) a	87,8 (±3.8) b	11,7 (±0.8) a
4	15,1 (±1.0) a	22,2 (±1.4) a	85,8 (±7.5) b	15,2 (±1.3) a
5	8,97 (±0.4) b	3,2 (±0.1) c	111,7 (±3.3) a	3,3 (±0.1) b

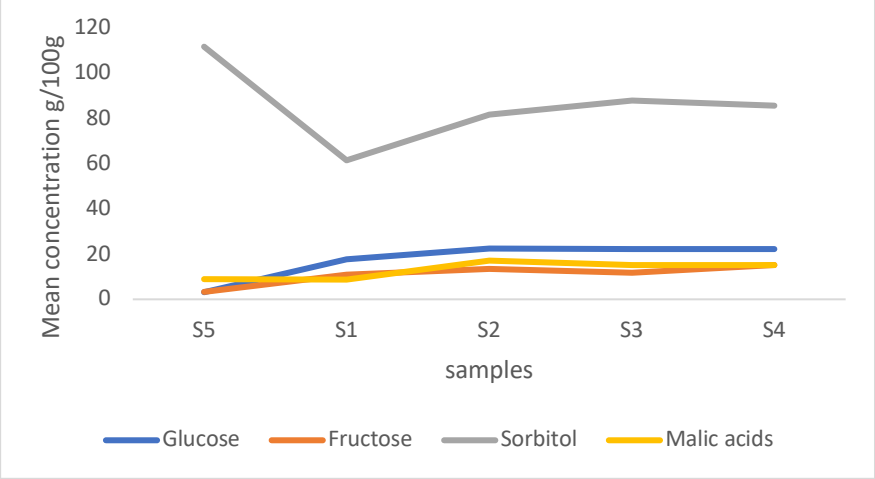


Figure 22. Plot of the concentration compounds in the five samples.

3.2. UV spectra results

3.2.1. Calibration with mixed standard solutions

Figure 23 shows all the UV spectra for the mixed standard calibration solutions obtained in the wavelength range of 200 to 450 nm. These 25 spectra allowed to define the following calibration equations of THBA, THCA, TF and TP contents (Paula et al., 2017):

$$[THCA] = 130 \times ABS_{325nm} - 81 \times ABS_{380nm}$$

$$[THBA] = 180 \times ABS_{275nm} - 141 \times ABS_{345nm}$$

$$[TF] = 178 \times ABS_{375nm}$$

$$[TP] = 66 \times ABS_{220nm} - 168 \times ABS_{345nm}$$

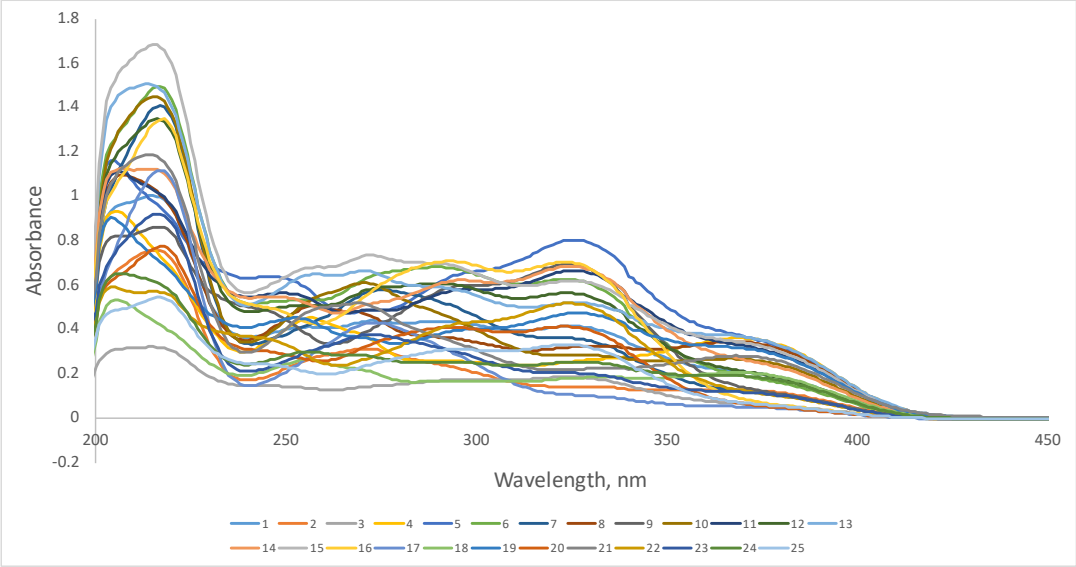


Figure 23. Spectra of the mixed standard calibration solutions of gallic acid, ferulic acid and quercetin.

In Table 6 is presented the determination coefficients, p-values and relative standard error of each obtained model. All these models presented correlation coefficients higher than 0.998 and p-values lower than 0.001, being all significant. The dynamic interval for these calibrations that used five levels of concentration of each standard was: 10 to 80 mg/L of gallic acid; 10 to 80 mg/L of ferulic acid; and, 10 to 60 mg/L of quercetin. All models were tested for the importance of the intercept in the equation, being all not significant.

Table 6. Results of the fitted models.

Compounds	R^2	P-value	RSE% *
Gallic acid	0.996	<0.001	2.89
ferulic acid	0.998	<0.001	2.89
Quercetin	0.995	<0.001	3.05
TP	0.998	<0.001	6

*) RSE % - percentage relative standard error.

The RES % for the models were low (RSE % <6.0%) but it should be noticed that and the RSE% for TP content was two times higher than the others.

3.2.2. Phenolic compounds extraction optimization

The optimization of the best analytical conditions for the extraction of 3 groups of compounds (THBA, THCA and TF contents), contributing also for the high levels of TP content, was studied based on the RSM, using two samples (sample 3 and sample 5, having different stages of maturation considering the fruit colour). A CCD was applied with two factors: ethanol percentage between 24.6 to 95.4% and pH of the aqueous HCl solution between 0.9 and 5.1. Figure 24 presents the CCD used with code values for each factor, with 3 center points.

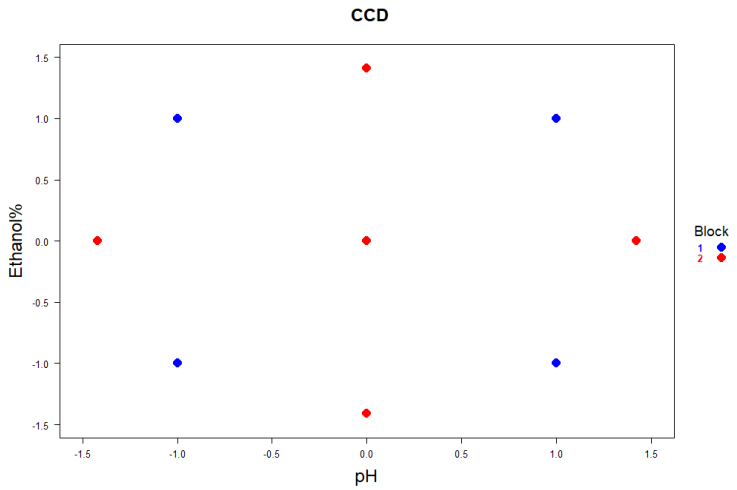


Figure 24. CCD with factors ethanol % and pH in the study of samples extraction.

Th pH values present the level -1 = 1.5 and level +1 = 4.5 and the factor ethanol %, the level -1= 35% and level +1= 85%. The star design presented extremes pH values of 0.88 and 5.12 and for the ethanol %, 24.6% and 95.4%.

As an example, Figure 25 shows all spectra obtained from the CCD assays using the sample 3. The spectrum was obtained from the spectrum solution prepared as described by Obied et al. (2005) methodology. The concentration levels obtained from the CCD assays are presented in Table 7.

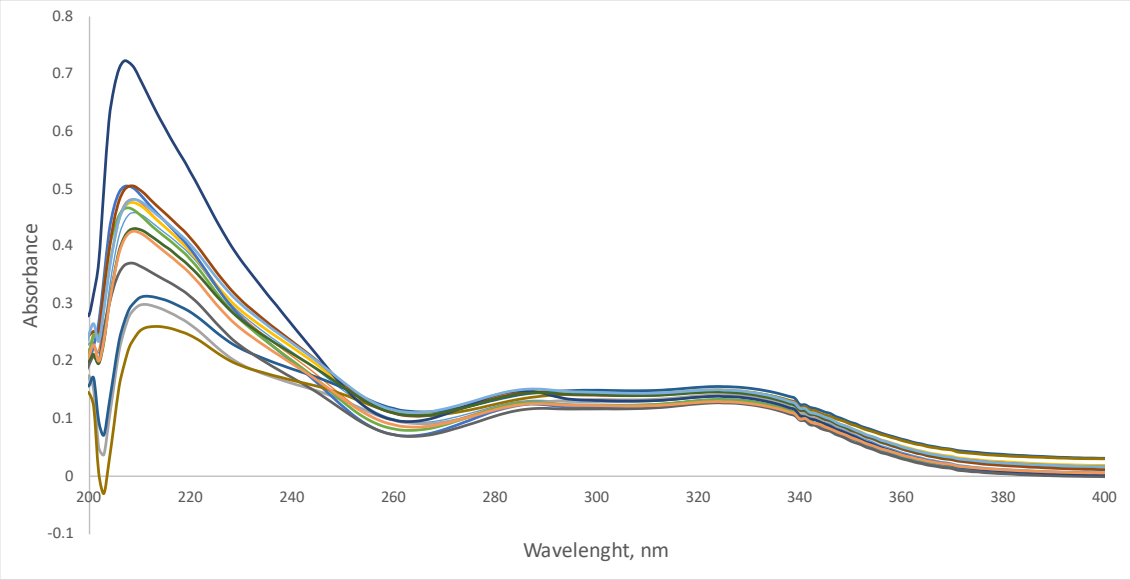


Figure 25. Spectra of all CCD assays for sample 3 of *Sorbus aucuparia L.* fruits.

The UV spectra of CCD extract assays did not have absorbance higher than 0.70.

Table 7. Concentration levels of THBA, THCA, TF and TP compounds in fruit extracts from *Sorbus aucuparia L.*

Parameters *	Concentration range
Hydroxybenzoic acids group, mg eq GA/L	[3.9; 9.7]
Hydroxycinnamic acids group, mg eq FA/L	[14.4; 18.1]
Flavonoids group, mg QC/L	[0.4; 7.9]
Total phenolic, mg TP/L	[29.8; 50.5]

*) GA – gallic acid; FA – ferulic acid; QC – quercetin; TP – total phenolic; eq – equivalent.

The results presented in Table 7 were used as responses in the RSM data treatment, as described in the following sections.

3.2.3. Flavonoids

The Table 8 shows the RSM results by fitting the response FT content with the two factors used in CCD (pH and ethanol %) for the sample number 3 and 5. For both samples the models were significant (p-values < 0.001) and presented R^2 values higher than 90%. Both models showed dependency only in the ethanol % factor but, with significant ethanol % squared term for the sample 3. However, the interaction term between the two factors was not significant in the model.

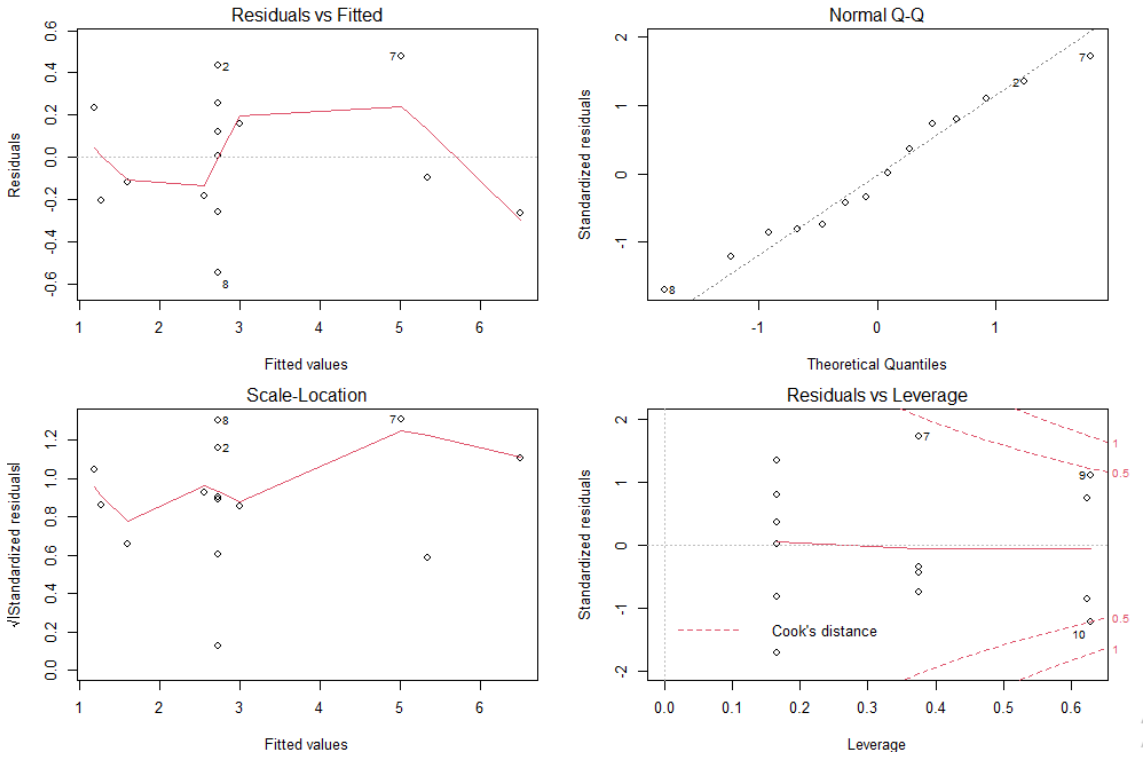
Table 8. Final model of TF content for the samples 3 and 5.

Response	Coefficient (±error)	p-value	R^2 model	p-value model
		Sample 3		
Intercept	2.74 (±0.14)	<0.001***	96.51%	<0.001***
pH	0.16 (±0.12)	0.226		
Ethanol %	1.87 (±0.12)	<0.001 ***		
pH^2	0.02 (±0.13)	0.865		
Ethanol $\%^2$	0.55 (±0.13)	<0.001 ***		
		Sample 5		
Intercept	3.00 (±0.22)	<0.001***	90.65%	<0.001***
pH	0.09 (±0.19)	0.648		
Ethanol %	1.71 (±0.19)	<0.001 ***		
pH^2	0.09 (±0.19)	0.642		
Ethanol $\%^2$	0.31 (±0.19)	0.139		

*) 0 – "***" – 0.001 – "**" – 0.01 – "*" – 0.05

The Figure 26 exhibits four plots for two samples that allows to validate the models. The plots revealed that the model's residues were random, following a normal distribution, which confirms the model fitting suitability to the data. The Cook's distance was lower than 0.5 for the sample number 3, indicative that there were no values excessively influential in the model. But this was not the case for the sample number 5 since, it has one value greater than 1, indicative of an influential data in the estimation of coefficients regression.

Sample 3



Sample 5

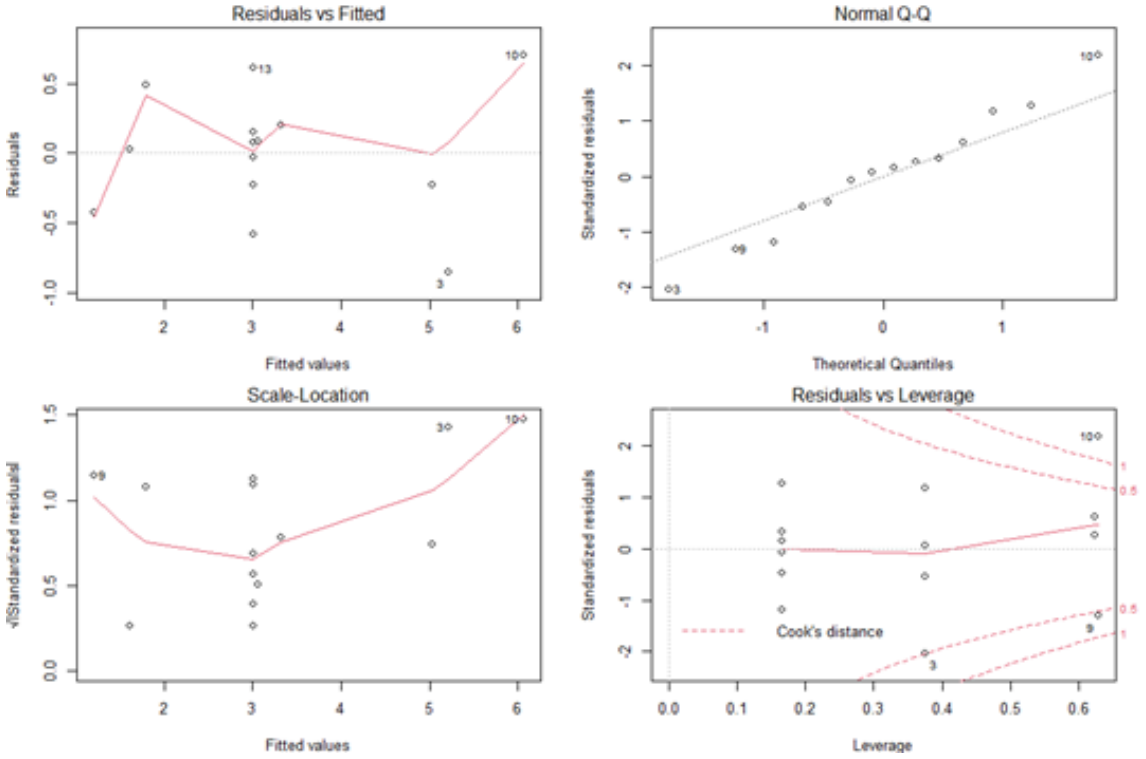


Figure 26. Plots of TF response model's validation for the samples 3 and 5.

In the case of the leverage values assessment, it was found results above 0.5 for the sample 5 which indicate the presence of influential value or an outlier. For this sample, more testing should be done to reduce model errors.

The Figure 27 presented the 3D response surfaces and 2D contour plots of TF content obtained from the RSM analysis, for the samples 3 and 5. These plots are representations of the regression equation between the response and the extract conditions and are the most useful approach in revealing their dependency.

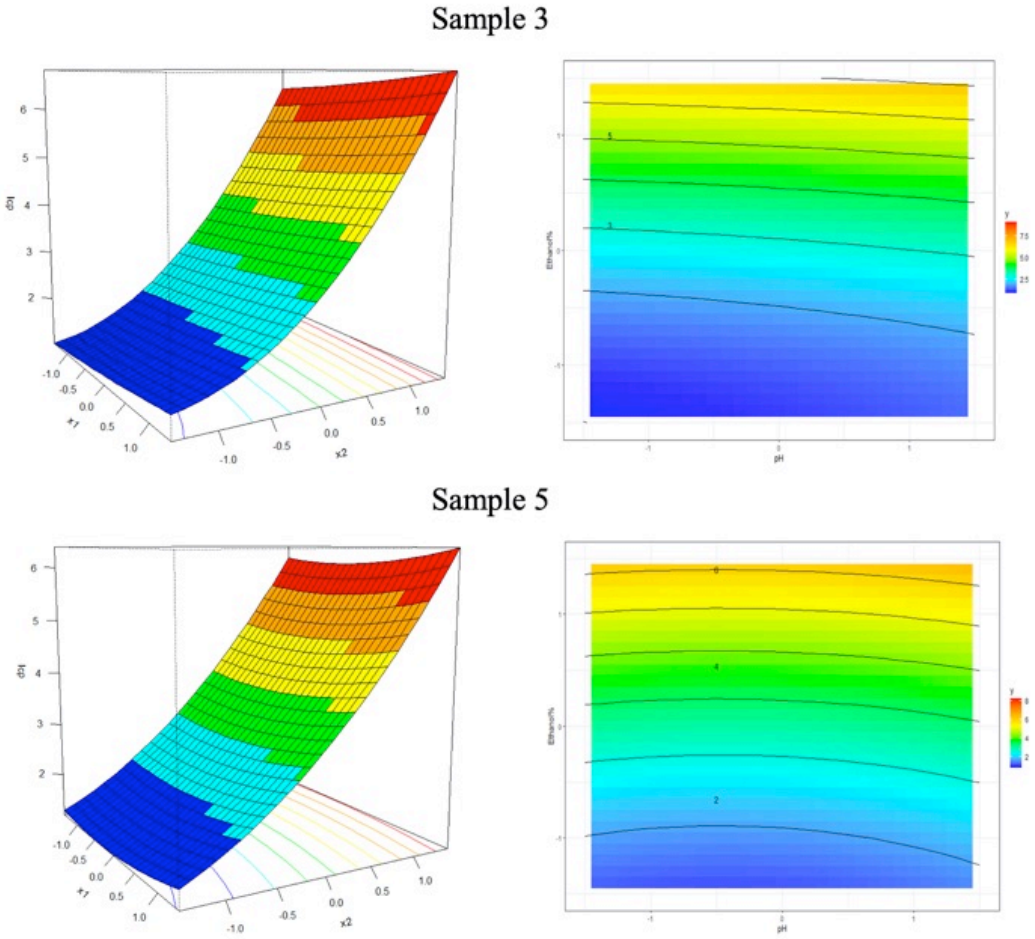


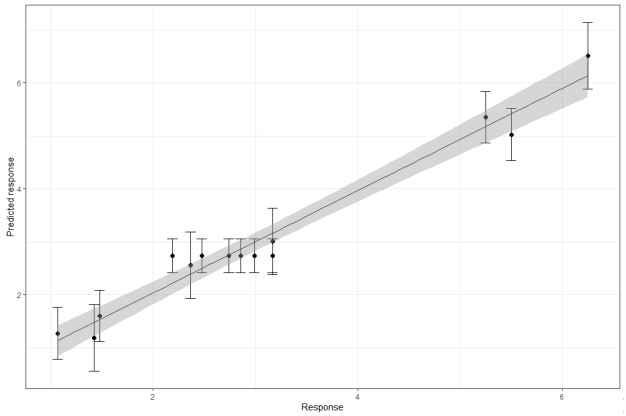
Figure 27. 3D surface and 2D contour plots of TF response for the samples 3 and 5.

The RSM results showed that flavonoids extraction from Tramazeira pulp in both samples was favoured at high levels of ethanol % (level higher than 1 means ethanol % higher than 85 %) and pH was not significant for the two samples. Figure 27 shows that the rate of flavonoids extract increases with increasing of ethanol % in both samples. So, it is clear that the optimal

point for flavonoids extraction would have ethanol% higher than 80%, for any pH value within the range of pH used.

In order to verify model's quality, Figure 28 shows the comparison establish between the model's predicted response and the experimental results for the flavonoid compounds. Both plots showed a straight line with slopes of 1.12 (±0.03) and 1.17 (±0.04), respectively, for the sample 3 and 5. These values are nearby 1 (theoretical expected value) and intercept was not significant, being considered as zero. In this linear relation, samples 3 and 5 presented correlation coefficients higher than 0.99 (0.995 and 0.991, respectively). Overall, these results showed that the obtained models allowed to predict correctly the total flavonoids content.

Sample 3



Sample 5

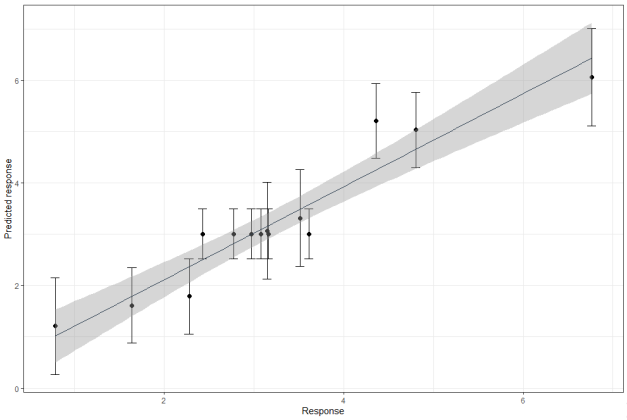


Figure 28. Predicted response for the TF content of the samples 3 and 5.

3.2.4. Hydroxybenzoic acids

The Table 9 shows the RSM results obtained using the concentrations of THBA as response for the two samples. The models were all significant (p-values < 0.001) and explained 91.95% of data variability present in the results for sample 3 and 96.56% for the sample 5. For both samples the extraction is dependent on the pH and ethanol % values but, in sample 3 the squared pH term was not significant, as it happened for sample 5.

Figure 29 allows to validate the obtained models since the plots confirm that the residuals show a random behavior and are distributed normally. However, there is some results with high Cook's distances and leverage values but did not affected significantly the results. This means that more assays should be done in order to reduce the experimental errors.

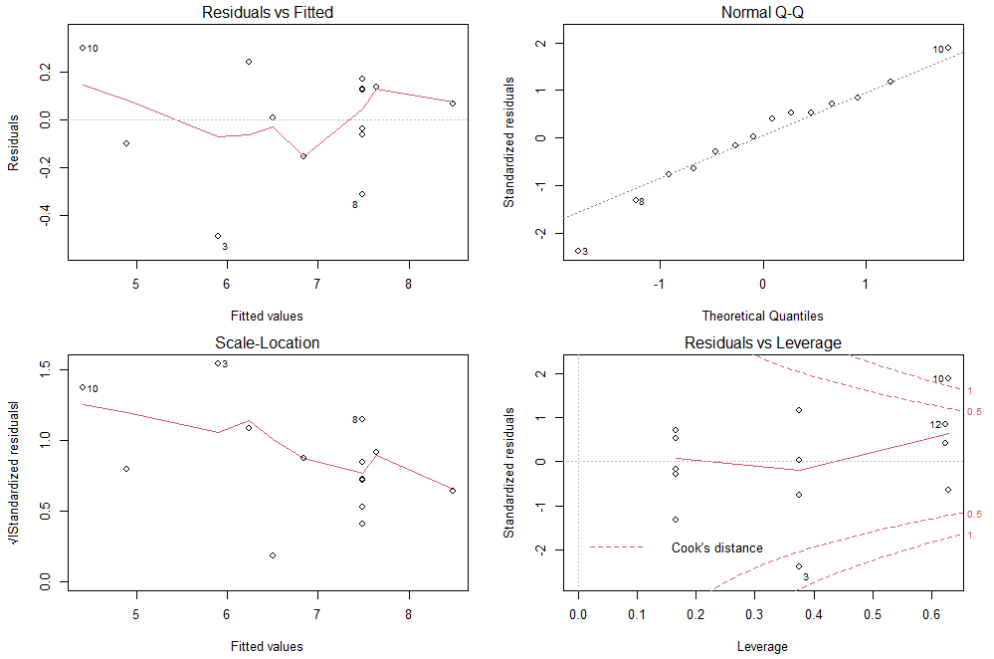
Table 9. Final model of THBA content for the samples 3 and 5.

Response	Coefficient (±error)	p-value	R^2 model	p-value model
		Sample 3		
Intercept	10.46 (±0.19)	<0.001***	91.95%	<0.001
pH	-0.39 (±0.17)	0.043 *		
Ethanol %	-0.59 (±0.17)	0.007 **		
pH^2	0.19 (±0.17)	0.304		
Ethanol $\%^2$	-1.56 (±0.17)	<0.001***		
		Sample 5		
Intercept	7.49 (±0.11)	<0.001***	96.56%	<0.001
pH	-0.30 (±0.1)	0.009 **		
Ethanol %	-0.17 (±0.1)	0.096 *		
pH^2	0.29 (±0.1)	0.014 *		
Ethanol $\%^2$	-1.41 (±0.1)	<0.001***		

*) 0 – "***" – 0.001 – "**" – 0.01 – "*" – 0.05

The response together with the two factors under study are represented in the Figure 30, as 3D surface and 2D contour plots. As can be seen, the optimal zone of extraction of hydroxybenzoic acids was similar for both samples, being the factor ethanol % the most influential. The middle zone of the surface and contour plots presents corresponds to the zone favored for the extraction of these compounds and, although with little influence of pH, it is favored at lower pH.

Sample 3



Sample 5

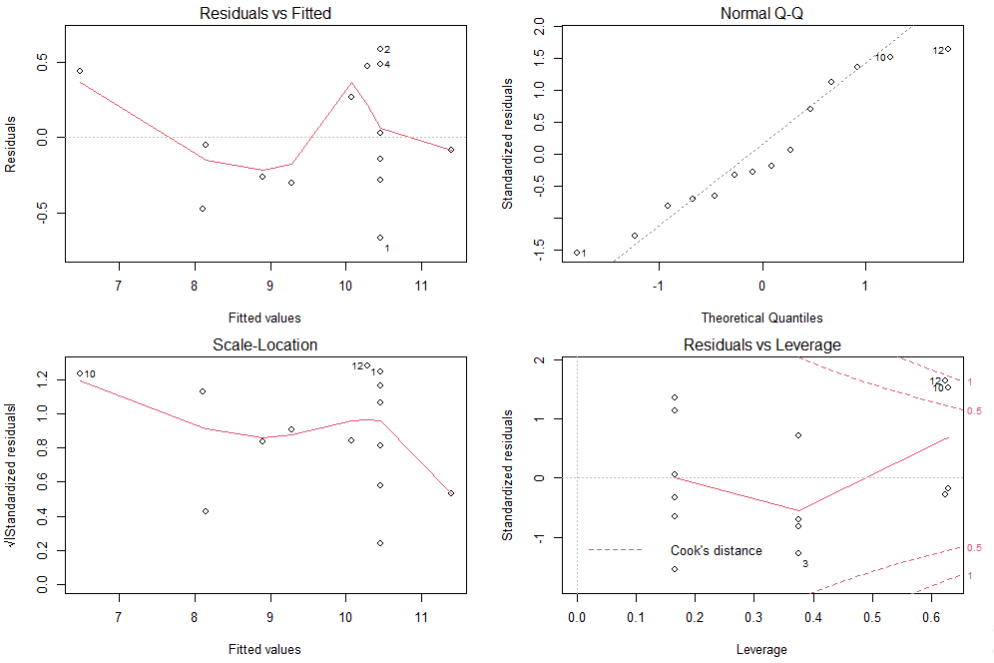
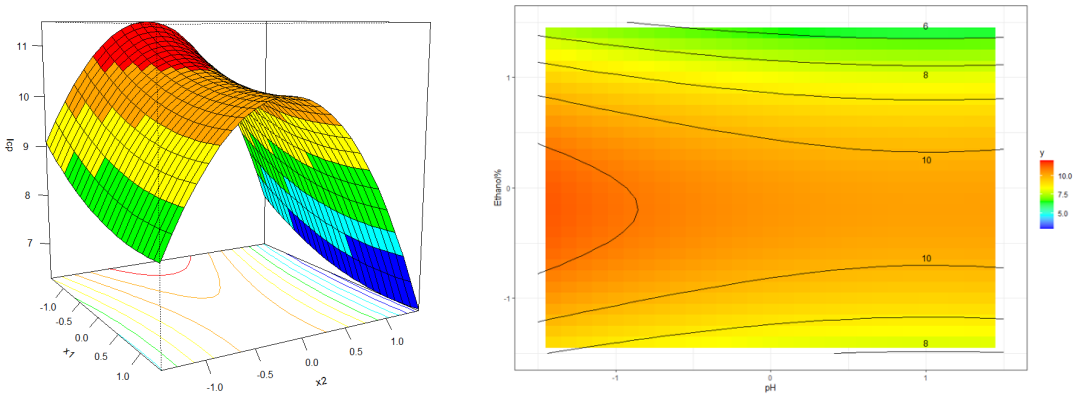


Figure 29. Plots of THBA response model's validation for the samples 3 and 5.

The Figure 31 bellow shows the satisfactory predicted response for both samples, where a straight line with slope 1.06 (±0.01) for sample 3 and 1.05 (±0.009) for sample 5, close to unity, were obtained. Also, both linear models had correlation coefficients higher than 0.99 and not

significant intercepts (therefore, considered zero value), meaning that the models allowed to predict correctly the THBA content.

Sample 3



Sample 5

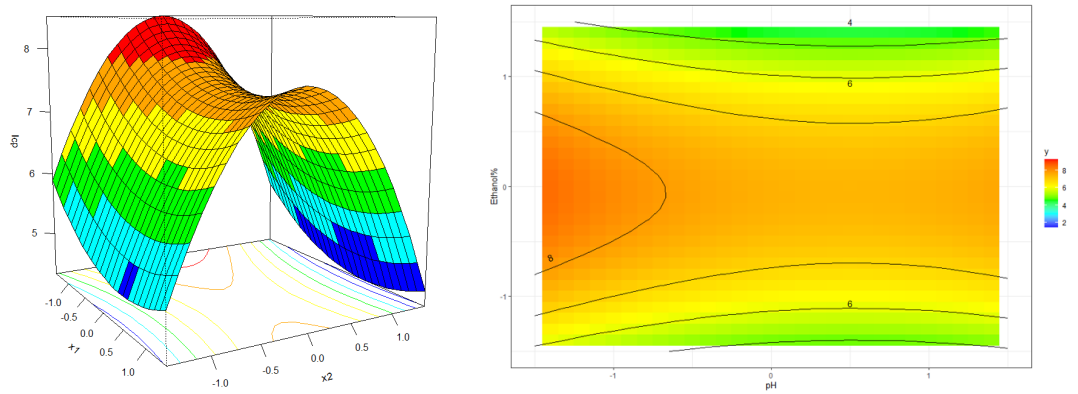


Figure 30. 3D surface and 2D contour plots of THBA response for the samples 3 and 5.

Sample 3 Sample 5

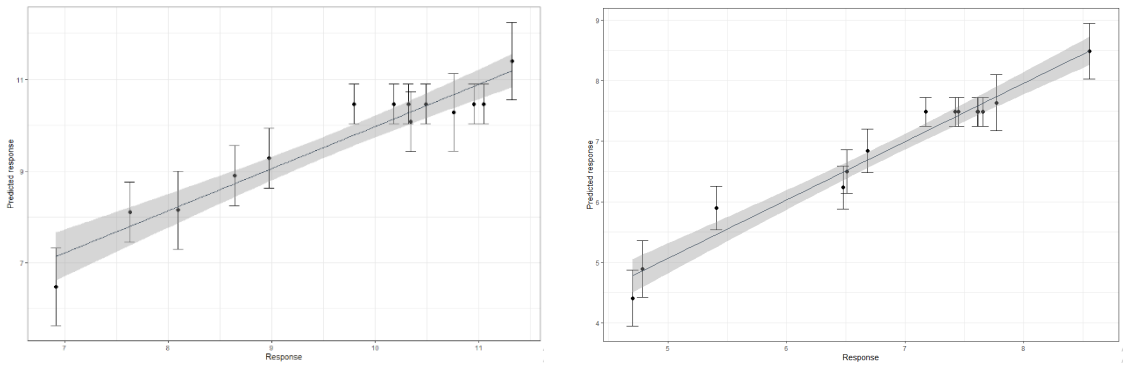


Figure 31. Predicted response for the THBA content for the samples 3 and 5.

3.2.5. Hydroxycinnamic acid

The Table 10 shows the RSM final results obtained using the concentrations of THCA as response for the two samples. The interaction term was not shown since it has no significance. The assays with sample 3 showed dependence on the term pH, but not in the sample 5. However, the squared ethanol % term was significant in both samples, but not its main effect term. The models were all significant (p-values <0.001) and had satisfactory determination coefficients, indicating that the models can explain more than 89% of the variability in the experimental data.

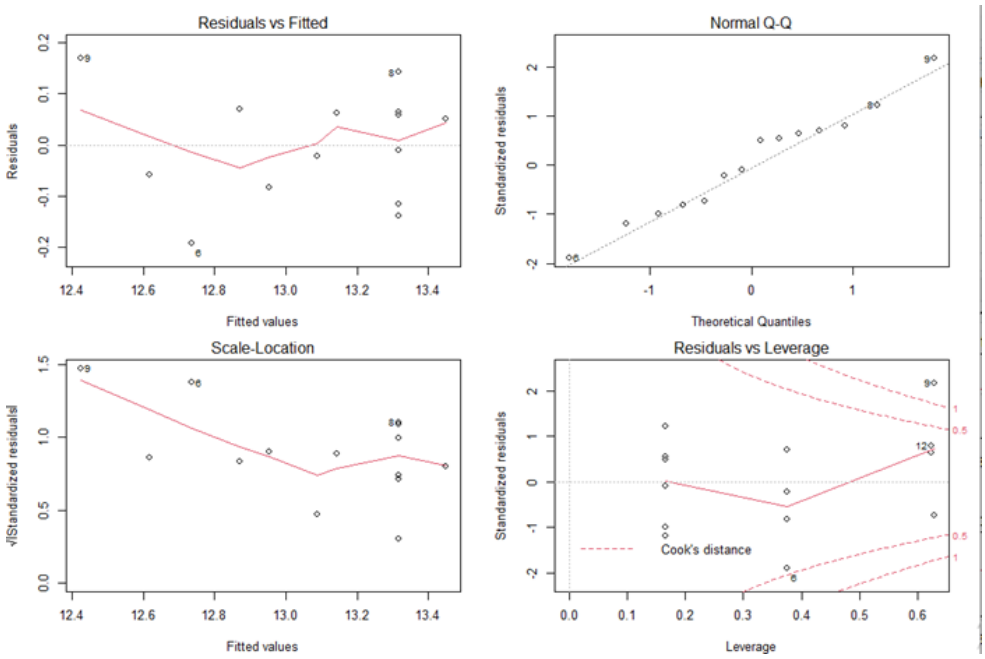
Table 10. Final model of THCA content for the samples 3 and 5.

Response	Coefficient (±error)	P-value	R^2 model	P-value model
		Sample 3		
Intercept	13.32 (±0.05)	<0.001***	89.82%	<0.001
pH	-0.11 (±0.05)	0.040		
Ethanol %	-0.07 (±0.05)	0.169		
pH^2	-0.01 (±0.05)	0.820		
Ethanol $\%^2$	-0.39 (±0.05)	<0.001***		
		Sample 5		
Intercept	17.32 (±0.09)	<0.001***	92.05%	<0.001
pH	-0.09 (±0.08)	0.279		
Ethanol %	-0.05 (±0.07)	0.501		
pH^2	-0.01 (±0.08)	0.877		
Ethanol $\%^2$	-0.82 (±0.08)	<0.001***		

*) 0 – "***" – 0.001 – "**" – 0.01 – "*" – 0.05

For THCA response model's validation, Figure 32 shows that the model for sample 3 had random residuals that followed a normal distribution, which confirms the model fitting suitability to the data. Only one result had Cook's distance greater than 1 and several with leverage values above 0.5, indicating the presence of an extreme results. However, considering the plot of predicted performance (Figure 33), where the errors of each test are shown, they were explained by having assays with high experimental errors.

Sample 3



Sample 5

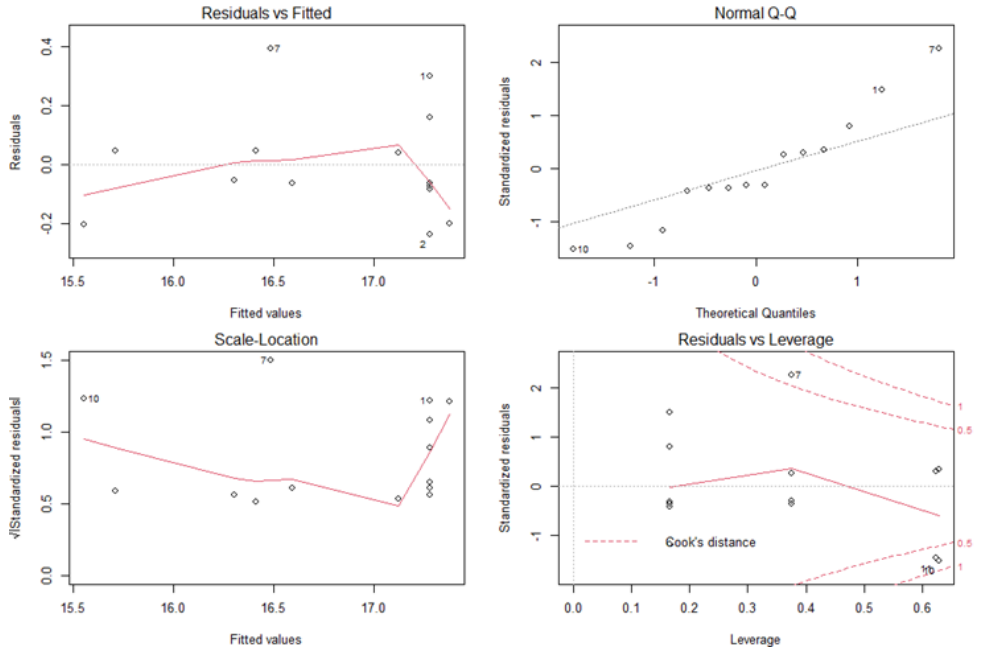


Figure 32. Plots of THCA response model's validation for the samples 3 and 5.

In sample 5, the residues were random but they showed problems in the normality plot, being satisfactory the values of Cook's distance. Again, the assays with high leverage values can also be explained by the plot prediction performance (Figure 33), where the measurement errors are shown to be high.

Sample 3 Sample 5

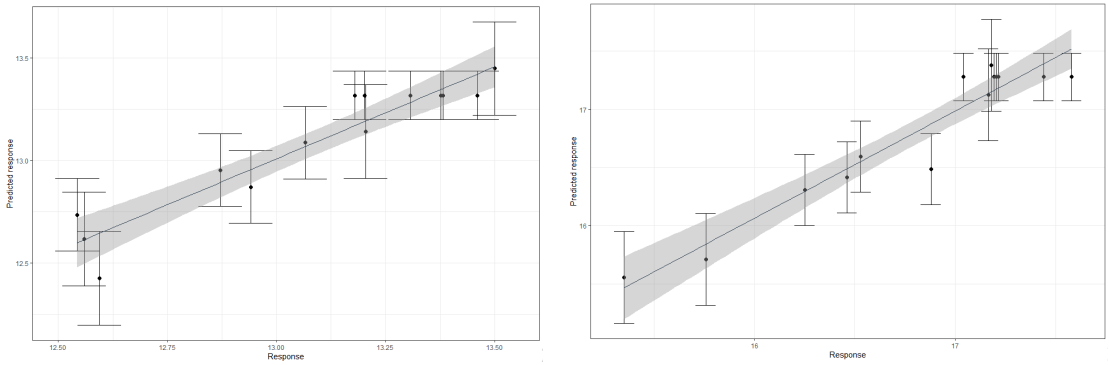
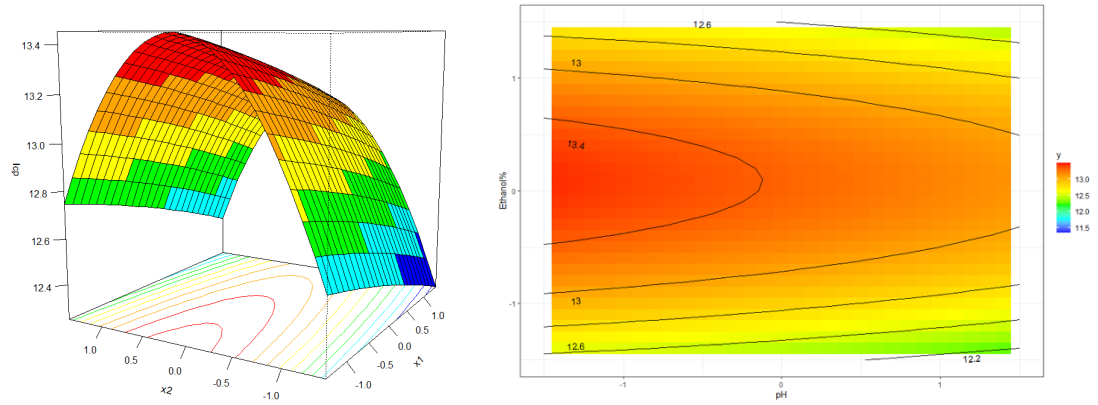


Figure 33. Predicted response for the THCA content of samples 3 and 5.

The 3D surface and 2D contour plots of THCA response for the two samples were present in Figure 34.

Sample 3



Sample 5

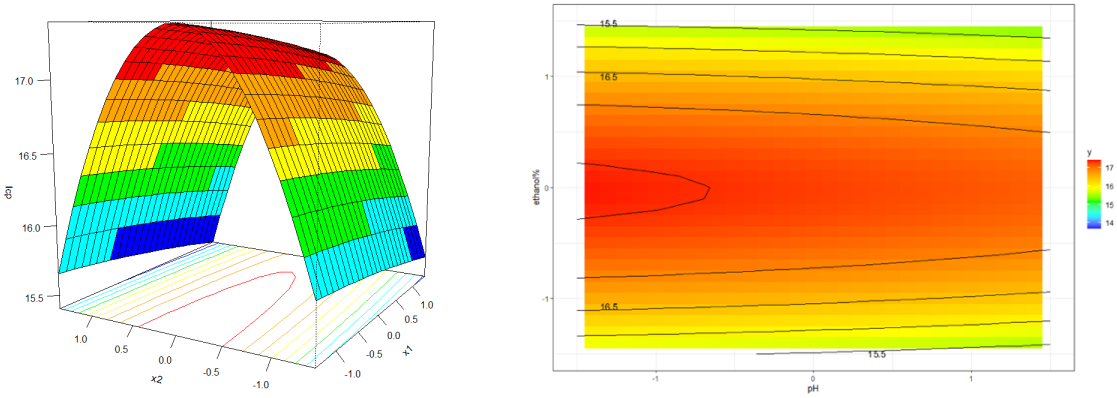


Figure 34. 3D surface and 2D contour plots of THCA response for samples 3 and 5.

The 3D surface and 2D contour plots of THCA response showed similar behavior to that obtained for the THBA response, where the middle zone in these plots was the optimal zone for extraction the hydroxycinnamic acids and, although with little influence of pH, it is favored at lower pH.

The overall results showed that the hydroxycinnamic acids content had higher experimental errors, but it should be noted that the levels obtained are close and therefore have less variability, which is reflected in the errors of the RSM model. However, Figure 33 showed that the values predicted by the models show a linear relationship with the experimental ones, whose slopes are close to unity: 1.01 (±0.002) for sample 3 (intercept = 13.32 (±0.05), not significant) and 1.02 (±0.003) for sample 5, with intercept= 17.32 (±0.09). The correlation coefficients were higher than 0.999, meaning that these models can predict correctly the THCA content.

3.2.6. Total phenolic compounds

Th RSM results from total phenolic compounds response are presented in Table 11, where the interaction term also has no significance.

Table 11. Final model of TP content for the samples 3 and 5.

Response	Coefficient (±error)	P-value	R^2 model	P-value model
		Sample 3		
Intercept	26.56 (±0.23)	<0.001***	90.26%	<0.001
pH	0.03 (±0.19)	0.872		
Ethanol %	-0.78 (±0.19)	0.003		
pH²	-0.32 (±0.21)	0.153		
Ethanol %²	-1.66 (±0.20)	<0.001***		
		Sample 5		
Intercept	3.00 (±0.22)	<0.001***	91.69%	<0.001
pH	-2.25 (±0.49)	0.001		
Ethanol %	-0.79 (±0.49)	0.144		
pH²	1.92 (±0.52)	0.005		
Ethanol %²	-3.87 (±0.51)	<0.001***		

*) 0 – "***" – 0.001 – "**" – 0.01 – "*" – 0.05

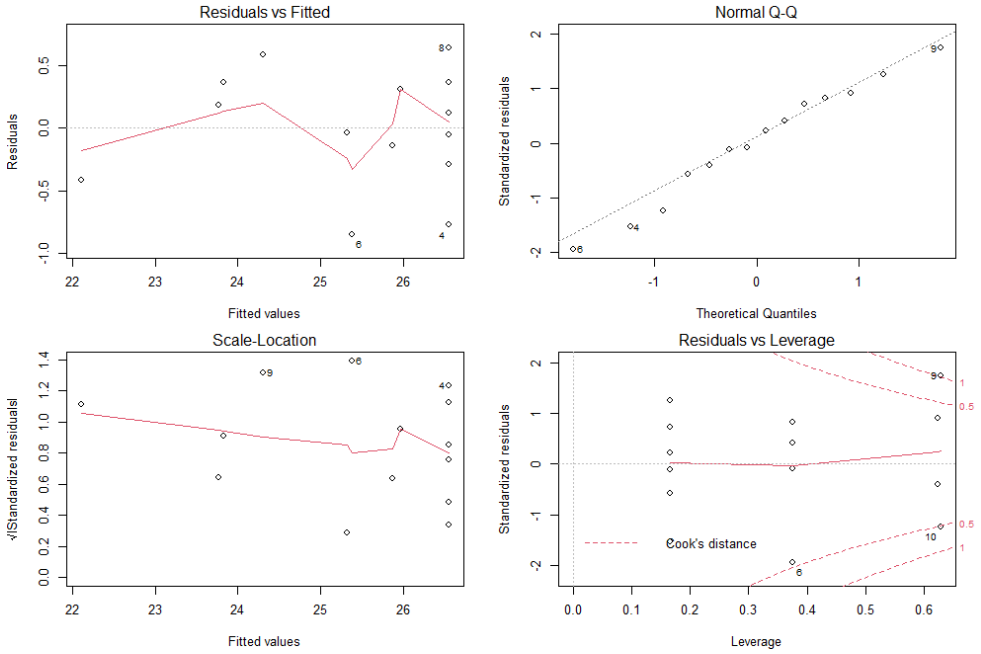
Table 11 shows different results for the two analysed samples, but representing significant models. The extraction of TP content in sample 3 is dependent on the main effect ethanol % term and the squared ethanol % term, allowing this model to explain 90 % of the variability of the data. However, in sample 5, the model includes the main effect pH term and the quadratic terms of pH and ethanol %, allowing to explain more than 91 % of the variability present in the data.

Representing the model's validation, the plots in Figure 35 indicate that the residuals had satisfactory randomness and normality and, although some leverage values were greater than 0.5, Cook's distance values were acceptable. The overall results confirmed the adequacy of the model in the adjustment of experimental data. The Figure 36 presents the plots of RSM model showing that for sample 3, TP content had higher levels in the middle zone of the factor ethanol percentage, over the entire range of tested pH values. However, for the sample 5, the TP content had higher concentration values at low pH values and center ethanol % values. So, the experimental assays indicated that the optimal extraction conditions would have pH in the range of 0.9 to 1.5 and ethanol % in the range of 40 to 80%

To evaluate the model's predictive performance, Figure 37 represents the linear relationship between the predictive values and the experimental values. The linear regression presented a straight line with slope 1.03 (±0.005) and value zero for intercept (not significant) for sample 3, as well a straight line for the sample 5, with slope of 1.05(±0.009) and intercept of 39.72 (±0.57). The two linear regressions presented correlation coefficients nearby 1 (0.9996 and 0.9989, respectively). In this case, the obtained results showed that the two models can predict correctly the TP compounds.

Considering all the presented results, the selected optimal hydroethanolic solution for the samples extraction had pH of 3 and ethanol % of 85 %.

Sample 3



Sample 5

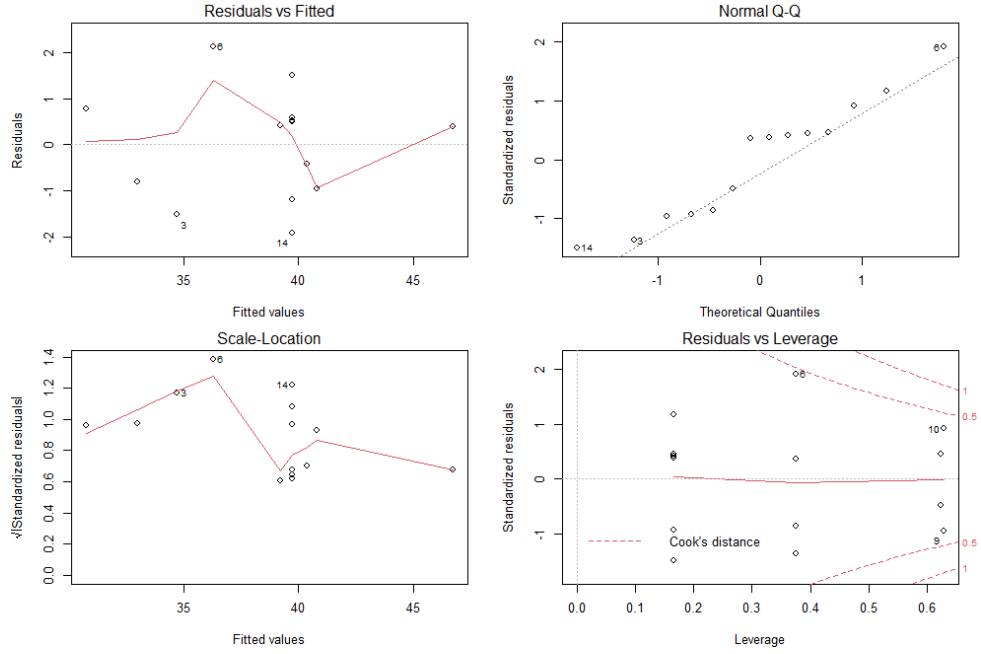
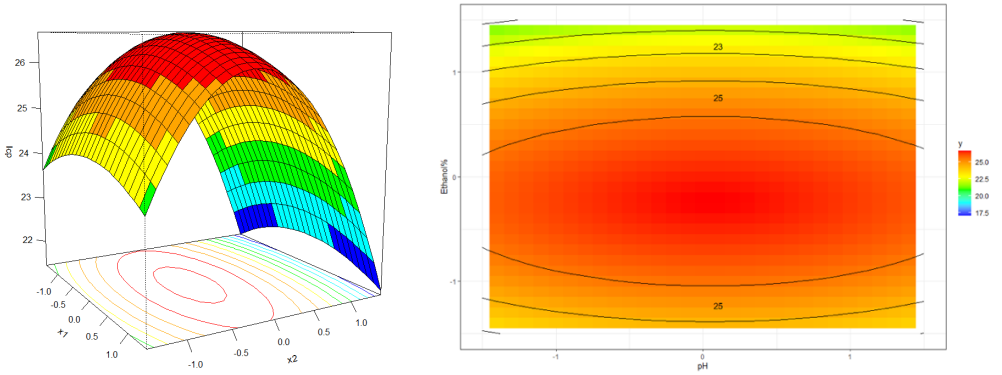


Figure 35. Plot for model's validation of total phenolic compounds for sample 3 and 5.

Sample 3



Sample 5

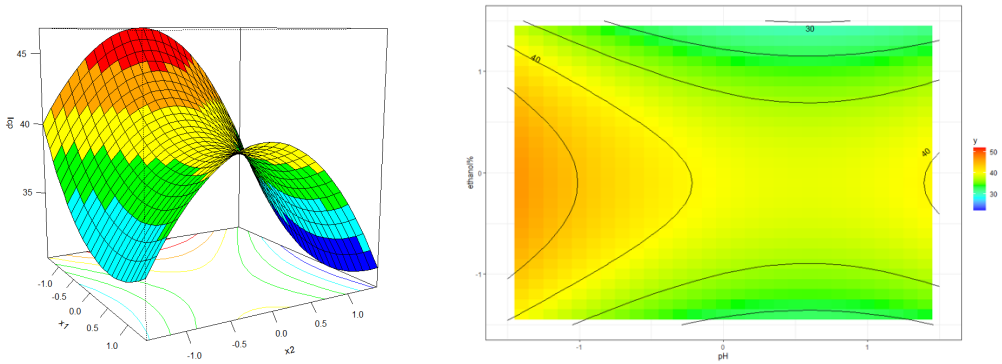


Figure 36. 3D surface and 2D contour plots of TP response for samples 3 and 5.

Sample 3 Sample 5

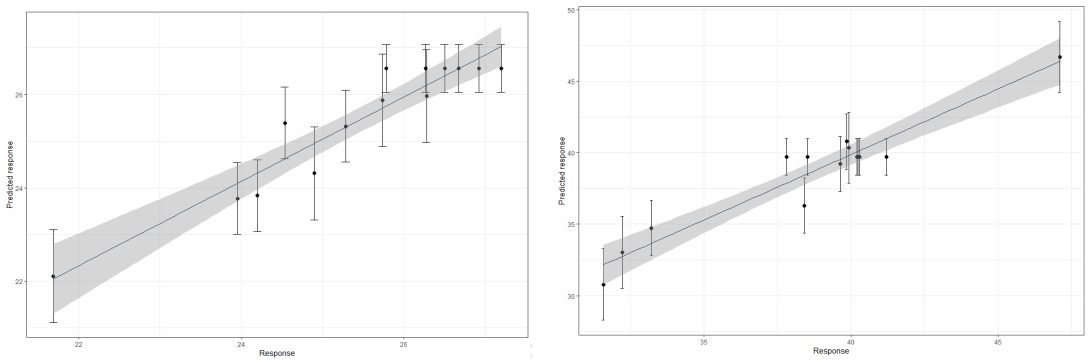


Figure 37. Predicted response for the TP compounds of sample 3 and 5.

3.2.7. UV Spectra analysis of extracts from five samples

The previous equations (section 5.2.1) were applied in the analysis of the five *Sorbus aucuparia L.* samples, using the selected optimal hydroethanolic extract solution. These extracts were prepared as described by Obied et al. (2005) methodology for the UV spectrum. Table 12

presents the results for the THBA, THCA, TF and TP contents in the extract obtained using the optimal hydroethanolic solution for sample 4.

Table 12. Concentration of the 4 compound groups in the sample 4 extract.

Compounds	Mean	s *	RSE%
Hydroxybenzoic acids, mg eq GA/L	14.4	0.6	4.2
Hydroxycinnamic acids, mg eq FA/L	22.7	0.4	1.9
Flavonoids, mg QC/L	6.1	0.3	4.7
Total phenolic content, mg TPC/L	40.6	0.6	1.5

*) s – standard deviation; RSE % - percentage relative standard deviation.

In the analysed sample, the class of THCA had the highest concentration (22.7 mg eq FA/L) followed by the hydroxybenzoic acids (14.4 mg eq GA/L), and, at lower levels, the class of flavonoids (6.1 mg QC/L). However, the RSE % values were lower than 5%, indicating that the established prediction models gave acceptable results.

3.3. The antioxidant activity results

For the analysis of TP content using Folin-Cicalteu method, TF content with $AlCl_3$ method, as well the DPPH test and FRAP test, calibrations curves were developed. The linear equations obtained were:

$$[TF] , mg/L = 0.028 x + 0.015; R^2 = 0.999.$$

$$[TP], mg/L = 0.0131 x + 0.081; R^2 = 0.999.$$

$$[DPPH], mg/L = 1.307 x - 2.83; R^2 = 0.997.$$

$$[FRAP] , mg/L = 0.026 x -0.056; R^2 = 0.999.$$

Table 13 shows the results from DPPH and FRAP tests, as well the TP and TF contents in all the five samples, measured in the respective extract, obtained with the selected hydroethanolic extract solution, which are presented by increased order of the sugar content (HPLC results). ANOVA results were also introduced to verify which sample mean values where statistically different (same letters in each column represent means that were not different statistically at significant levels of 0.05).

As we can see in the Table 13, the results of DPPH obtained by the UV spectra shows that the samples 2 and 3 were similar, which is the same case for the samples 4 and 2. It should be noticed that there is no great variability within the four compound groups results. The FRAP results showed that the samples 1 and 2 had similar values, as well the samples 4 and 5, a behaviour that happens in the results of TP content. Overall, sample2 showed the highest TP compounds and FRAP concentrations, followed by the sample1. For TF content, the results were not similar within samples 1 and 2 (having the highest content) followed by samples 4 and 5. This means that hydroxybenzoic acids and hydroxycinnamic acids have higher concentrations than the flavonoid compounds in the Tramazeira extract samples.

Table 13. Results of the five samples: DPPH and FRAP tests; TF and TP concentrations *.

Samples	DPPH (mg/100g)	FRAP (g/100g)	TP compounds (g/100g)	TF compounds (mg/100g)
5	5.436 (±0.000) d	1.50 (±0.14) b	0.64 (±0.05) b	3.94 (±0.26) ab
1	5.460 (±0.003) a	1.83 (±0.08) a	0.797 (±0.006) a	4.60 (±0.14) a
2	5.446 (±0.000) bc	2.06 (±0.25) a	0.81 (±0.04) a	2.95 (±0.44) ab
3	5.450 (±0.001) b	0.95 (±0.12) c	0.39 (±0.02) c	4.75 (±0.35) a
4	5.445 (±0.001) c	1.36 (±0.05) b	0.60 (±0.02) b	1.92 (±0.96) c

*) DPPH - 2, 2-diphenyl-1-picrylhydrazyl; FRAP - ferric reducing antioxidant power; TP – total phenolic; TF – total flavonoid.

The work of Mraihi et al. (2013) showed results of TP and TF compounds for two varieties of *Crataegus* pulp fruit (Tunisia), *C. monogyna* and *C. azarolus*, The pulp extract had lower levels of TP compounds (< 125 mg eq gallic acid/100 g dried weight) and high levels of TF compounds (> 150 mg eq rutin/100 g dried weight) in comparison with the results obtained from Tramazeira pulp fruit. The same comparison was obtained for the Alothman et al. (2009)

work, which analysed the tropical fruits from Malaysia (pineapple, banana pisang mas and guava), and Sochor et al. (2010) work, which presented results for the content of phenolic compounds and antioxidant capacity of several fruits of apricot genotypes. However, the results obtained for DPPH a and FRAP are lower than those obtained in these referenced works (Mraihi et al., 2013; Alothman et al., 2009; Sochor et al., 2010), which may be related to the low levels of flavonoid compounds extracted from the Tramazeira pulp fruit.

3.4. Microbiological results

3.4.1. Minimum inhibitory concentration

Two different microbial species were used to screen the possible antimicrobial activity of Tramazeira extract. Of the species used, *Staphylococcus aureus* is one of the most common gram-positive bacteria causing food poisoning, (Musher et al. 1977). As gram-negative bacteria, we have *Escherichia coli* that we can find in foods that have been contaminated by humans, as it belongs to the normal flora of humans (Wang.et al. 2009; Bettelheim et al. 1994).

The Tramazeira fruit extract (additive) was effective against all of the microorganisms evaluated, including a gram-positive strain (*S. aureus*) minimum inhibitory concentration (CMI) was 0.081 g/mL and a gram-negative strain (*Eshericia coli*) was 0.040 g/mL. For the concentration minimum bactericidal (CMB), both bacteria had the same result, 0.65 g/mL. However, unlike reported by other investigators gram-negative bacteria are more sensitive than gram-positive bacteria. Accordingly, the bacterial cell wall, particularly in gram-negative bacteria, was an effective barrier against candidate drug molecules. This barrier is strongly polar and contains efflux pumps that act as a resistance mechanism, ejecting the compounds that pass through the outer membrane (Brown et al. 2014). Al-Fatimi et al. (2007) and Sahreen et al. (2013) reported that plant extracts effective against gram-negative bacteria contained polar compounds that could interact with the chemical composition of the bacterial cell wall structure, thus promoting its effects.

3.4.2. Microbiological analysis results

The microbiological analyses performed were: quantification of yeasts and fungi, total mesophilic bacteria, coagulase positive *Staphylococcus aureus*, total Enterobacteriaceae and sulphite-reducing clostridium spores. These analyses were made on three different orange juices (J1, without Tramazeira extract; J2, with 0.04 g/mL and J3 with 0.08 g / mL of Tamazeira extract) under refrigeration (4 °C ± 2 °C) at different times T0 (day zero of adding the additive) and T2 (after two days of adding the additive). The results of microbiological analyses are shown in Table 26.

Total mesophilic microorganisms showed the same results as yeast and fungi for the T0 (<10 log CFU/mL) while for the T2 showed low counts for all samples 2.63, 3.51 and 3.15 log CFU/mL for the samples J1, J2 and J3 respectively.

Table 14. Microbiological results for the three different experiments with orange juices.

Parameters	Yeast and fungi (log CFU/mL)	Total mesophilic microorganisms (log CFU/mL)	*Staphylococcus aureus* (log CFU/mL)	*Enterobacteriaceae* (log CFU/mL)	Clostridium sulphite reducing spores (log CFU/mL)
T0-J1	<10	<10	Absent	<1	Absent
T0-J2	<10	<10	Absent	<1	Absent
T0-J3	<10	<10	Absent	<1	Absent
T2-J1	<10	2.63	Absent	<1	Absent
T2-J2	3.38	3.51	Absent	<1	Absent
T2-J3	3.11	3.15	Absent	<1	Absent

*) TO= day zero of adding the additive; T2= after two days of adding the additive; J1= without Tramazeira extract; J2= with 0.04 g/mL; J3= with 0.08 g/mL of Tramazeira extract.

During the two days storage time in the fridge, all samples showed the absence of coagulase positive for *Staphylococcus aureus*, sulphite-reducing *Clostridium* spores and for total Enterobacteriaceae (<1 log CFU/mL).

Yeast and fungi were not detected in the three samples for the T0 (<10 log CFU/mL) and for the T2 the samples J2 and J3 showed a low microorganism count (3.38 and 3.11 log CFU/mL respectively).

4. Conclusion

Mountain-ash (*Sorbus aucuparia L.)* have been shown to be a rich source of phenolic compounds and antioxidants. Using a new methodology based on a single run UV spectrum, the total concentration of three classes of phenolic compounds (total flavonoids, hydroxybenzoic acids, and hydroxycinnamic acids) and total phenolic content in Tramazeira extract were determined. All results indicate that the analytical method applied in this work exhibits an overall acceptable quality in the analysis of standard solutions and samples. This study also showed that the hydroethanolic solution with 85% of ethanol and with pH of 3, established with aqueous HCl solution, can extract high phenolic content representative of *Sorbus L.* fruit.

Tramazeira fruit extract was used as an additive in orange fresh juice, showing antimicrobial activity against gram-positive and gram-negative bacteria. Tramazeira extract was more effective against gram-positive bacteria for the CMI and for the CMB, both results were similar for two bacterias in study (*S. aureus* and *E. Coli*). For the trial time and for the concentrations of the Tramazeira fruit extract tested, the extract did not influence the microbiological quality of the orange juice. However, the results suggest that the juices obtained with the highest concentration are more stable from a microbiological point of view than the low concentration.

In future works, it is pretended to use higher concentrations of Tramazeira fruit extracts, test on other food products and check if the extracts improve the bioactive properties of the products where they will eventually be used.

References

- Aas G, Maier J, Baltisberger M, Morphology, isozyme variation, cytology, and reproduction of hybrids between Sorbus aria (L.) Crantz and S. torminalis (L.) Crantz Bot Helv, 104, 195-214, 1994.
- Addai Z, Abdullah A, Sahilah AM, Effect of extraction solvents on the phenolic content and antioxidant properties at two papaya cultivars, J Med Plant Res, 7, 3354-3359, 2013.
- Al-Fatimi M, Wurster M, Schroder G, Lindequist U. Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. J Ethnopharmacol, 111: 657-666, 2007.
- Alothman M, Bhat R, Karim AA, Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents, Food Chem, 115, 785–788, 2009.
- AOAC, 2012, Association of Official Analytical Chemistry. Official methods of analysis. (19th ed).(1-3000). Gaithersburg, MD: Author.
- Berker KI, Guclu K, Tor I, Apak R, Comparative evaluation of Fe (III) reducing power-based antioxidant capacity assays in the presence of phenanthroline, batho-phenanthroline, tripyridyltriazine (FRAP), and ferricyanide reagents, Talanta, 72, 1157-1165, 2007.
- Berna L, Kampuse S, Dukalska L, Murniece I, The chemical and physical properties of sweet rowanberries in powder sugar. In Proceedings of the 6th Baltic Conference on Food Sci Techmol, Jelgava, Latvia, 2011.
- Bettelheim KA, Biochemical characteristics of Escherichia coli in domestic animals and humans, Gyles C (edi.), Wallingford (United Kingdom) Med, 3-30, 1994.
- Boncler M, Golanski J, Golanski J, Lukasiak M, Redzynia M, Dastych J, Watala C, A new approach for the assessment of the toxicity of polyphenol-rich compounds with the use of high content screening analysis, PLoS ONE, 12, 1–26, 2017.
- Box GEP, Behnken DW, Some new three level designs for the study of quantitative variables, Technometrics, 2, 455-475, 1960.
- Bozkurt-Guzel C, Serbtei T, Kultur S, Cytotoxic activities of some Turkish medicinal plants against HeLa cells in vitro, Indian J Tradit Knowl, 17, 43–49, 2018.
- Brown DG, Lister T, May-Dracka TL. New natural products as new leads for antibacterial drug discovery. Bioorg. Med. Chem. Lett., 24: 413-418, 2014.
- Carmichael PC, Effect of fruit maturation and ripening potential for optimum eating quality of 'Forelle' pears, Master thesis, University of Stellenbosch, 2011.
- Chávez-González ML, Sepúlveda L, Verma DK, Luna-García HA, Rodríguez-Durán LV, Ilina A, Aguilar CN, Conventional and Emerging Extraction Processes of Flavonoids, Processes, 8, 434, 2020.

- Costa R, Silva LR, Health benefits of nongallated and gallated flavan-3-ols: A prospectus, Review, in Recent Advances in Gallate Research, 1-50, 2014.
- Dai J, Mumper RJ, Plant phenolic: Extraction, analysis and their antioxidant and anticancer properties, Molecules, 15, 7313–7352, 2010.
- Ekin HN, Gokbulut A, Aydin ZU, Donmez AA, Orhan IE, Insight into anticholinesterase and antioxidant potential of thirty-four Rosaceae samples and phenolic characterization of the active extracts by HPLC, Ind Crops Prod, 91, 104–113, 2016.
- Fraser MH, Cuerrier A, Haddad PS, Arnason JT, Owen PL, Johns T, Medicinal plants of Cree communities (Québec, Canada): antioxidant activity of plants used to treat type 2 diabetes symptoms, Can J Physiol Pharmacol, 85, 1200–1214, 2007.
- Garcia-Salas P, Morales-Soto A, Segura-Carretero A, Fernández-Gutiérrez A, Phenolic-compound-extraction systems for fruit and vegetable samples, Mol, 15, 8813-8826, 2010.
- Grussu D, Stewart D, McDougall GJ, Berry Polyphenols Inhibit-Amylase in vitro: Identifying Active Components in Rowanberry and Raspberry, J Agric Food Chem, 59, 2324–2331, 2011.
- Guerrero-Analco JA, Martineau L, Saleem A, Madiraju P, Muhammad A, Durst T, Haddad P, Arnason JT, Bioassay-guided isolation of the antidiabetic principle from Sorbus decora (Rosaceae) used traditionally by the eeyou istchee Cree first nations, J Nat Prod, 73, 1519–1523, 2010.
- Hatano T, Kagawa H, Yasuhara T, Okuda T, Two new flavonoids and other constituents in licorice root: their astringency and radical scavenging effects, Chem Pharm Bull, 36, 2090-2097, 1988.
- Hukkanen AT, Polonen SS, Karenlampi SO, Kokko HI, Antioxidant capacity and phenolic content of sweet rowanberries, J Agric Food Chem, 54, 112–119, 2006.
- Isaikina NV, Kalinkina GI, Razina TG , Zueva EP, Rybalkina OY, Ulirich AV, Fedorova EP, Shilova AB, *Sorbus aucuparia L.* fruit is a source of the drug for increasing the efficiency of tumor chemotherapy, Russ J Bioorgan Chem, 44, 899–905, 2018.
- ISO 15213:2003, Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions.
- ISO 21527-1:2008, Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 1: Colony count technique in products with water activity greater than 0, 95.
- Jankun A, Znaczenie apomiksji w ewolucji rodzaju Sorbus (Rosaceae), Fragm Florist Geobot, 38, 627-686, 1993.
- Kahkonen MP, Hopia AI, Heinonen M, Berry phenolics and their antioxidant activity, J Agric Food Chem, 49, 4076–4082, 2001.

- Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M, Antioxidant activity of plant extracts containing phenolic compounds, J Agric Food Chem, 47, 3954–3962, 1999.
- Khan S, Itrat F, Kazmi MH, Abdul M, Nighat A, Lubna I, Mehreen L, Cashmins A and B, Potent Antioxidant Coumarins from Sorbus cashmiriana, Chem Nat Compd, 51, 626–629, 2015.
- Kim H, Song MJ, Ethnomedicinal practices for treating liver disorders of local communities in the Southern Regions of Korea, Evid Based Complement Altern Med, 2013, 1–11, 2013.
- Klensporf-Pawlik D, Przybylski R, Antioxidant activity of selected wild Canadian prairie fruits, Acta Sci Pol Technol Aliment, 14, 357–366, 2015.
- Krivoruchko EV, Andrushchenko OA, Kononenko AV, Carboxylic acids from Sorbus aucuparia and S. aria, Chem Nat Compd, 49, 742–743, 2013.
- Kylli P, Nohynek L, Puupponen-Pimiä R, Westerlund-Wikström B, McDougall G, Stewart D, Heinonen M, Rowanberry Phenolics: Compositional Analysis and Bioactivities, J Agric Food Chem, 58, 11985–11992, 2010.
- Lacombe A, Vivian C.H.W., The potential of berries to serve as selective inhibitors of pathogens and promoters of beneficial microorganisms, F Q S, 1, 3–12, 2017.
- Lee TK, Roh HS, Yu JS, Kwon DJ, Kim SY, Baek KH, Kim KH, A novel cytotoxic activity of the fruit of Sorbus commixta against human lung cancer cells and isolation of the major constituents, J Funct Foods, 30, 1–7, 2017.
- Lenth RV, Response-Surface Methods in R, Using rsm, J Stat Softw, 32, 1–17, 2009.
- Liepina I, Nikolajeva V, Jakobsone I, Antimicrobial activity of extracts from fruits of Aronia melanocarpa and Sorbus aucuparia, Environ Exp Biol, 11, 195–199, 2013.
- Loef M., Walach H., Fruit, vegetables and prevention of cognitive decline or dementia: A systematic review of cohort studies, J nutr health aging, 16, 626-630, 2012.
- Łuczaj Ł, Kohler P, Piroznikow E, Graniszewska Ma, Pieroni A, Gervasi T, Wild edible plants of Belarus: from Rostafiński's questionnaire of 1883 to the present, J Ethnobiol Ethnomed, 9, 1–21, 2013.
- Magalhães AS, Branca SM, Pereira JA, Andrade PB, Valentão P, Carvalho M, Protective effect of quince (Cydonia oblonga Miller) fruit against oxidative hemolysis of human erythrocytes, Food Chem Toxicol, 47, 1372-1377, 2009.
- Manach C, Donovan JL, Pharmacokinetics and Metabolism of Dietary Flavonoids in Humans, Free Radic Res, 38, 771–785, 2004.
- Maroco J, Análise Estatística com Utilização do SPSS (3rd edi), Lisboa: Silabo, Lda, 2007.
- McCune LM, Johns T, Antioxidant activity relates to plant part, life form and growing condition in some diabetes remedies, J Ethnopharmacol, 112, 461–469, 2007.

- Mertz C, Gancel AL, Gunata Z, Alter P, Dhuique-Mayer C, Vaillant F, Perez AM, Ruales J, Brat P, Phenolic compounds, carotenoids and antioxidant activity of three tropical fruits, J Food Compos Anal, 22, 381-387, 2009.
- Mikilic-Petkovsek M, Schmitzer V, Slatnar A, Stampar F, Veberic R, Composition of sugars, organic acids, and total phenolics in 25 wild or cultivated berry species, J Food Sci, 77,1064–1070, 2012.
- Mikulic-Petkovsek M, Krska B, Kiprovski B, Veberic R, Bioactive Components and Antioxidant Capacity of Fruits from Nine Sorbus Genotypes, J Food Sci, 82, 647–658 , 2017.
- Molla Y; Nedi T; Tadesse G; Alemayehu H; Shibeshi W, Evaluation of the in vitro antibacterial activity of the solvent fractions of the leaves of Rhamnus prinoides L'Herit (Rhamnaceae) against pathogenic bactéria. BMC Complement, Altern Med, 16, 1–9, 2016.
- Montgomery DC, Design and Analysis of Experiments: Response surface method and designs. New Jersey: John Wiley and Sons, Inc 2005.
- Moure A, Cruz JM, Franco D., Dominguez JM, Sineiro J, Dominguez H, Nunez MJ, Parajo JC, Natural antioxidants from residual sources, Food Chem, 72, 145–171, 2001.
- Mraihi F, Journi M, Chérif JK, Sokmen M, Sokmen A, Trabelsi-Ayadi M, Phenolic Contents and Antioxidant Potential of Crataegus Fruits Grown in Tunisia as Determined by DPPH, FRAP, and β-Carotene/Linoleic Acid Assay, J Chem, Article ID 378264, 2013.
- Mrkonjić ZO, Nađpal JD, Beara IN, Aleksic-Sabo VS, Cetojevic-simin DD, Mimica-Dukic NM, Lesjak MM, Phenolic profiling and bioactivities of fresh fruits and jam of *Sorbus* species, J Serbian Chem Soc, 82, 651–664, 2017.
- Musher DM, M.D.; McKenzie SOMD, Infections due to staphylococcus aureus, Med, 56, 383-410, 1977.
- Naczk M, Shahidi F, Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis, J Pharm Biomed Anal, 41, 1523-1542, 2006.
- Nakatani N, Antioxidants from spices and herbs: In Natural antioxidants – Chemistry, health effects and applications, Edited by: Shahidi F, 65–75, 1996.
- Navarro JM, Flores P, Garrido C, Martinez V, Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. Food Chem, 96, 66-73, 2006.
- Neves JM, Matos C, Moutinho C, Queiroz G, Gomes LR, Ethnopharmacological notes about ancient uses of medicinal plants in Trás-os-Montes (northern of Portugal), J Ethnopharmacol, 124, 270–283, 2009.
- Nishiumi S, Miyamoto S, Kawabata K, Ohnishi K, Mukai R, Murakami A, Ashida H, Terao J, Dietary flavonoids as cancer-preventive and therapeutic biofactors, Review Frontiers in bioscience, 3, 1332-62, 2011.

- NP 4400-1:2002, Food microbiology, General guidance for the enumeration of coagulate positive Staphylococci (*Staphylococcus aureus* and other species), Part 1: Technique with colonies confirmation (Routine method).
- NP 4405: 2002, Food microbiology, General guidance for the enumeration of microrganisms, Colony count technique at 30ºC.
- Obeid HK, Allen MS, Jr Bergood DR, Prenzler PD, Robards K, Investigation of Australian olive mill waste for recovery of biophenols, J Agric Food Chem, 53, 9911-20, 2005.
- Olszewska M, Separation of quercetin, sexangularetin, kaempferol and isorhamnetin for simultaneous HPLC determination of flavonoid aglycones in inflorescences, leaves and fruits of three *Sorbus* species, J Pharm Biomed Anal, 48, 629–635, 2008.
- Olszewska MA, Kolodziejczyk-Czepas J, Rutkowska M, Magiera A, Michel P, Rejman MW, Pawel N, Owczarek A, The effect of standardised flower extracts of Sorbus aucuparia L. on proinflammatory enzymes, multiple oxidants, and oxidative/nitrative damage of human plasma components in vitro, Oxid Med Cell Longev, 1–18, 2019.
- Olszewska MA, Michel P, Antioxidant activity of inflorescences, leaves and fruits of three Sorbus species in relation to their polyphenolic composition, Nat Prod Res, 23, 1507–1521, 2009.
- Olszewska MA, Variation in the phenolic content and in vitro antioxidant activity of Sorbus aucuparia leaf extracts during vegetation, Acta Pol Pharm Drug Res, 68, 937–944, 2011.
- Paula VB, Estevinho LM, Dias LG, Quantification of three phenolic classes and total phenolic content of propolis extracts using a single UV-vis spectrum, J Apic Res, 56, 569–580, 2017.
- Phipps JB, Robertson KR, Smith PG, Rohrer JR, A checklist of the subfamily Maloideae (Rosaceae), Can J Bot, 68, 2209-2269, 1990.
- Poyrazoglu E.S., Changes in ascorbic acid and sugar content of rowanberries during ripening, J Food Qual, 27, 366–370, 2004.
- Pranskuniene Z, Ratkeviciute K, Simaitiene Z, Pranskunas A, Bernatoniene J, Ethnobotanical study of cultivated plants in Kaišiadorys District, Lithuania: possible trends for new herbal based medicines, Evid Based Complement Altern Med, 2019, 1–15, 2019.
- Prati S, Baravelli V, Fabbri D, Schwarzinger C, Brandolini V, Maietti A, Tedeschi P, Benvenutti S, Macchia M, Marotti I, Bonetti A, Catizone P, Dinelli G, Composition and content of seed flavonoids in forage and grain legume crops, J Sep Sci, 30, 491-501, 2007.
- Raspe O, Findlay C, Jacquemart AL, Sorbus aucuparia L., J Ecology, 88, 910-930, 2000.
- Raudonis R, Raudone L, Gaivelyte K, Viskelis P, Janulis V, Phenolic and antioxidant profiles of rowan (Sorbus L.) fruits, Nat Prod Res, 28, 1231–1240, 2014.

- Razina TG, Zueva EP, Ulrich AV., Rybalkina O.Yu, Chaikovskii A.V., Isaikina N.V., Kalinkina G.I., Zhdanov V.V., Zyuz'kov G.N., Antitumor Effects of Sorbus aucuparia L. Extract Highly Saturated with Anthocyans and Their Mechanisms, Bull Exp Biol Med, 162, 93–97, 2016.
- Ross KA, Beta T, Arntfield SD, A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods, Food Chem, 113, 336-344, 2009.
- Sahreen S, Khan MR, Khan RA, Shah NA. Estimation of flavoniods, antimicrobial, antitumor and anticancer activity of Carissa opaca fruits. BMC Complement Altern Med, 13: 372, 2013.
- Sak K, Jurisoo K, Raal A, Estonian folk traditional experiences on natural anticancer remedies: from past to the future, Pharm Biol, 52, 855–866, 2014.
- Sarv V, Venskutonis PR, Bhat R, The Sorbus spp.—Underutilised Plants for Foods and Nutraceuticals: Review on Polyphenolic Phytochemicals and Antioxidant Potential, 9, 813, 2020.
- Simirgiotis MJ, Caligari PDS, Schmeda-Hirschmann G, Identification of phenolic compounds from the fruits of the mountain papaya Vasconcellea pubescens A. DC. Grown in Chile by liquid chromatography-UV detection-mass spectrometry, Food Chem, 115, 775-784, 2009.
- Singleton VL, Orthofer R, Lamuela-Raventos RM, Analysis of total phenols and other oxidation substrates ans antioxidants by means of Folin-Ciocalteu reagent, Meth Enz, 299, 152-178, 1999.
- Singleton VL, Rossi JA, Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents, Am J Enol Vitic, 16, 144-158, 1965.
- Smith-Warner SA, Spiegelman D, Yaun SS, Adami HO, Beeson WL, Van Den Brandt PA, Folsom AR, Fraser GE, Freudenheim JL, Goldbohm RA, Graham S, Miller AB, Potter JD, Rohan TE, Speizer FE, Toniolo P, Willett WC, Wolk A, ZeleniuchJacquotte A, Hunter DJ, Types of dietary fat and breast cancer: a pooled analysis of cohort studies, Int J Cancer, 92, 767-74, 2001.
- Sochor J, Zitka O, Skutkova H, Pavlik D, Babula P, Krska B, Horna A, Adam V, Provaznik I, Kizek R, Content of Phenolic Compounds and Antioxidant Capacity in Fruits of Apricot Genotypes, Mol, 15(9), 6285-6305, 2010.
- Sohn EJ, Kang DG, Mun YJ, Woo WH, Lee HS, Anti-atherogenic Effects of the Methanol Extract of Sorbus Cortex in Atherogenic-Diet Rats, Biol Pharm Bull 28, 1444–1449, 2005.
- Tamkute L, Gil BM, Carballido JR, Pukalskiene M, Venskutonis PR, Effect of cranberry pomace extracts isolated by pressurized ethanol and water on the inhibition of food pathogenic/spoilage bacteria and the quality of pork products, Food Res Int, 120, 38–51, 2019.

- Tapiero H., Tew K. D., Nguyen Ba G., Mathé, G., Polyphenols: do they play a role in the prevention of human pathologies?, Biomed Pharmacoth, 56, 200-207, 2002.
- Tardio J, Pardo-de-santayna M, Morales R, Ethnobotanical review of wild edible plants in Spain, Bot J Linn Soc, 152, 27–71, 2006.
- Termentzi A, Kefalas P, Kokkalou E, Antioxidant activities of various extracts and fractions of Sorbus domestica fruit at different maturity stages, Food Chem, 98, 599–608, 2006.
- Tunde J, Jiri S, Jiri M, Stefan B, Borivoj K, Mojmir B, Sezai E, Ozturk YS, Polyphenolic profile of interspecific crosses of rowan (Sorbus aucuparia L.), Ital J Food Sci, 26, 317–324, 2014.
- Urbanaviciut I, Liaudanskas M, Bobinas C, Šarkinas A, Rezgien A, Viskelis P, Japanese Quince (Chaenomeles japonica) as a potential source of phenols: optimization of the extraction parameters and assessment of antiradical and antimicrobial activities, Foods, 9, 1132, 2020.
- Vujanović M, Majkić T, Gökhan Z, Beara I, ,Cvetanović A, Mahomoodally FM, Radojković M, Advantages of contemporary extraction techniques for the extraction of bioactive constituents from black elderberry (Sambucus nigra L.) flowers, Review Ind Crop prod, 136, 93-101, 2019.
- Wang H, Feng H, Liang W, Luo Y, Malyarchuk V, Effect of Surface Roughness on Retention and Removal of Escherichia coli O157:H7 on Surfaces of Selected Fruits, J food sci , 74, 8-15, 2009.
- Woisky RG, Salatino A, Analysis of propolis: some parameters and procedures for chemical quality control, J Apric Res, 37, 99-105, 1998.
- Wu CFJ, Hamada MS, Experiments: Planning, Analysis, and Optimization, 2nd Edi, John Wiley & Sons, Inc, 2011.
- Xiaojin Y., Zhenyu W., Zunpeng S., Zhengqing L., Yuan N., Keli Y., Haina B., Ruihai L; Wenli L., Effect and mechanism of Sorbus pohuashanensis (Hante) Hedl. Flavonoids protect against arsenic trioxide-induced cardiotoxicity, Biomed Pharmacother, 88, 1–10, 2017.
- Yin MH, Kang DG, Choi DH, Kwon TO, Lee HS, Screening of vasorelaxant activity of some medicinal plants used in Oriental medicines, J Ethnopharmacol, 99, 113–117, 2005.
- Yongxia Y., Yanguo Z., Haijun L., Yan Z., Enbi C., Hongyan Z., Pingya L., Jinping L., Triterpenoids from fruits of Sorbus pohuashanensis inhibit acetaminophen-induced acute liver injury in mice, Biomed Pharmacother, 109, 493–502, 2019.
- Yu T, Lee YJ, Jang HJ, Kim AR, Hong S, Kim TW, Kim MY, Lee J, Lee YG, Cho JY, Anti-inflammatory activity of Sorbus commixta water extract and its molecular inhibitory mechanism, J Ethnopharmacol, 134, 493-500, 2011.

- Zhongxiang F, Min Z, Linxiang W, HPLC-DAD-ESI-MS analysis of phenolic compounds in bayberries (Myrica rubra Sieb. et Zucc.), Food Chem, 100, 845-852, 2007.