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« FOOD CHEMISTRY »

MASTER THESIS

Method development for the determination of benzoic acid, sorbic acid and four parabens in fruit juices and soft drinks using HPLC-DAD and application on products of Greek origin

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ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

Ανάπτυξη μεθόδου για τον προσδιορισμό βενζοϊκού οξέος, σορβικού οξέος και τεσσάρων parabens σε χυμούς φρούτων και αναψυκτικά χρησιμοποιώντας HPLC-DAD και εφαρμογή της μεθόδου σε προϊόντα ελληνικής παραγωγής

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ABSTRACT

In this master thesis, an analytical method was developed, optimized and validated for the qualitative and quantitative detection and determination of six preservatives: benzoic acid, sorbic acid, methyl paraben, ethyl paraben, propyl paraben and butyl paraben in three food substrates: fruit juices, soft drinks and energy drinks. High Performance Liquid Chromatography coupled with DAD detector was used. The chromatographic separation was carried out by gradient elution using an NUCLEOSIL C_{18} column (150mm x 4.6 mm, 5μm) which was maintained at ambient temperature. The mobile phase, composed of solvent A (ammonium acetate buffer pH=4.2) and solvent B (acetonitrile), was set at a stable flow rate of 0.8 mL/min. The target analytes were detected and identified at 230 nm (benzoic acid) and 254 nm (sorbic acid and parabens) and standard addition calibration curves were used for quantification. The method development and optimization involved testing different parameters such as the mobile phase, flow rate, the solvent of the standards and sample preparation parameters.

The optimized method was then validated in terms of linearity, accuracy, precision and detectability and was applied to market samples of Greek origin (fruit juices and soft drinks) and energy drinks of foreign origin, in order to evaluate compliance with the European legislative framework. Several preservatives were detected, proving the effectiveness and usefulness of the method to the analysis of real market samples. Of the 22 fruit juice samples, 1 sample was found positive for added benzoate. Of the 15 soft drinks, half of the samples contained benzoic acid, while 13 tested positive for sorbate, none of which exceed the legal limit. Finally, of the 7 energy drinks, 4 contained benzoic acid (3 of which contained higher quantity than the legal limit) and 5 sorbic acid. Parabens were not detected in any of the 44 samples analyzed.

Topic Area: Food Chemistry, Analytical Chemistry, Instrumental Analysis

Keywords: Benzoic acid, Sorbic acid, Parabens, HPLC-DAD, Beverages

ΠΕΡΙΛΗΨΗ

Στην παρούσα μεταπτυχιακή εργασία αναπτύχθηκε, βελτιστοποιήθηκε και επικυρώθηκε μια αναλυτική μέθοδος για την ποιοτική και ποσοτική ανίχνευση και προσδιορισμό έξι συντηρητικών: βενζοϊκό οξύ, σορβικό οξύ και τέσσερις εστέρες του *p*-υδροξυβενζοϊκού οξέος (μέθυλ-, αίθυλ-, πρόπυλ-, βούτυλ-, ή αλλιώς parabens) σε: χυμούς φρούτων, αναψυκτικά και ενεργειακά ποτά. Χρησιμοποιήθηκε υγρή χρωματογραφία υψηλής απόδοσης σε συνδυασμό με ανιχνευτή DAD. Ο χρωματογραφικός διαχωρισμός πραγματοποιήθηκε με βαθμιδωτή έκλουση, χρησιμοποιώντας τη στήλη NUCLEOSIL C_{18} (150 mm x 4.6 mm, 5 μm) η οποία διατηρήθηκε σε θερμοκρασία περιβάλλοντος. Η κινητή φάση, που αποτελείτο από το διάλυμα Α (ρυθμιστικό διάλυμα οξικού αμμωνίου σε pH=4.2) και το διάλυμα Β (ακετονιτρίλιο), ρυθμίστηκε σε σταθερό ρυθμό ροής 0.8 mL/min. Τα εν λόγω αναλυόμενα συστατικά εντοπίστηκαν και προσδιορίστηκαν στα 230 nm (βενζοϊκό οξύ) και 254 nm (σορβικό οξύ και parabens) και για την ποσοτικοποίηση χρησιμοποιήθηκαν καμπύλες γνωστής προσθήκης. Η ανάπτυξη και βελτιστοποίηση της μεθόδου περιλάμβανε τη δοκιμή παραμέτρων, όπως τη σύσταση της κινητής φάσης, το ρυθμός ροής, το διαλύτη των προτύπων και την προκατεργασία του δείγματος.

Η βελτιστοποιημένη μέθοδος επικυρώθηκε σε όρους γραμμικότητας, ακρίβειας, πιστότητας και ανιχνευσιμότητας και εφαρμόστηκε σε δείγματα ελληνικής προέλευσης (χυμοί φρούτων και αναψυκτικά) και ενεργειακά ποτά, προκειμένου να αξιολογηθεί η συμμόρφωση της ελληνικής αγοράς με το ευρωπαϊκό νομικό πλαίσιο. Πολλά συντηρητικά εντοπίστηκαν, αποδεικνύοντας την αποτελεσματικότητα της μεθόδου για την ανάλυση πραγματικών δειγμάτων αγοράς. Από τους 22 χυμούς φρούτων, 1 δείγμα βρέθηκε θετικό για προσθήκη βενζοϊκού οξέος. Από τα 15 αναψυκτικά, τα μισά δείγματα περιείχαν βενζοϊκό, ενώ 13 βρέθηκαν θετικά για σορβικό οξύ, χωρίς να υπερβαίνουν το νόμιμο όριο. Τέλος, από τα 7 ενεργειακά ποτά, 4 περιείχαν βενζοϊκό (από τα οποία 3 παραβίασαν το νόμιμο όριο) και 5 σορβικό οξύ. Τα parabens δεν εντοπίστηκαν σε κανένα από τα 44 αναλυθέντα δείγματα.

Θεματική περιοχή: Χημεία Τροφίμων, Αναλυτική Χημεία, Ενόργανη Ανάλυση

Λέξεις-κλειδιά: Βενζοϊκό Οξύ, Σορβικό οξύ, Εστέρες του *p*-υδροξυβενζοϊκού οξέος, HPLC-DAD, Μη αλκοολούχα ποτά

To my beloved grandfather, Elias, who has always been my number one supporter and is watching over me, lately, from above.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF TABLES

FOREWORD

The purpose of this master thesis was to develop and validate a new reliable and rapid method for the simultaneous determination of six preservatives (benzoic acid, sorbic acid, methyl-, ethyl-, propyl- and butyl- paraben) used in the food and beverage industry: in samples of fruit juices, soft drinks and energy drinks. The supply of the samples (44 samples) that were analyzed in this study was done through retail purchase in local Greek stores and other supermarket chains. The experimental work was carried out in the Food Chemistry laboratory of the Chemistry Department of the National and Kapodistrian University of Athens.

CHAPTER 1
INTRODUCTION

1.1 Preservatives: Purpose, Properties and Legislation

The development of civilizations and the constant demand to meet their basic needs, such as feed, have led the scientific community to search for solutions to extend the shelf life of food and to avoid socioeconomic impacts on a global scale. As it is well known, foods are excellent substrates for microbial growth, when the right conditions are present (pH, temperature and water activity). The most modern approach of the last decades is the addition of preservatives, compounds that have the ability to maintain the quality of the food, its physicochemical properties and the functionality of its nutrients without affecting its organoleptic characteristics. Their addition is carried out during the stages of preparation, processing, packaging and transport of foodstuffs, directly or indirectly. [1, 2]

Their main mechanism of action is the inhibition of the growth of unwanted microorganisms, such as bacteria, fungi and yeasts, which are participating in certain chemical reactions or are inhibiting useful enzymes in microbial cells. In order to achieve this, preservatives, particularly in the food industry, have to comply with certain conditions. In particular, the same ones or their metabolites must not be toxic, must be stable at high temperatures, have antimicrobial properties within the pH range of the specific food, must not affect the sensory properties of the products and not react with other ingredients. It is mandatory that preservatives do not cause negative effects on the health of consumers and that is why ADI (Acceptable Daily Intake) was established, in particular for sensitive consumer groups, and the use of the minimum quantity possible is suggested in each case. Due to the characteristics of the food product or because the preservatives themselves may be altered, it may be necessary to add a second preservative to stabilize the first or to enhance a specific function (synergistic action). [2,3]

Preservatives can be divided into two categories, natural compounds, such as lacto- peroxidase and flavonoids, which are components of food or can also be obtained as a result of fermentation processes, and synthetic ones, such as benzoic acid, sorbic acid and esters of para-hydroxybenzoic acid or their salts, which will be studied in this master thesis. [1, 2, 3]

Their maximum amount of addition, their chemical identification and purity are controlled by European Union legislation and the European Food Safety Authority (EFSA, EU 1333/2008) and by the Codex Alimentarius Committee (CAC), which was established by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). The European Regulation is in agreement with CAC, with a very few exceptions. Revisions of the permitted addition limits are constantly being for the protection of consumers' health. Each additive is identified by a coded name (E) and three or four digits and this numbering system follows the guidelines of the International Numbering System (INS) as defined by the Codex Alimentarius Commission. For preservatives, the name includes the numbers E200-E297. [2, 4]

The following section includes the three categories of preservatives relevant to this thesis, their physicochemical properties, their mechanism of action against microbes and the legislation surrounding their addition to various substrates. The three groups are benzoic acid (BA), sorbic acid (SA) and four of the most important parabens (PHBs) that are used in the industry.

1.2 Benzoic Acid

1.2.1 Structure and physicochemical properties

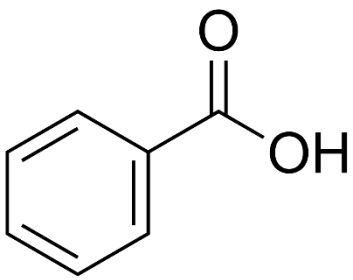

Figure 1*:* Structure of Benzoic Acid.

Benzoic acid (BA) is an organic weak acid and its structure is shown in Figure 1. In the solid phase, it has a transparent and monoclinic crystal structure. It has a relatively low solubility in water, but its solubility increases in solvents such as benzene, carbon tetrachloride, acetone and alcohols. It may occur naturally in organic tissues such as apple, berries, tomatoes or is formed through fermentation progresses by lactic acid bacteria in dairy products. The pKa of the acid is 4.2, which makes it a suitable preservative for acidic substrates, such as carbonated beverages. Benzoic acid acts as a preservative in the undissociated form only, and it is therefore necessary to maintain a specific acidity to exclude the possibility of dissociation. It is known, moreover, that at pH = 4, 60 % of this compound is not dissociated, whereas at pH equal to 6, only 1.5 % is undissociated. To extend its application to food industry, the following water-soluble salts have been formed by neutralisation, sodium benzoate (E211), potassium benzoate (E212) and calcium benzoate (E213), with sodium salt being the first permitted preservative by the FDA. [1, 5, 6, 7, 8]

1.2.2 Mechanism of action and use in food industry

In food industry, benzoic acid and its salts, sodium, potassium and calcium, with the coded numbers E210-E213, have been identified as preservative and antioxidant compounds. Their main mechanism includes the inhibition of the proliferation of fungi, yeasts and bacteria, avoiding, therefore, degradation of food or toxin development. It is known that inhibition of the growth of yeast and fungi is carried out by adding 0.05%-0.1% of the undissociated acid, while as regards the inhibition of the growth of sporogenic bacteria, addition of 0.01-0.02% of undissociated acid is, only, needed. [9]

One hypothesis for the mechanism of action of benzoic acid and salts for this inhibition is the interfering of permeability of the cell membrane, causing disruption of both the substrate transport and oxidative phosphorylation by the electron transport system. [9]

For example, in *Bacillus subtilis*, transmembrane transport of amino acids is interrupted, resulting in cell starvation. This inhibition was caused by the destruction of the proton motive force by the continuous transport of protons into the cells by benzoic acid. In this way, the inhibitory power of this acid can be determined from the distribution of organic/water phase ratio, the pK value and the ability of the benzoic acid molecules to delocalizate the negative charge of ions inside the cells and thus increase the mobility in their membrane. [9]

Another example is the effect of benzoic acid on the growth of the microorganism *E.Coli*, with its action as preservative being the reduction of the intracellular pH through the undissociated form. At the same time, the uptake of amino acids by the microorganisms *Penicillium chrysogenum, B. Subtilis* and *Pseudomonas aeruginosa* takes place via the undissociated form of the acid, which diffuses freely through the cell membrane and then ionises, producing protons that acidify the alkaline internal environmental of the microorganism cell. [9]

Finally, another mechanism of action of this preservative is the inactivation of enzymes, such as alpha-ketoglutarate, electrolyte dehydrogenase, 6-

phosphofructo-2-kinase and lipase, important in Krebs cycle and oxidative phosphorylation. [4, 10]

Typically, benzoic acid is used in food industry, in combination with other preservatives, particularly in acidic substrates (pH = 4 - 4.5), due to the strong dependence of the acid on pH. The maximum antimicrobial activity occurs in the range of pH = 2.5 - 4, while further increase of the pH of culture medium reduces the effectiveness of this preservative. [9, 11]

Most industrial companies characterize benzoic acid (E210) and its salts (E211-213) as very important preservatives, particularly in the production of soft drinks, fruit, vegetables and juices, meat products, jams and confectionery.[4] In a comparative experimental study carried out on a turkey product, the biocidal activity against *Listeria monocytogenes* at temperatures lower than 14 °C, was greater when using benzoic acid, rather than sorbic or propionic acid.[10] Only 75 mg/L sodium benzoate is needed, in order to inhibit the germination of ascospores and subsequent growth of the microorganism *Neosartorya fischeri,* which is one of the most heat-resistant fungi that cause spoilage in fruit juices. [9]

However, extended uncontrolled addition of the acid could damage certain nutrient components of the food. In particular, excessive use of benzoic acid leads to destruction of vitamin B_1 and in this way, the calcium ion becomes insoluble and makes it difficult for human body to absorb it.[12] It is further recommended the avoidance of addition of this preservative in fruit juices with high content of ascorbic acid, as the interaction between benzoic acid and ascorbic acid, under certain conditions, forms benzene, a toxic molecule, at a detectable level. Essentially, the hydroxyl radicals that are formed from the reduction of oxygen or hydrogen peroxide by ascorbic acid, a reaction catalyzed by metal ions Cu(II) and Fe(III), decarboxylate benzoic acid, resulting in the formation of benzene. The formation of benzene depends on many factors, such as the pH of the solution, the concentration of the preservative, the antioxidants (ascorbic acid), the metal ions, the sugars, the hydroxyl precursors, temperature, UV radiation and storage time. It should be noted, however, that the estimated dietary exposure to benzene from

beverages is low, namely 8 ng/kg, indicating a low risk to public health, as opposed to inhalation of this compound. However, only 50% of inhaled benzene is absorbed by the human body, in contrast to 100% of benzene absorbed from the consumption of beverages, which is why the formation of this molecule in foodstuffs is a very important field for research. [6, 13, 14, 15]

1.2.3 Legislation

Although the preservative action of benzoic acid against microorganisms such as fungi and bacteria is important for food industry, its levels need to be controlled, because of its negative effects on the human body, by establishing permissible limits.

Excessive intake of benzoic acid or its sodium salt could lead to stomach disorders, intolerance, allergic reactions, abdominal pain, and even inhibition of intermediate metabolic processes in the body. [6, 11]

Experimental studies, mainly in rats, are conducted from time to time to test and monitor the effect of this additive on growth, carcinogenesis and genotoxicity, in the short and long term. The results, in terms of development, demonstrate that consumption of benzoic acid affects on weight and height reduction, not only of the laboratory animal, but also of the fetus that this animal gestates, compared to the control group, while for short-term effects, there were no health effects up to the level of consumption of 1000 mg/kg acid per kg body weight per day. At the same time, the Joint Committee FAO/WHO Joint Expert Committee reviewed all previous studies concerning the long-term effects, carcinogenicity and genotoxicity and rejected the association of benzoic acid and its salts with these health problems, making them among the safest food additives. [6, 8]

However, it is necessary, despite the safety of this preservative, to set the value of Acceptable Daily Intake (ADI), which is 20 mg/kg per kg body weight per day, expressed as benzoic acid, for the preservatives E210-E213, in accordance with the Joint Committee FAO/WHO Joint Expert Committee, which rejected the previous ADI of 5 mg/kg per kg body weight per day at the last meeting in 2022.

Particularly for food categories, where the addition of benzoic acid is very frequent, a maximum level has been set, in order to reduce the negative effects during consumption of the food. In particular, the European Regulation 1333/2008, for fruit juices, specify the maximum level for benzoic acid and its salts to 200 mg/L, while for non-alcoholic flavoured drinks (soft drinks) to 150 mg/L. For energy drinks, the maximum level for this additive is 250 mg/kg, according to the revised version of the Codex Alimentarius Commission of 2021. The permitted limits, summarized in Table 1, demonstrate the importance of monitoring this group of food consumed on a daily basis, particularly by more sensitive age groups, such as children. [2, 6, 16, 17, 18]

Table 1: Maximum Permissible Limits of Benzoic Acid in substrates related to this thesis.

Substrates	Maximum Permissible Limit	Regulation
Fruit Juices	200 mg/L	EU 1333/2008
Soft Drinks	150 mg/L	EU 1333/2008
Energy Drinks	250 mg/kg (450 mg/kg if pH>3.5)	CODEX STAN 192-1995 (Revised in 2021)

1.3 Sorbic Acid

1.3.1 Structure and physicochemical properties

On the other side, sorbic acid (SA) does not have a ring structure such as benzoic acid, but it is a monocarboxylic *α,β*-unsaturated straight-chain acid, the structure of which is illustrated in Figure 2. Some of its properties are molar mass (112.13 g/mol), pKa (4.75), while in solid phase sorbic acid consists of numerous small white crystals. The solubility of sorbic acid in water at room temperature is only 0.15 g per 100 mL, but with changes, specifically an increase in temperature, pH or both, its solubility in the medium will eventually increase. Even higher solubility appears in solvents such as alcohols, especially ethanol, and acetic acid. Its salts, namely potassium sorbate finds a variety of applications in the food industry, due to its high solubility in water and exhibits an antimicrobial activity of 74%. The acid shows maximum absorbance, in aqueous solutions, at 254 nm, due to the coupled double bonds of carbonyl system in its molecule, and this is the most common wavelength chosen for its determination, as will be shown below. The conjugated double bonds of sorbic acid are susceptible to various reactions and this property directly affects the antimicrobial activity and the quality and safety of products where sorbic acid is added. [19, 20]

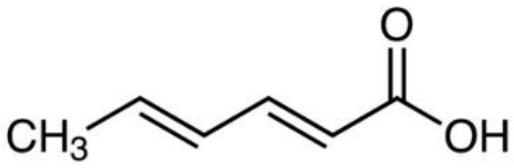

Figure 2: Structure of Sorbic Acid.

1.3.2 Mechanism of action and use in food industry

Sorbic acid and its salts (sodium, potassium and calcium) belong to the category of preservatives and are characterized by the coded names E200-E203, respectively. Their antimicrobial activity is stronger against yeasts and fungi than bacteria and over time, there have been many cases about the mechanism in which this preservative help with the inhibition of microorganisms' growth, similar to those mentioned above for benzoic acid.

First, it is mentioned the involvement of acid in the inhibition of spore germination in the binding reactions, through its action on spore membranes or on the protease enzymes involved in germination. The permeability of cell membranes is altered and becomes more fluid during this germination process and thus the membrane becomes sensitive to the acid. In addition, as spore reproduction involves the action of proteases and sorbic acid has the ability to inhibit enzymes with sulfhydryl groups, it can affect, in this way, the spore binding reactions. As follows, morphological changes in the cell and alterations in their membrane quality upon exposure of the microbial spores to the cells of sorbic acid are taking place, without the mechanism being fully known, leading to inhibition of microbial growth. Finally, many mechanisms have been proposed linking cell metabolism, transport functions and nutrient uptake to the action of sorbic acid and the inhibition of the electron transport system, ATP synthesis or action of transport enzymes. However, none of these researches have reached a decisive conclusion about the mechanism of action of sorbic acid as a preservative against microorganisms and further research is required. [20, 21]

Some examples of inhibited microorganisms by sorbic acid, are the yeasts and fungi genus *Brettanomyces, Candida, Cryptococcus*, *Alternaria, Aspergillus* and the bacteria genus *Acetobacter* and *Enterobacter*. More specifically, a previous study showed that 0.1% sorbic acid solution in vegetables, such as cucumber, is sufficient to inactivate the growth of yeasts, but also to inhibit the production of acids by the acetic bacteria, while concentration of 0.075% sorbic acid solution could proceed against the proliferation of *E. coli, Salmonella typhimurium* and

Pseudomonas spp. [9, 20] However, caution is needed, as many microorganisms can metabolize sorbic acid, under certain conditions, as a fatty acid through *β*-oxidation, or use it as a carbon source. [20]

Sorbic acid and its salts have been used as a food preservative for over 60 years on a variety of substrates. Some of these are dairy products, such as cheese and yogurt, fruits, vegetables and their juices, wines, carbonated drinks and meat products. In some cases, sorbates can partially or completely replace benzoate in foods with low pH, to avoid unpleasant taste caused by high benzoate concentration. Especially, for fruit juices, which is one of the main food substrates studied in this master thesis, the addition of 0.02%-0.05% sorbic acid solution is effective for high moisture content fruits, while the lower the moisture level, the smaller the quantity of sorbate required for food preservation. For soft drinks, the addition of 0.02%-0.10% sorbate solution is able to improve the shelf life of the product. [19, 20] For yeasts and other fungi, such as mold, the use of this particular preservative is important in products with low pH and/or intermediate water activity (a_w), such as carbonated beverages, salad dressings, syrups, tomato products, jams, jellies and chocolate syrup. [20]

As proven, the antimicrobial activity of sorbates is affected by a set of factors, such as composition, processing, pH, a_w and temperature, which may act either synergistically or competitively. Specifically, the antimicrobial activity increases as pH of substrate decreases, approaching its dimension constant (pKa = 4.75), and as aw decreases. Finally, the control and prediction of sorbic acid losses during food storage is important, as they directly affect the preservation of the product and these losses depend on sorbate levels, nature and pH of the food, amino acids, metal ions, light, antioxidants, moisture content, other present preservatives, packaging material and storage temperature. [9, 20]

1.3.3 Legislation

The most general assessment of sorbic acid and its salts is that they belong to GRAS category (Generally Recognized As Safe) by the FDA (Food and Drug Administration) of the United States, as the experiments on laboratory animals have demonstrated low acute, short-term and chronic toxicity, while do not show an effect on carcinogenesis and mutagenesis. The only observation is the rare causing of urticaria and dermatitis. [22, 23]

However, as with any food additive, it is necessary to determine the Acceptable Daily Intake (ADI) and for sorbic acid, this value is 25 mg/kg per body weight per day. However, some revisions have been made over time to this limit established by the joint FAO/WHO Committee of 1973 and should be noted. Specifically, in 2015, the European Food Safety Authority (EFSA), following a research into reproductive two-generation toxicity in laboratory animals after consumption of sorbic acid, set a provisional value of ADI at 3 mg/kg per body weight per day, for the acid and its potassium salt. From this value, the calcium salt is excluded, as there is a lack of genotoxicity data. This decision reviewed in 2019, and through an extended one-generation reproductive toxicity study (EOGRTS), the provisional ADI value is, finally, set at 11 mg/kg body weight per day. [24, 25]

The maximum limit for its addition to various food substrates is defined by the European Regulation 1333/2008, and is set at 500 mg/L for fruit juices, in which sugars have been added in quantity more than 200 g/L, at 300 mg/L for soft drinks and at 250 mg/L for soft drinks, when benzoic acid is also present, while for energy drinks the maximum value is set at 500 mg/kg, according to the 2021 revised version of the Codex Alimentarius Commission. Table 2 includes these permissible limits, which need continuously research, in an effort to improve both the maintenance of the product, but also any health effect of this additive to the consumer. [17, 18, 22]

Table 2: Maximum Permissible Limits of Sorbic Acid in substrates related to this thesis.

Substrates	Maximum Permissible Limit	Regulation
Fruit Juices	500 mg/L (sugars more than 200 mg/L)	EU 1333/2008
Soft Drinks	300 mg/L, 250 mg/L (if benzoic acid is present)	EU 1333/2008
Energy Drinks	500 mg/kg	CODEX STAN 192-1995 (Revised in 2021)

1.4 Parabens (Esters of p-hydroxybenzoic acid)

1.4.1 Structure and physicochemical properties

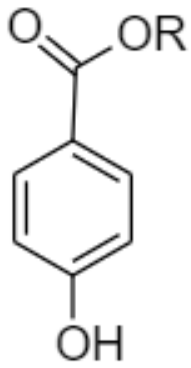

Figure 3: Structure of ester of p-hydroxybenzoate.

The last category of preservatives, studied in this master thesis, is the esters of *para*-hydroxybenzoate or 4-hydroxybenzoic acid or otherwise parabens. These esters represent a group of aromatic carboxylic acids, where there is a hydroxy group at position 4 with various alkyl radicals as side chains. Their structure is illustrated in Figure 3, where depending on the R group, the corresponding ester is formed. The most important of these esters are the following: Methyl p-hydroxybenzoate (MP, E218), Ethyl ester (EP, E214), Propyl ester (PP, E216) and Butyl ester (BP) (Figure 4). They are a stable, odorless and non-volatile category of compounds with white crystalline structure and exhibit a sufficient water solubility to act as preservatives. By increasing the alkyl chain length, the solubility of esters in organic solvents increases, which directly affects the desirable activity against microorganisms. In particular, propyl ester exhibits a remarkable solubility behavior in aqueous solution, with about 50% reduced solubility in mixtures containing the corresponding ethyl ester. Parabens show resistance to hydrolysis in cold and hot water, but also in acidic solutions, which depends directly on the pH. Above from pH=7, hydrolysis takes place, producing PHBA and the corresponding alcohol, where the corresponding carboxylic acid is ionized. Their antimicrobial activity is not so common and is limited to a certain number of food substrates, because of some doubts in their use

that arise, but they are important preservatives not only in the food industry, but also in cosmetics and medicines. [26, 27]

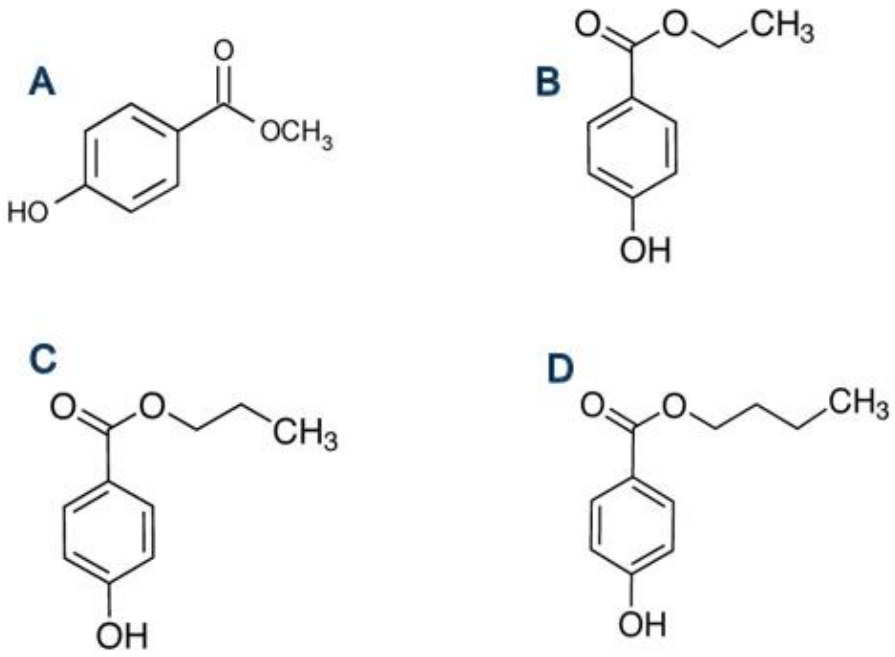

Figure 4: The structure of the best-known parabens. A: Methyl-(MP), B: Ethyl-(EP), C: Propyl-(PP), D: Butyl-(BP)

1.4.2 Mechanism of action and use in food industry

In food industry, the main parabens, as shown above, have the code names E214-E219, except for butyl ester, which is prohibited as food preservative in European Union according to Regulation 1333/2008, nor can it be used for plastic materials that come into contact with food (Food Contact Materials, FCM) according to Regulation 10/2011. [17, 28] Butyl-paraben is allowed to be used in food industry, only as a synthetic flavor, with good manufacturing practices in place, according to C.F.R. Regulation 172.515. [29]

Their action is antimicrobial and more effective against fungi than bacteria, and especially in gram-positive bacteria. Because their antimicrobial activity against gram-negative bacteria is limited, a second biocide, in addition to parabens, is

often added to food substrates. More specifically, studies have been reported the inhibition of *E. coli*, *Pseudomonas aeruginosa*, *Aspergillus niger*, and *Candida albicans* from parabens, and in this way this group of compounds plays an important role as preservative. [30]

The mechanism of inhibition of microorganisms' growth by parabens is probably related to the decomposition of the lipid bilayer membrane by inhibiting DNA/RNA synthesis or certain enzymes, such as ATPase and phosphotransferases, in certain species of bacteria. In this way, the transport processes and the function of the mitochondria are impeded. In particular, Propyl p-hydroxybenzoate (PP) is considered more active against most bacteria compared to the corresponding Methyl ester (MP), due to its higher solubility and permeability of the bacterial membrane, but further research is needed on the antimicrobial activity of parabens, as the high solubility can trigger the leakage of intracellular components. [1, 30, 31]

The antimicrobial activity of parabens depends in a variety of factors, such as the length and the branching of the ester chain. For example, if the length of the ester chain is increasing, there is an increased preservative effect, while any type of branching reduces the effectiveness of the action. However, caution is needed, as the longer the alkyl chain, the lower the water solubility of the compound, and since microbial replication generally occurs in the aqueous phase in the organic and aqueous phase distribution, the amount of paraben in the aqueous phase determines the effectiveness of the preservatives. Regarding the effect of pH, parabens have maximum antimicrobial activity at pH=4-8, while beyond the value of 8, ester hydrolysis can occur, which reduces the effectiveness of the action. [30, 32]

The existence and addition of parabens in the food industry is extensive in various food substrates and the amount varies from species to species, while packaging materials can be a source of parabens. Typical examples of food substrates where addition of parabens takes place are alcoholic or non-alcoholic beverages, fruit juices, cereals, meat and dairy products, while they are naturally present at low

levels in some food products, such as barley, strawberries, raisins, carrots and onions. More specifically, a research carried out by Liao's group (Liao et al., 2013) in the United States of America and China showed that the highest percentage of parabens was found in cereals (average value: 18.6 ng/g), followed by beverages (14.2 ng/g), while the lowest concentrations were found in fats and oils (0.27 ng/g) and fruits (0.83 ng/g), with Methyl p-hydroxybenzoate (MP) accounting for the largest percentage of 42-78% of all parabens. A study in Spain (Gálvez-Ontiveros et al., 2021) showed similar results, where the most frequently detected paraben was MP at 49%, followed by EP at 15.5% and PP at 10.2% in a total of 56 different types of samples. Regarding BP, it was detected in 13 food groups (jelly, black sesame powder, tea powder and coffee powder, etc.) with an average concentration of 0.005 ng/g, while its presence in beverages reaches 12.1%. [30]

1.4.3 Legislation

As it is mentioned above, according to Food and Drug Administration (FDA), Methyl *p*-hydroxybenzoate (MP) and Propyl *p*-hydroxybenzoate (PP), under Regulations 21 CFR 184.1490 and 21 CFR 184.1670 respectively, have been recognized as safe preservatives in food, for use at a level not exceeding 0.1 percent in accordance with good manufacturing practice, while the above parabens and Ethyl *p*-hydroxybenzoate (EP) are allowed to be used as antifungals in food packaging materials without limits or restrictions (Regulations 21 CFR 181.23 and 21 CFR 175.105). Conversely, the use of Butyl *p*-hydroxybenzoate (BP) as food preservative is prohibited. The permitted categories for the addition of parabens are defined by the European Union and are the following five: nuts, confectionery, liquid food supplements, jellies and coatings of meat products, while for other products, their use is constantly reviewed. [29, 34, 35, 36, 37, 39]

Due to possible adverse health issues parabens may cause in humans, research is taking place for the definition of a clear limit for their use and consumption by the different groups of people in the market. More specifically, concerns have been raised about the safety of parabens due to their potential of endocrine disruption, as it has occurred in both *in vitro* and *in vivo* studies. In addition, methyl, ethyl, and

propyl parabens have shown estrogenic activity *in vitro* experiments, but not *in vivo* assays, using high-dose oral or subcutaneous dispensations in mice and rats. The results were positive, however, when butyl paraben or isobutyl paraben, which are not used as food additives, were used for this experiment. [27, 38]

Regarding the reproductive system, several studies have demonstrated the influence of propyl paraben in male rats, even at the lowest dose of 10 mg / kg per kilogram of body weight per day. This amount has been defined as the Lowest Observed Adverse Effect Level (LOAEL) for this particular paraben by the Scientific Committee on Food (SCF). In contrast, methyl and ethyl paraben showed no effect at doses up to 1000 mg/kg body weight per kg body weight per day. Therefore, 1000 mg/kg bw/day was considered as the threshold dose (No Observed Adverse Effect Level, NOAEL) for both methyl paraben and ethyl paraben and the Acceptable Daily Intake (ADI) was established as 0-10 mg/kg per kilograms of body weight for the sum of methyl and ethyl esters and their sodium salts. The value does not include propyl ester and other parabens with longer alkyl side chains due to their potential endocrine disruption, their undefined NOAEL and their effect on the reproductive system. In 2015, the European Medicines Agency (EMA) reported evidence of adverse health effects associated with consumption of propyl paraben and set an ADI value of 1.25 mg/kg per kilogram of body weight per day. [38, 40]

In addition, Cantox Health Sciences International noted that parabens have no genotoxic, carcinogenic, or teratogenic effects and are rapidly hydrolyzed to *p*-hydroxybenzoic acid and excreted through the urine. [27]

Permissible limits for their use in food industry, have been set by Codex Alimentarius Commission and are set exclusively for energy drinks at 500 mg/kg for MP and EP, while there is no reference to other substrates related to this thesis. [18] It is important, although the effect of these additives has been determined to a point, that further research is carried out on the safe use of parabens in the food section for the protection of the consumers' health.

CHAPTER 2
LITERATURE REVIEW

2.1 Methods for the determination of benzoic acid, sorbic acid and four parabens in fruit juices, soft drinks and energy drinks

As it was seen from the previous chapter, the use of these three categories of food additives is necessary and very common. For this reason, the methods of analysis for the qualitative and quantitative determination of benzoic acid, sorbic acid and parabens in a number of matrices have been extensively studied. Some of them incorporate UV-Vis radiation, Calorimetry, Electrophoresis, Gas Chromatography (GC), Liquid chromatography coupled to Mass Spectrometry (LC-MS), with the most widespread being the use of High Performance Liquid Chromatography coupled to Diode Array Detector (HPLC-DAD), which was also used in this particular master thesis. Table 3 includes the literature review carried out for the methodologies achieving the simultaneous determination of the compounds of interest or their individual identification in fruit juices, soft drinks and energy drinks using HPLC-DAD. It contains the characteristics of the mobile and stationary phase, the wavelength in which the compounds were determined, limits of detection (LOD) and quantification (LOQ), linearity range, recovery rates and the sample preparation procedure. With these data, the goal was to develop and validate a new method for the simultaneous determination of these additives with better analytical parameters and to check compliance with the EFSA legislative limits in different Greek products (Chapter 2.3).

Table 3: Literature Review, where SB: Sodium Benzoate, PS: Potassium Sorbate, BA: Benzoic acid, SA: Sorbic acid, MP, EP, PP, BP: Methyl-, Ethyl-, Propyl-, Butyl Paraben. [41-69]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
1	Soft Drinks	SB	Luna C18 (250 x 4.60 mm, 5µm, 100 Å)	Isocratic elution, Acetic Acid:Isopropanol:H_2O (12:2:86)	254 nm	**LOD**=31.61(mg/L)	0-500 mg/L, R^2 = 0.9975	94.53-96.05%	1.Filtration 2.Ultrasonic bath, Voltrex, Stirring	[41]
2	Soft Drinks, Fruit Juices	SB, PS	Eclipse-XDB C18 (150 × 4.6 mm, 5 µm).	Isocratic elution, Ammonium Acetate Buffer (pH = 4.2): ACN (80:20)	225 nm	**SB: LOD**=0.6 **, PS: LOD**= 0.5, (mg/kg)	3.125–50 mg/kg, R^2=0.9	**SB**= 99.2-100.6%, **PS**=99.1-99.7%	1.Dissolution in deionized H_2O. 2.Addition IS, dilution with mobile phase. 3.Filtration	[42]
3	Fruit Juices	BA, SA, MP, PP	Capcell pak C18 (150 mm × 4.6 mm, 5µm)	Isocratic elution, MeOH: Ammonium Acetate Buffer (pH=4.4) (50:50)	254 nm	**LOD: BA**=5.46, **SA**= 1.08, **MP**= 3.65, **PP**= 1.60 (mg/L)	**BA**:20-170 ppm **SA**: 12-42 ppm, **MP** and **PP**: 10-60 ppm, R^2>0.992	98-100%	1.Dissolution in Mobile phase. 2.Centrifugation 3. Filtration	[43]
4	Soft Drinks, Fruit Juices	SB, PS, MP, PP	Supelcosil LC-18 (25cm × 4.6mm, 5µm)	Gradient elution, A: MeOH: Zinc Acet. Buffer (pH=4.4) (70:30), B: MeOH:	225 nm: **SB**, 254 nm: other	**LOD=** 0.2, (mg/L)	1-100 mg/L, R^2>0.9907	90.8-105.1%	1.Addition MeOH, NaOH, Water, Carrez I, Carrez II. 2.Ultrasonic bath,	[44]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
				Zinc Acet. Buffer (pH=4.4) (35:65).					Centrifugation, Filtration.	
5	Soft Drinks, Fruit Juices	BA, SA, MP, EP, PP, BP	Waters Symmetry C18 (4.6×75mm, 3.5µm)	Gradient elution, MeOH and 0.02M Ammonium Acetate Buffer	238 nm	**LOD: BA**=0.01, **SA**=0.003, **MP,EP,PP,BP**=0.01 (g/kg)	0.01-0.1mg/ml. R^2>0.9996.	**BA**: 93.5-102.9%, **SA**:94.1-101.5%, **MP**:95.3-101.2%, **EP**:94.8-103.8%, **PP**:95.7-100.6%	**Fruit Juice**: 1. Addition potassium ferricyanide, zinc acetate and H_2O. 2.Centrifugation,Filtration.	[45]
6	Soft Drinks	SB, SA	LiChrosorb RP18, (250 x 4.6 mm I.D., 10µm)	Gradient elution, 0.1 M KH_2PO_4 Buffer (pH=4): MeOH (95:5)	235nm for **BA**,250 nm for **SA**	**LOD**: 0.1–3.0 (mg/L)	5–500 mg/L, R^2>0.99	**BA**: 96.9%, **SA**:97.2%	1.Degassing 2. Filtration	[46]
7	Soft Drinks, Pomegranate juice	SB, PS	(ODS-) 3V (250mm × 4.6 mm, 5µm)	Gradient elution, 0.025M Sodium Acetate Buffer (pH=6):ACN.	230 nm	**LOD: PS**=0.12, **SB**=0.10, (µg/ml).	5–25 µg/ml, R^2>0.9985	97.67%-105.56%.	1.Degassing in ultrasonic bath 2. Dilution with Milli-Q H_2O and Filtration	[47]
8	Soft Drinks	SB, PS	HS LiChroCART RP-18 (30 mm x 4 mm; 3 µm)	Isocratic elution, MeOH:5m M	225.4 nm for **SB**. 255.4	**LOD:** 0.003 mg/L	**SB**:30-180 mg/L, **PS**: 62.6-374 mg/L	**SB**: 90.5-99.32%, **PS**: 86.46-96.05%	Filtration	[48]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
				Dipotassium Phosphate Buffer (pH=3.70) (20:80)	nm for **PS**.		R^2>0.9869			
9	Energy drinks and Soft Drinks	BA, MP, EP, PP, BP	Capcell Pak C18 (150mm × 4.6 mm i.d., 5 μm)	Gradient elution, ACN and aqueous solution of sulfuric acid (pH=2.3)	265 nm	**LOD: BA**= 2.70, **MP=**0.215, **PP=** 0.207, **BP=** 0.199, (ppb)	**BA** 100-300ppm, **MP** 16-24ppm, **PP** 16-24ppm, **BP** 16-24ppm, R^2>0.990	**BA:**>99.8 %, **MP:**>99.1 %, **PP:**>99.1 %, **BP:**>99.1 %	Filtration	[49]
10	Soft Drinks, Orange Juice	MP, EP, PP, BP	Inertsil/Wondasil-C18 (250x4.6 mm, 5 mm)	Isocratic elution,MeOH:0.02M Amonium Acetate Buffer (pH=4) (70:30)	254 nm	**LOD: MP=**1.52, **EP=**1.06, **PP=**0.32, **BP**=0.17 (ng/mL)	5–1000 ng/mL, R^2>0.9970	89.8-118.1%	Extraction Method: DLLME-SFO.	[50]
11	Soft Drinks	SB, PS, MP, EP, PP	Separon SGX C_{18} (150 mm × 3 mm i.d.; 5 μm)	Isocratic elution, 5mmol/L acetic acid, 40% ACN, (pH=5)	254 nm	**LOD: SB**:2.8, **PS**:0.25, **MP**:0.46, **EP**:0.5, **PP**:0.54 (mg/L)	**SB**:$5x10^{-5}$-$2x10^{-3}$mol/, **PS,MP,EP,PP**:$1x10^{-5}$-$2X10^{-4}$mol/L	100±5%	1.Degassing 2.Filtration	[51]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
12	Soft Drinks	SB, PS	RP18 (250 mm × 4.6 mm, 5 µm)	Isocratic elution, ACN:5 mmol/L Ammonium Acetate Buffer (40:60)	254 nm	**LOD: SB:** 0.1, **PS:** 0.08 (µg/ml)	1–500µg/mL, **BS:** R^2=0.999, **PS:** R^2=0.998	95.6%-111.2%	Extraction Method: DLLME.	[52]
13	Apple Juice	BA, SA, MP, EP, PP, BP	COSMOSIL 3C18-AR II (100 mm x 3.0 mm i.d., 3µm)	Gradient elution, A: MeOH:ACN:5 mmol/L Citrate Buffer(1:2:7),B:MeOH/ACN(1:2).	230 nm: BA, SA, 260 nm: PHBs	**LOQ: BA,SA**= 10, **PHBs**= 5 **(mg/kg)**	0.5-10 µg/mL (**BA, SOA**), 0.25-5 µg/mL (**PHBs**), R^2>0.999	>95%	Extraction Method: Quencher method	[53]
14	Soft Drinks, Fruit Juices	BA, SA	WondaSil C_{18} (200 mm× 4.6 mm i.d., 5µm)	Gradient elution, A: H_2O with 20 mmol/L Ammonium Acetate, B: ACN	223 nm **BA**, 252 nm **SA**. **ATLD method**: 210 nm	**ATLD method: LOD: BA=**42.4, **SA=**4.8, (ng/ml)	0-12µg/ml, R^2>0.9977	**ATLD method:BA:** 92.3%, **SA:** 95.7%	**Soft Drinks** 1.Degassing 2. Dissolution in MeOH 3. Filtration. **Fruit Juices** 1. Centrifugation And Dissolution of supernatant in MeOH 3.Filtration	[54]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
15	Soft Drinks, Fruit Juices	BA, SA	Supleco C_{18} (250× 4.6 mm, 5µm)	Gradient elution: A: 0,05 mol/l Am. Acetate Buffer (pH 4.5) and B: ACN.	230nm for BA, 260nm for SA	**LOD: BA=**0.72, 5.31 µg/ml, **SA=**3, 8.97 µg/ml respectively	25-750µg/ml	**-**	1.Degassing, Dissolution in deionized H_2O: MeOH 1:2(v/v). 2.Stirring and Filtration	[55]
16	Fruit Juices	BA, SA	C_{18} (ACE 5 mm, 4.6 x 250 mm)	Isocratic elution, Acetic Acid:MeOH (pH=4.74, 65:35)	250 nm	**LOD: BA=**0.017, **SA=**0.025 (mg/kg)	0.2–10.0 mg/mL, **BA:** R^2= 0.9999, **SA:** R^2= 0.9998	**BA:** 98.5±2.3%, **SA:** 99.4±4.5%	1.Addition 35% MeOH. 2.Ultrasonic bath, Addition 35% MeOH. 3.Filtration	[56]
17	Orange Juice, Soda	SB, PS	ACE-121-1504 C_{18} (15 cm x 3.9 mm I.D., 5 µm).	Isocratic elution, Ammonium Acetate Buffer:ACN (72:28)	**SB:** 225 nm, **PS:** 255 nm	**LOD: SB=**0.023, **PS=**0.012, (mg/L)	0.1-100 mg/L, R^2= 0.9999	**SB:** 95–96%, **PS:** 93-94%	1.Centrifugation 2. Dissolution supernant in Mobile phase. 3.Filtration	[57]
18	Fruit Juices	BA	Supelco C_{18} (250 mm × 4.6 mm, 5 µm)	Isocratic elution, Ammonium Acetate Buffer (0.05 M): MeOH (70:30)	230 nm	**LOD:** 0.15, (mg/L)	0.5–50 mg/L, R^2=0.996	93-117%	Extraction Method: HA-LLME	[58]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
19	Fruit Juices	SB, PS	Supelcosil LC-18 (25 cm x 4.6 mm, 5 mm)	Isocratic elution, Ammonium Acetate Buffer 5mM (ph=4.2):ACN (90:10)	**SB:** 225 nm, **PS:** 255 nm	**LOD:** 10mg/L for both	2.5-100 mg/L, R^2=0.994-0.998	**SB**: 86-96%, **PS:** 82-93%	1.Dissolution in Mobile phase. 2.Filtration	[59]
20	Soft Drinks	BA, SA, MP, PP	Supelco 516 C_{18} (15 cm×4.6 mm, 5μm)	Gradient elution, Ammonium Acet.Buffer 50mM (pH=4.4): MeOH	254 nm	**LOD: BA=**0.5 **SA=**0.1 **MP=**0.3 **PP=**0.1 (mg/L)	**BA:** 5.0–120, **SA:** 1.0–75, **MP:** 3.0–100, **PP:** 1.0–75 (mg/L)	**BA:** 112%, **SA:** 105%, **MP:** 109%, **PP:** 106%	1.Dissolution in MeOH. 2.Ultrasonic bath and Voltrex 3.Filtration	[60]
21	Fruit Juices	SB, PS	PerfectSil Target C_{18} (250 × 4.6 mm, 5 μm)	Isocratic elution, ACN: Am. Acetate 5 mM (pH=4.4) (40:60)	254 nm	**LOD: SB=**0.1, **PS=**0.08. (μg/mL)	1-500 μg/ml **SB:** R^2=0.9990, PS**:** R^2=0.998	**SB:** 108.5% **PS:** 103.7%	1.Centrifugation, supernatant collection and Dissolution in H_2O. 2.DLLME	[61]
22	Soft Drinks, Fruit Juices	BA, SA, MP, EP, PP, BP	Inertsil ODS-SP (250 mm x 4.6 mm, 5 mm),C_{18} guard column (25 mm x 4.6 mm, 5 mm)	Gradient elution, A: Ammonium Acetate Solution 0.1M (pH=7.2), B: mix	230 nm	**LOD: BA=**0.011, **SA,MP,EP,PP=**0.005, **BP=**0.007 (μg/mL)	**BA, SA:** 0.1–300μg/mL **MP:** 0.1–297μg/mL **EP:** 0.1–306μg/mL **PP:** 0.1–	**-**	Modified Chinese official analytical method GB 7718-2004	[62]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
				MeOH:ACN (90:10).			300μg/mL R^2=0.99			
23	Soft Drinks	MP, EP, PP, BP	Agilent C_{18} column (150 mm× 4.6 mm, 3.5 μm)	Gradient elution, A: MeOH B: H_2O	260 nm	**LOD: MP=**0.0030, **EP=**0.0023, **PP=**0.0016, **BP=**0.0052 (mg/L)	1–500 mg/L, R^2=0.9997-0.9998	85.1%–95.4%	1.Addition NaCl,Ammonium Acetate buffer and ACN. 2. Collect of ACN phase, repeat process and evaporation 3.Purification με LC-C_{18}. 4.Filtration	[63]
24	Cola	BA, SA	YMC ODS-Pack AM (250 x 4.6 mm I.D., 5μm	Isocratic elution, ACN: AmmoniumAcetate Buffer 0.005 M (pH=4.0) (15:85)	**BA:** 225 nm, **SA:** 256 nm	**LOD: BA:** 2.4 (μg/g)	**BA:** 6.25–75 μg/mL, R^2=0.9996	**BA:** 98%	1.Degassing 2. Dilution in Mobile phase.	[64]
25	Fruit Juices	BA, SA	MC-Triart C_{18} (250 × 4.6 mm, 3 μm)	Gradient elution, A: Sodium formate 25 mM(pH=4.4), B: ACN.	235 nm	**LOD: BA:** 0.0364, **SA:** 0.0367 (μg/mL)	0.1-10 mg/L**, BA:** R^2=0.9987, **SA:** R^2=0.9975	**BA:** 100.7–102.6%, **SA:** 98.42–104.1%	1.Centrifugation, Dissolution in H_2O and adjustment to pH=2 with HCl. 2.DLLME	[65]

	Substrate	Specified compounds	HPLC Column	Mobile phase	λ (nm)	LOD/LOQ	Linearity Range, R^2	Recovery rates (%)	Sample preparation	Reference
26	Soft Drinks	BA, SA, MP, EP, PP, BP	Capcell pak MF-C_8 (4.6 × 150 mm, 5 µm)	Gradient elution, A: 0.1% TBA-OH (0.1% phosphoric acid), B: ACN	217 nm	**LOD: MP, EP:** 0.1, **SA,BA:** 0.11, **PP,BP:** 0.12 (mg/L)	1-50 mg/L, R^2=0.9999	81.1-113.7%	1.Dissolution in extraction solvent,Ultrasonic bath. 2.Addition Carrez I and II, extraction solvent. 4. Collection of supernatant. 5.Filtration.	[66]
27	Sport Drinks	BA, SA	Eclipse- XDB C_{18} (150 × 4.6 mm, 5 µm).	Isocratic elution, Ammonium Acetate (pH = 4.2) and ACN (80:20 v/v)	225 nm	**LOD: SB:** 0.6413, **PS:** 1.1224, (mg/L)	R^2=0.999	**SB:** 99-102%, **PS:** 98-100%	1.Filtration 2.Ultrasonic bath	[67]
28	Beverages	BA, SA	CAPCELL PAK MF-C8 (Shiseido, SG 804.5 µm, 4.6×150 mm),	Gradient elution, A: TBAOH 0.1%, B: ACN	217 nm	**LOD: BA:** 0.03, **SA:** 0.07 (µg/mL)	0.47-30 µg/mL, R^2=0.99	-	1.Addition of H_2O, 15% tartaric acid, NaCl, H_2O 2. Distillation, dissolution in 1% NaOH, Filtration.	[68]
29	Beverages	SA, BA, MP, EP, PP	CLC-ODS (250mm×4.6mm i.d., 5.0 µm) with guardian column	Isocratic elution, ACN/Acetate(pH =4.2) (40:60)	254 nm, except BA	**LOD:** 0.04-0.11 (mg/kg)	0.05 to 50 mg/L,R^2= 0.99	80-110%	1.Degassing 2. QuEChERS	[69]

2.2 Literature Review Conclusions

As it is shown in Table 3, the determination of the 6 preservatives (benzoic acid, sorbic acid and parabens) is done by using High Performance Liquid Chromatography coupled to DAD and specifically Reversed Phase Chromatography (RPC), either using isocratic or gradient elution. In RPC, the stationary phase is less polar than the mobile, and for this reason stationary phase is a silica which has been surface-modified with RMe_2SiCl, where R is usually a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17} (C_{18} or C_8 column respectively) or other various groups such as phenyl, diols, amino groups, cyano groups, etc. The mobile phase consists of mixtures of organic solvents [mainly, methanol (MeOH) and acetonitrile (ACN)] with aqueous buffer solutions. Examples of these buffers are ammonium/zinc/sodium acetate buffer, monopotassium and dispotasium phosphate buffer, sodium citrate and sodium formate buffer.

In terms of the wavelength that is chosen for the determination, it is the one at which the target compound shows maximum absorbance. In this context, detection of benzoic acid is performed at 225, 230, 238 and 254 nm, of sorbic acid at 225 and 254 nm and of parabens at 254 and 260 nm, as it is reported at Table 3.

Sample preparation plays an important role at the detection and determination of analytes using liquid chromatography. Various methods for the preparation of fruit juice samples, soft drinks samples and energy drinks samples are mentioned in the literature, some of which involve a simple extraction with stirring, centrifugation and filtration, or the use of DLLME-SFO (Dispersive Liquid–Liquid Microextraction based on the Solidification of Floating Organic drop) or the use of QuEChERs method.

Finally, method's performance is verified by certain validation parameters that have to be within certain limits. These performance characteristics are the following: Accuracy (Recovery rate), Precision (Repeatability and Reproducibility), Detectability (Limit of Detection, LOD and Limit of Quantification, LOQ), Linearity (Linearity Range), Specificity and Robustness. The first four were calculated during the validation of our method and were the ones that were targeted for improvement

in relation to the values found in the literature review (Table 3). In the majority of the literature references the column temperature was not considered as critical parameter, so the analyses took place at ambient temperature.

Therefore, after consulting and carrying out an extensive study of the available literature, the use of gradient elution was chosen, where mobile phase is a mixture of ammonium acetate buffer and ACN and stationary phase is column C_{18}. The wavelength for the targeted compounds were the following: 230 nm for benzoic acid and 254 nm for sorbic acid and the four parabens.

2.3 Aim of Master thesis

It is clear that the use of preservatives in the food industry must be controlled and minimized as much as possible. The three aforementioned categories of preservatives: benzoic acid (Subsection 1.2), sorbic acid (Subsection 1.3) and the four most used parabens (Subsection 1.4) strongly participate in this effort to maintain food safety and quality, however due to some potential complications to the consumers' health, certain levels of their addition and/or natural presence in various products have been set.

Bearing in mind the high and widespread consumption of fruit juices, soft drinks and energy drinks, especially by more sensitive groups, such as children, the aim of this thesis is to develop, optimize and validate a method, according to the European protocols, for the qualitative and quantitative determination of preservatives in these three matrices. The goal of this work is to find a quick and accurate method for the determination of these preservatives and to check for potential disregard of the legal limits in products of Greek origin using Reversed Phase High Performance Chromatography coupled with Diode Array spectrophotometry (RP-HPLC-DAD), taking into consideration the conditions and the method validation parameters of the previous researches (Table 3).

CHAPTER 3
INSTRUMENTATION AND REAGENTS

3.1 High Performance Liquid Chromatography coupled with Diode Array Detector (HPLC-DAD)

The technique that was used as part of this research was High Performance Liquid Chromatography (HPLC) coupled with Diode Array Detector (DAD). The most important principles of HPLC are mentioned below.

One of the ways to separate, identify and determine related compounds in complex mixtures is the use of chromatography, which plays an important part in instrumental analysis. More specifically, the sample is dissolved in a mobile phase, which could be a gas, liquid, or supercritical fluid. Through an immiscible stationary phase, which is immobilized in a column or on a solid surface, the mobile phase is forced to pass. The separation and distribution of the analytes between the two phases are depending on the type of interaction the compounds have with these phases and this is why the choice for the stationary and mobile phase is an important step in method development. When there is strong retention of substances in the stationary phase, the analytes move slowly into the mobile phase, while the opposite happens in the case of weak retention by the stationary phase. The quantification of the solutes arise from the difference in migration rates i.e. amount of time a compound spends in the stationary phase relative to the time it spends in the mobile phase. [70]

Liquid chromatography refers to all chromatographic techniques in which the mobile phase is a liquid. Depending on the type of separation, liquid chromatography can be separated as follows: [70]

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Molecular exclusion Chromatography
- Affinity chromatography

In this thesis, the interest will be focused on Partition Chromatography (Section 3.1.1).

3.1.1 Partition Chromatography

In partition chromatography, the stationary and mobile phase are both in liquid form and specifically, the stationary phase should not react with the liquid mobile phase. There are two categories in partition chromatography: liquid-liquid and liquid-bonded-phase chromatography. In the first case, the stationary phase is held on the packaging particles by physical adsorption and in the latter by chemical bonding. Over time, the use of bonded-phase methods becomes more frequent because of their greater stability and compatibility with gradient elution. [70]

The most used supports for this type of packings are prepared from silica, and especially modified silica compositions. These are siloxanes, that are formed by reaction of the hydrolyzed surface with an organo-chlorosilane and they are characterized by a R group, which is an alkyl group or a substituted alkyl group. [70]

There are two basic categories, in which partition chromatography could be divided into, based on the relative polarities of the mobile and stationary phases:

- Normal phase chromatography: when stationary phase is highly polar (triethylene glycol or water) and the mobile phase is less polar (hexane or i-propyl)
- Reversed phase chromatography: the mobile phase (water, MeOH, ACN, or tetrahydrofuran) is more polar than the stationary (hydrocarbon).

In this way, depending on the type of the chromatography, the relative polarity is changing and so the elution time is varying. Due to the high polarity of the stationary phase in normal phase chromatography, the least polar component is eluted first and while increasing the polarity of the mobile phase, the elution time is decreased. With reversed-phase chromatography, however, the opposite is happening. [70]

3.1.2 Reversed Phase Chromatography

The separation mechanism in reversed phase chromatography is based on the hydrophobic binding interaction between the molecule, dissolved in the mobile phase, and the immobilised hydrophobic ligand, i.e. the stationary phase. Below, the main characteristics of stationary and mobile phases in reversed-phase chromatography are summarized.

3.1.2.1 The medium of Reversed Phase Chromatography

The medium of a reversed phase chromatography is mainly comprised of hydrophobic ligands, which are chemically bonded to a porous, insoluble beaded matrix. The base matrix should be characterized by chemical and mechanical stability and the most used reversed phase media are made of silica or a synthetic organic polymer such as polystyrene. The most ordinary ligand on the surface of a silica-based reversed phase medium is the octadecyl group (Fig. 5). [70, 71]

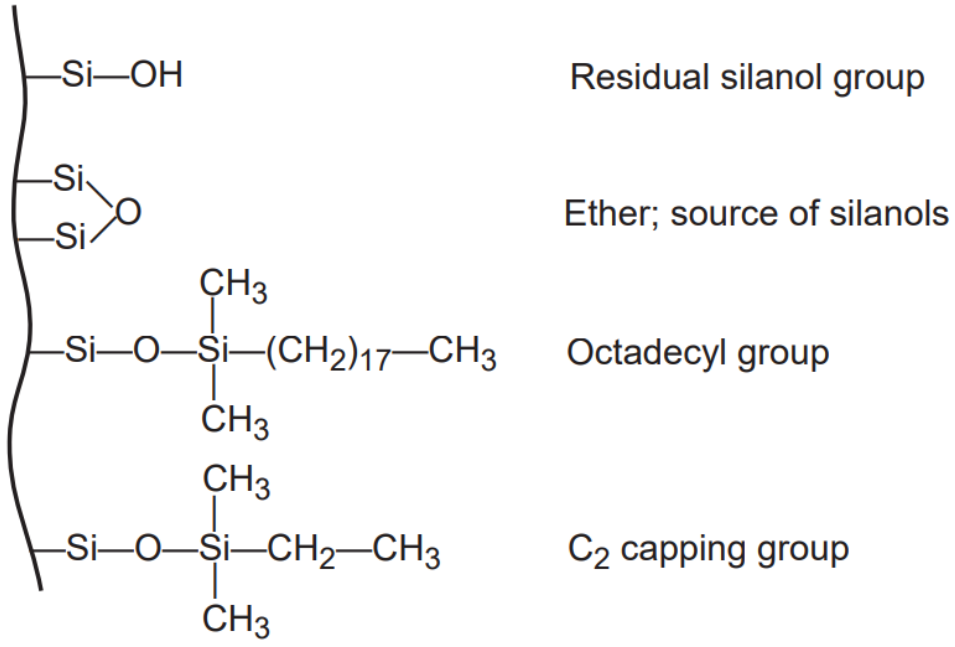

Figure 5: Most used ligands for a silica-based reversed phase medium. [70]

There are plenty of factors that affect the selectivity of silica-based media and some of them are:

- properties of the ligand
- mobile phase composition
- different processes for production of silica-based matrices

As for the composition of the silica gel, there could be simple derivatisation with ligands, containing various carbon chain lengths. In this way the carbon content, the surface density and distribution of the immobilised ligands are able to be controlled during the synthesis part. Regarding of other properties of the matrix, the silica gel matrix is sensitive at higher pH than 7.5, as it could dissolve. [71]

As it is mentioned before, polysterene is an alternative choice for the matrix of reversed phase media and in contrast with silica gel matrix, polystyrene is stable, regardless of the pH value. Therefore, its use is important, in cases where, separations are performed above pH 7.5, resulting in greater retention selectivity. [71]

Lastly, the porosity and the particle size of the matrix beads are crucial points for the determination of the available capacity for solute binding by the medium. More specifically, when pore sizes are approximately 100 Å, these mediums are used predominately for small organic molecules and peptides, and when pore sizes are 300 Å or greater, it is for the purification of recombinant peptides and proteins. As for the particle size of the beads, 3 and 5 μm beads are chosen for small analytical scale, while for larger scale preparative applications particle sizes of 15 μm and greater are usually performed with. [71]

3.1.2.2 The ligands of the medium in Reversed Phase chromatography

The type of ligand grafted to the surface of the medium affects greatly the selectivity of the chromatography and some of the most popular ones are linear hydrocarbon chains (n-alkyl groups) (Fig. 6). In order to achieve adequate separation, the more hydrophilic molecules are, the more hydrophobic immobilised ligands are required. [70, 71]

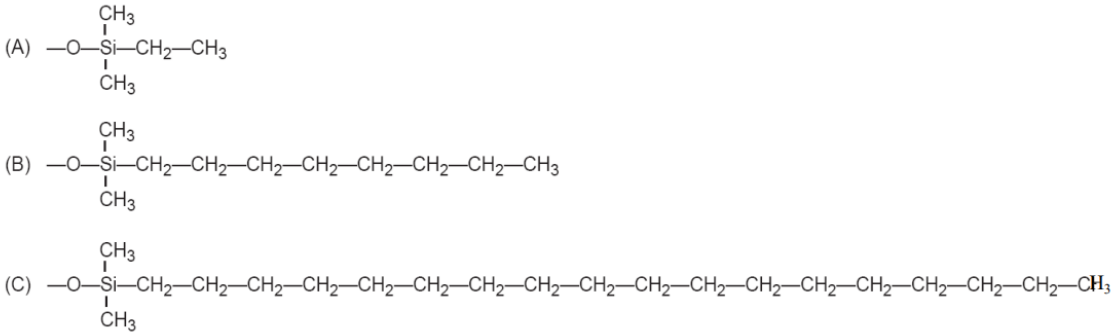

Figure 6: n-alkyl hydrocarbon ligands. (A) Two-carbon capping group, (B) C_8 ligand, (C) C_{18} ligand. [70]

The coupling of the alkyl groups on the surface of the silica gel takes place through silanol groups using chlorotrialkylsilane reagents (Fig. 7). All silicified phases have some percentage of residuals of unreacted silanols and acts as secondary site for other interactions, i.e extraction or retention of highly polar analytes or impurities, but can also bind irreversibly other analysts of interest. More specifically, the steric hindrance of C_{18} and C_8 reagents prevents complete derivatisation of all the available silanol groups. In order to reduce these damaging side effects of the unreacted silanol sites, the residual silanol groups could react with other smaller alkylsilane reagents, such as chlorotrimethyl- and chlorotriethylsilanes and in this case the steric effects do not interfere with complete coverage of the silanol groups remaining on the surface of the silica gel (“end-capping”). [70, 71]

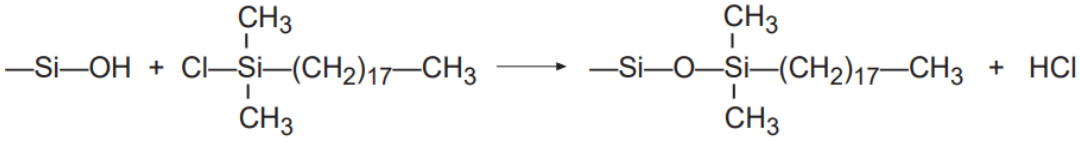

Figure 7: Substitution of silica with C_{18} chains with chlorotrialkylsilane reagent. [70]

3.1.2.3 Mobile phase in Reversed Phase Chromatography

In Reversed Phase Chromatography, mobile phase is more polar than the stationary phase and is usually a mixture of an aqueous buffer solution and an organic modifier.

The proper choice of buffer solution could result, in terms of buffering species, ionic strength and pH, in irreproducible retention and tailing in separation of polar and ionizable compounds. In this way, pH and its effects on analyte retention, solubility in the organic modifier and its effect on detection, are very important. The preferred pH usually implies the buffer choice. Since buffers control pH best at their pKa, it is crucial that the buffer possess a pKa near to the target pH, and this must be between 2 and 8 for silica-based packings. Moreover, the buffer must be adequately transparent in this area for conventional UV detection. The most used buffer solutions are phosphate, formate and acetate. [70,72]

The aqueous mobile phase's polarity is decreased by the addition of the organic solvent (modifier). In reversed phase chromatography, the mobile phase's eluting strength increases with decreasing polarity. Properties including miscibility with water, polarity, UV cut-off, viscosity, and safety must all be taken into account when choosing an organic solvent. Tetrahydrofuran (THF) and isopropanol (IPA), for instance, both have high elution strengths and can be helpful. However, the use of IPA is constrained by the substance's high viscosity, which results in poor performance and high back pressures. In contrast, THF can damage pump seals as well as peek tubing and fittings, and it must be stabilized with substances like BHT to avoid peroxide production. The two organic modifiers of preference for many reversed-phase applications are ACN and MeOH. However, each solvent has benefits and drawbacks that must be considered. ACN, for instance, is far more expensive than MeOH, which is also less harmful. However, the advantage

of ACN, having a lower UV cut-off than MeOH (190 nm vs 205 nm), is making it more suitable for the most applications that are requiring low UV detection wavelengths. Lastly, ACN/water mixes have lower viscosity than MeOH /water mixes and therefore lower back pressures across the column are going to be generated. [70,72]

3.1.3 Instrumentation of HPLC

Modern HPLC essentially comprises of the following main components namely, as it is shown in Fig 8.: [70]

- Solvent reservoir

  They contain the mobile phase, which is used to carry the sample through the column. The solvents should, first, be filtered through an inlet filter in order to withdraw any damaging particles that could possibly cause blockage of the column.
- Degassing system

  Dissolved gas will be removed through degassing system and therefore bubble formation is avoided and improved quantitative analysis is expected.
- Pump

  The dual function of the pump contains the passing of the mobile phase through the column at high pressure or at a constant a controlled flow rate. About mobile phase pump and elution system two categories are distinguished: Isocratic and Gradient elution. In the first case, constant solvent blend to separate and elute a sample's components, unlike the latter case, the solvent ratio is changed during separation in a programmed manner (continuously or step by step).
- Sample injection system

  They permit the introduction of the sample into the HPLC system and could be divided into three basic categories: loop, septum and stop flow injector. The most common is the loop injector, which enables the loading of the sample solution into the loop with the help of syringes. In the second category, the sample is injected by inserting the needle through the rubber

septum and in the latter case, the sample introduced at the top of the packing, when the column reaches an ambient pressure.

- Chromatographic Column

  As it was mentioned in Section 3.1.2.1, an analytical column allows the primary sample separation to occur, based on the differential attraction of the sample components for the mobile phase and the packing material within the column. Sometimes, a guard column is often included just prior to the analytical column, in order to chemically remove components of the sample that would otherwise block the main column.

- Detector

  After the analytical column, the separated components pass through the detector. The main function of the detector is the monitoring of the mobile-phase coming out of the column, which in turn emits electrical signals that are directly proportional to the characteristics either of the solute or the mobile-phase. Some of the most important types of detectors are: Refractive-index detectors, UV-detectors, Fluorescence Detectors and Amperometric detector. There can be many times a combination of multiple detectors. The one discussed below is Diode Array Detector-UV detector.

- Strip-chart recorder

  Its fuction is to record signals from the detector over time using a potentiometric recorder.

- Data handling device and microprocessor control.

  Lastly, the information is properly taken care of and saved in the computer system. It is common to use microprocessors in analytical equipment nowadays, which help with modernizing and automating processes, as well as making sophisticated devices easier to use.

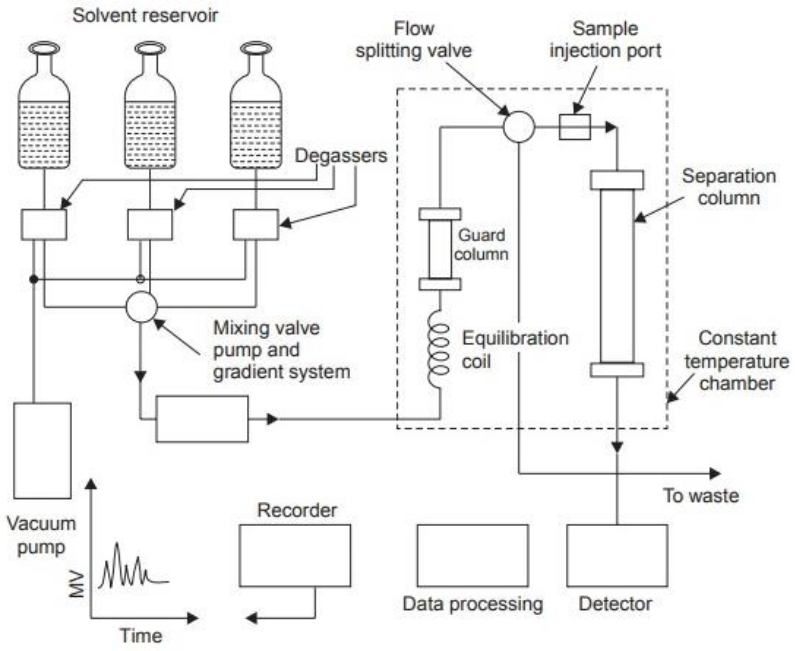

Figure 8: HPLC Instrumentation Flow Chart. [70]

3.1.4 Diode Array Detector (DAD)

The detector that was equipped the chromatographic system that used in the present work, was a Diode Array Detector (DAD). In this particular detector, two lamps are used as a light source: deuterium discharge lamp (D_2 lamp) with the wavelength of its light ranging from 190 to 380 nm (ultraviolent spectrum) and a tungsten lamp (W lamp) for further detection (visible spectrum). DADs differ from UV-VIS detectors in that light from the lamps is shone directly onto the flow cell, passes through the flow cell and is dispersed by the diffraction grating. For each wavelength, the amount of the dispersed light is estimated in the photodiode arrays (Fig. 9). [73]

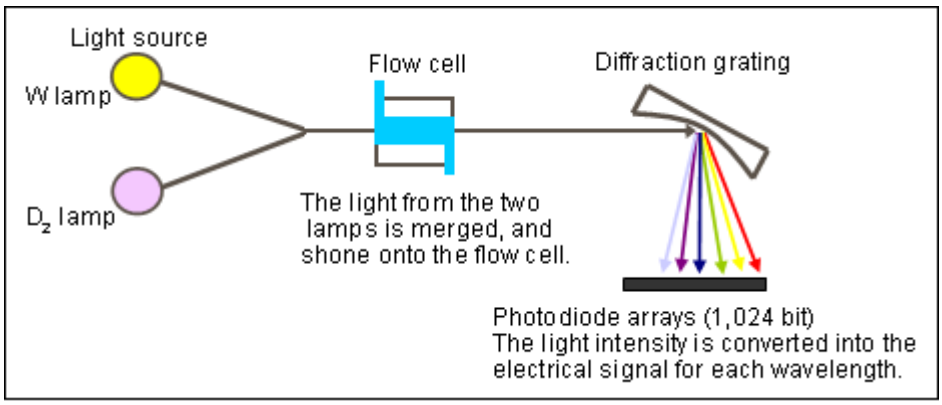

Figure 9: Diagrammatic illustration of DAD system. [71]

To address the needs of this master thesis, a Shimadzu Corporation system was used and was equipped with the below components:

- Degassing Unit DGU-20A5R
- LC-20AD Solvent Delivery Unit with possibility of gradient elution of four solvents
- Sample Injector Rheodyne 7725i (228-32210-93) (20 μL)
- SPD-M20A Photodiode Array Detector (PDA)
- LG Computer with LC Solution software for system programming and operation, as well as for processing of the chromatograms
- NUCLEOSIL (Machery-Nagel) 100-5 C_{18} column (150mm x 4.6 mm, 5μm)
- Hamilton syringe, 700 series, fixed needle (50 μL)

3.2 Laboratory Equipment

The laboratory equipment that was used includes:

- Calibrated analytical precision balances up to four decimal places (KERN & SOHN GmbH)
- Vortex Mixer (Velp Scientific)
- Centrifuge Basic Mod (NEYA 8)
- Degassing machine Elmasonic S 15 (H)
- Microliter pipettes for aqueous solvents 1000-5000 μL, 100 - 1000 μL, 10 - 100 μL (Witeg)

- Pipette tips (FL medical)
- HI2002-02 edge®- pH/ORP meter (Hanna Instruments)
- Volumetric flasks 5 mL, 10 mL, 1 L
- Volumetric cylinders of 10 mL, 50 mL,100 mL, 500 mL, 1 L
- Pasteur pipettes (Labbox)
- Lab spatulas
- Falcon centrifuge tubes 50 mL
- Beakers 25 mL, 50 mL, 100 mL
- Eppendorf tubes of 1.5 and 2 ml
- PVDF Syringe Filter (0.45 μm pore size) (Membrane Solutions)

3.3 Solvents and reagents

- ACN HPLC gradient grade (J.T. Baker™)
- Water HPLC gradient grade (Macron Fine Chemicals™)
- Ammonium Acetate (Penta Chemicals Unlimited)
- Acetic acid glacial (Sigma-Aldrich)
- Magnesium Sulphate anhydrous pure (PanReac AppliChem)
- Sodium Chloride (Lach-Ner)
- Tri-sodium Citrate dihydrate AGR (Labkem)
- Disodium hydrogen citrate sesquihydrate (Glentham)
- For the method development, MeOH HPLC (Honeywell) grade was also used.

3.4 Standards

Standard solutions were used for each analyte:

Benzoic acid (99.9%), Sorbic acid (99.9%), Methyl Paraben (99.6%), Ethyl Paraben (99.84%), Propyl Paraben (99.9%) were purchased form HPC Standards GmbH. Butyl Paraben (>99%) was provided from Thermo Scientific.

3.5 Preparation of stock and working standard solutions

A 1000 μg/mL concentration standard stock solution was prepared for each analyte by weighing 0.010 g into a 10 ml volumetric flask and adding suitable solvent, as follows:

- Benzoic Acid: 0.010 g in 10 mL H_2O
- Sorbic Acid: 0.010 g in 10 mL H_2O
- Methyl Paraben: 0.010 g in 10 mL ACN
- Ethyl Paraben: 0.010 g in 10 mL ACN
- Propyl Paraben: 0.010 g in 10 mL ACN
- Butyl Paraben: 0.010 g in 10 mL ACN

Standard stock solutions were kept in dark amber vial and stored at -20 °C, in order to remain stable for a long period of time. Working standard mix solutions for calibration curves ranging from 0.5 to 100 μg/mL (BA, SA) and from 0.25 to 50 μg/mL (PHBs) were prepared from the intermediate standard solution [BA, SA (250 μg/mL) and PHBs (125 μg/mL) in 50/50 ACN/H_2O] and diluted in a final volume of 80:20 H_2O:ACN, using pipettes for aqueous solvents. Working standard mix solutions for checking the matrix matched recovery [BA, SA (0.25-5 μg/mL each) and PHBs (0.125-2.5 μg/mL each) in 80:20 H_2O:ACN] were, also, prepared by serial dilutions from intermediate standard solutions [BA, SA (10 μg/mL) and PHBs (5 μg/mL)]. Lastly, for the determination of LODs, working standards mix solutions [BA (0.2 μg/mL), BP (0.26 μg/mL) and the rest (0.1 μg/mL) in 80:20 H_2O:ACN] for fruit juices and [BA (0.2 μg/mL), SA, PHBs (0.1 μg/mL) in 80:20 H_2O:ACN] for soft drinks were prepared by serial dilutions from intermediate standard solutions [BA, SA (100 μg/mL) and PHBs (50 μg/mL)].

CHAPTER 4
METHOD DEVELOPMENT AND OPTIMIZATION

The liquid chromatographic (LC) conditions were optimized, as to achieve sufficient retention for all analytes and separate them from the other matrix constituents, minimizing matrix effects. In this Chapter, the process and the conditions, that have been ultimately chosen for the method development and validation for the determination of the six preservatives: benzoic acid, sorbic acid and four parabens in fruit juices, soft drinks and energy drinks are listed in detail.

4.1 Stationary Phase characteristics

As it was observed in the literature review (Chapter 2), the most frequently used stationary phase is silica which has been surface-modified with RMe_2SiCl, where R is usually a straight chain alkyl groups, such as $C_{18}H_{37}$ or C_8H_{17} (C_{18} or C_8 column respectively). Therefore, this kind of column is what it was used in this thesis. Two different dimensions of C_{18} columns were tested:

- 5 μm particle size, L × I.D. 250 mm × 4.6 mm (NUCLEOSIL Machery-Nagel)
- 5 μm particle size, L × I.D. 150 mm × 4.6 mm (NUCLEOSIL (Machery-Nagel)

Finally, the column NUCLEOSIL (Machery-Nagel) 100-5 C_{18} column (150mm x 4.6 mm, 5μm) was selected, as to increase the speed of analysis.

4.2 Optimization of Mobile Phase composition

Optimization of chromatographic parameters is of major importance to achieve the most noteworthy sensitivity of the method as well as the separation of the analytes. In liquid chromatography, the most determining parameter is the composition of the mobile phase and therefore it is the first parameter to be optimized.

The mobile phase in Reversed Phase Chromatography is, usually, a mixture of an aqueous buffer solution and an organic modifier (Subsection 3.1.2.3). Studying the work of previous research groups (Table 3), 230 nm was selected for the determination of benzoic acid and 254 nm for the five other analytes (Subsection 4.5), as well as 5mM ammonium acetate (pH = 4.2) was chosen as the aqueous

buffer solution. The pH was close or lower than the pKa of the identified compounds, so that they are in the undissociated form for better detection and quantification. As for the organic modifier, two solvents were tested for the method development: MeOH and ACN.

Elution Programs with MeOH

Initially, MeOH was tested in various elution programs, in order to choose the one with the best results. Stationary phase was 5 μm particle size, L × I.D. 250 mm × 4.6 mm (NUCLEOSIL Machery-Nagel) and the mix standards (10 and 100 μg/mL) for the method development were dissolved in 50:50 MeOH:H_2O. Below, brief descriptions of each elution program, where MeOH was used, as well as characteristic chromatograms are presented.

- Elution Program 1: A: Ammonium Acetate 5mM Ammonium acetate (pH = 4.2) and B: MeOH in Isocratic elution (50:50), 1 mL/min, 38 min.

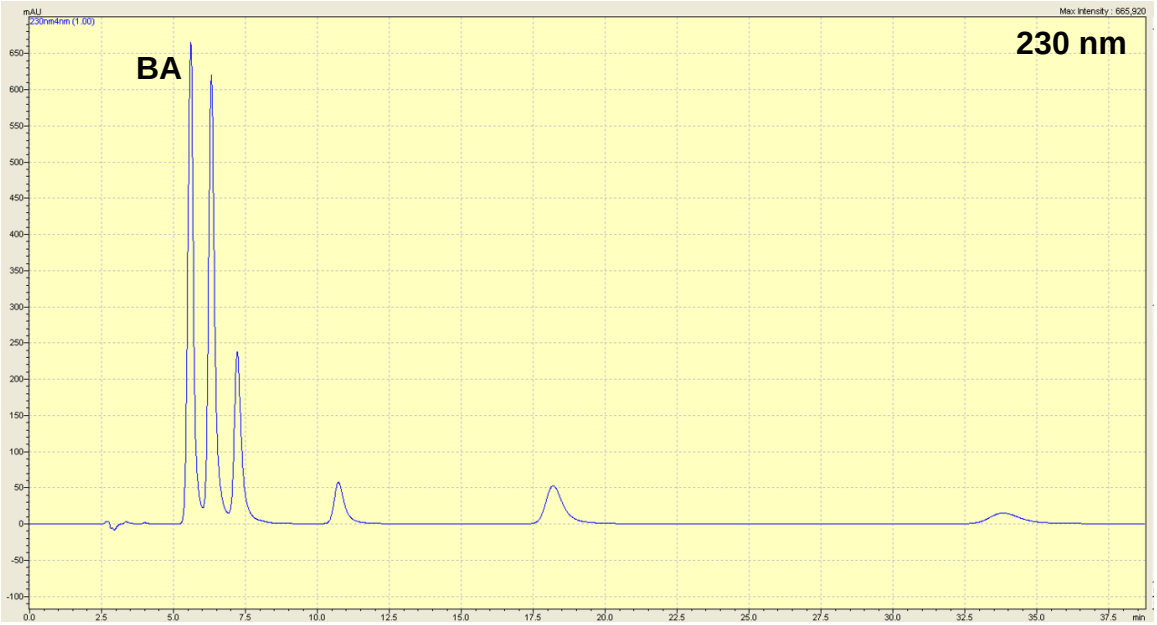

Figure 10: Chromatogram of Mix standard (10 μg/mL in 50:50 MeOH:H_2O), Elution Program 1 in 230 nm.

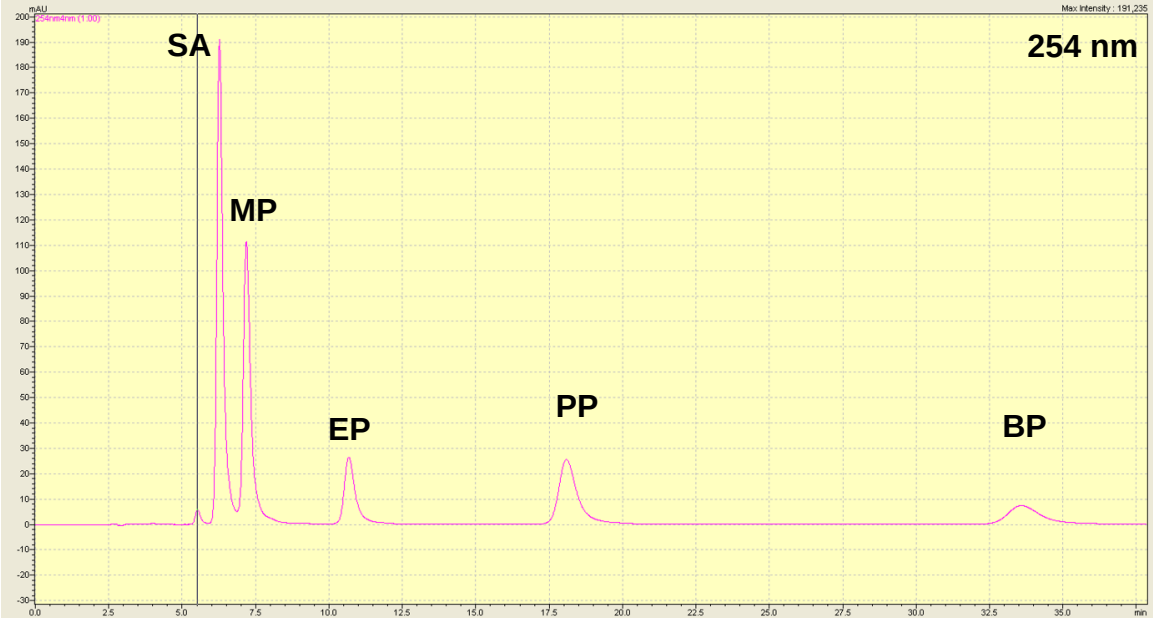

Figure 11: Chromatogram of Mix standard (10 µg/mL in 50:50 MeOH:H_2O), Elution Program 1 in 254 nm.

As it is shown in the chromatograms (Fig. 10 and 11), it is evident that the specific isocratic conditions could not achieve separation of BA, SA and MP and the analysis duration is prolonged. In this way, gradient elution program was necessary to test.

- Elution Program 2: A: Ammonium Acetate 5mM (pH = 4.2) and B: MeOH in gradient elution (Table 4), 1 min/mL, 30 min.

Table 4: Gradient Elution Program 2.

Time (min)	Mobile Phase Composition
0-20 min	Gradual increase of 30% B to 70 % B
20-30 min	Return to initial conditions

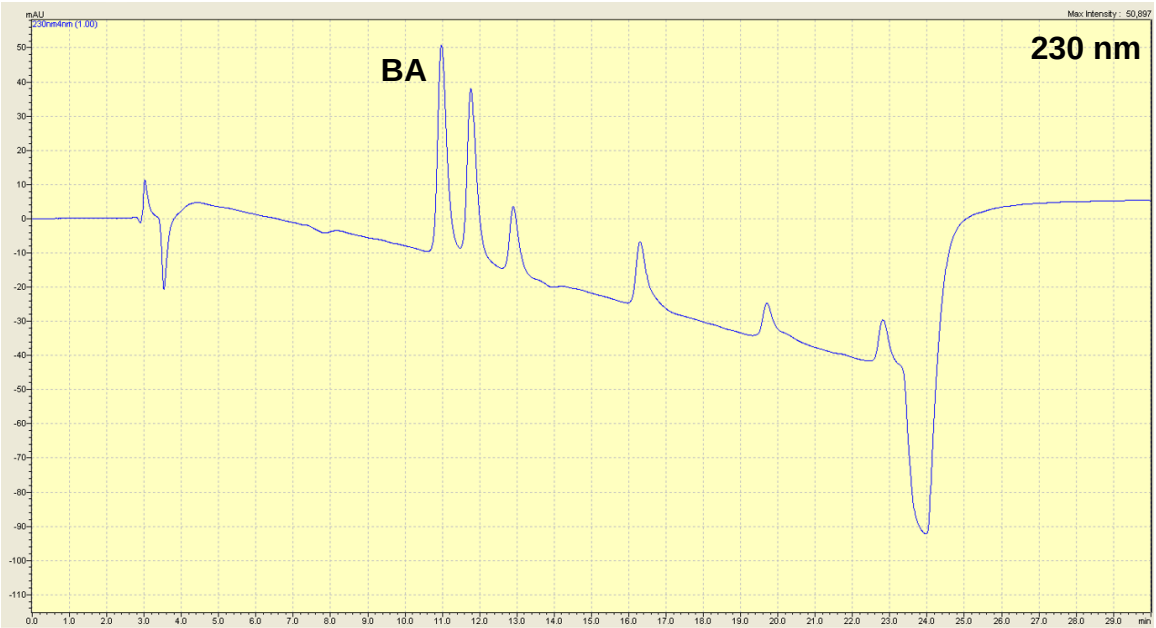

Figure 12: Chromatogram of Mix standard (10 µg/mL in 50:50 MeOH:H_2O), Elution Program 2 in 230nm.

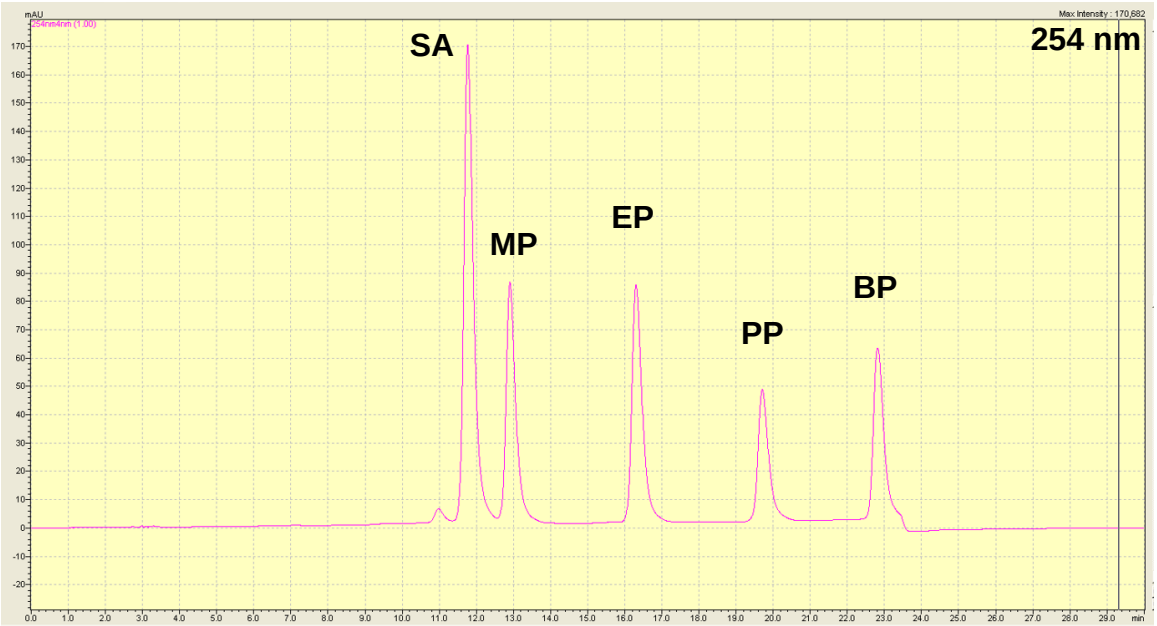

Figure 13: Chromatogram of Mix standard (10 µg/mL in 50:50 MeOH:H_2O), Elution Program 2 in 254 nm.

Regarding the above chromatograms (Fig. 12 and 13), the separation of BA, SA and MP is, still, not sufficient, elution time is prolonged, while BP is eluted after returning the composition of the mobile phase in initial conditions. For this reason, an increase in the percentage of organic solvent at the beginning (to separate the three analytes) as well as an increase in the percentage of MeOH at the end was implemented (Table 5).

- Elution Program 3: A: Ammonium Acetate 5mM (pH = 4.2) and B: MeOH in gradient elution (Table 5), 1 min/mL, 25 min.

Table 5: Gradient Elution Program 3.

Time (min)	**Mobile Phase Composition**
0-15 min	Gradual increase of 40% B to 80 % B
15-25 min	Return to initial conditions

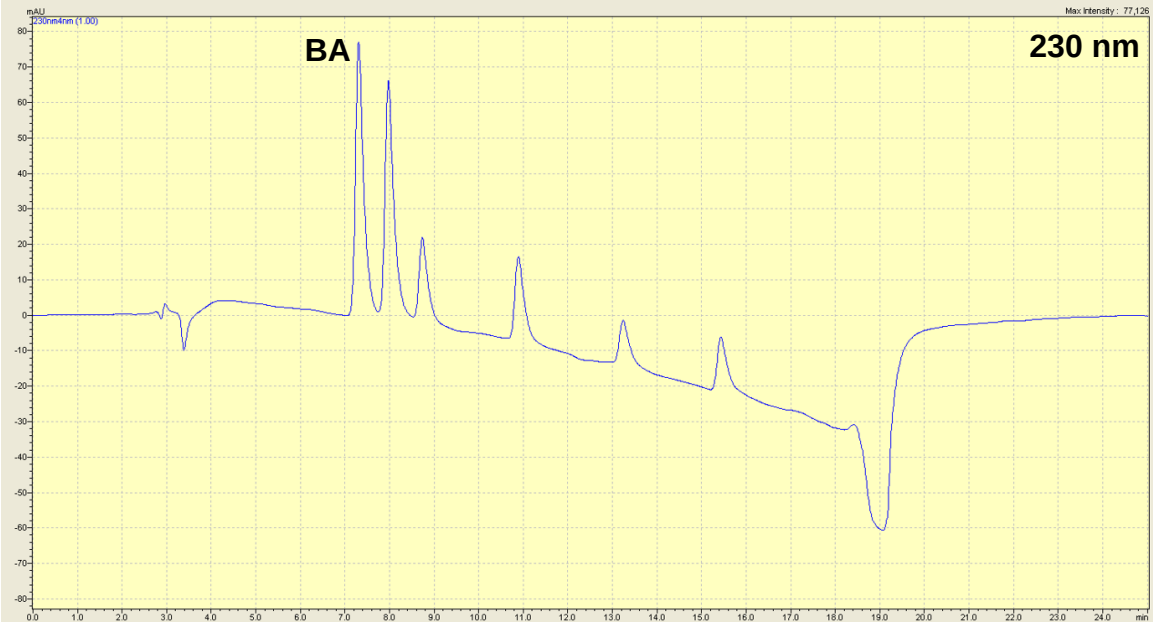

Figure 14: Chromatogram of Mix standard (10 µg/mL in 50:50 MeOH:H_2O), Elution Program 3 in 230 nm.

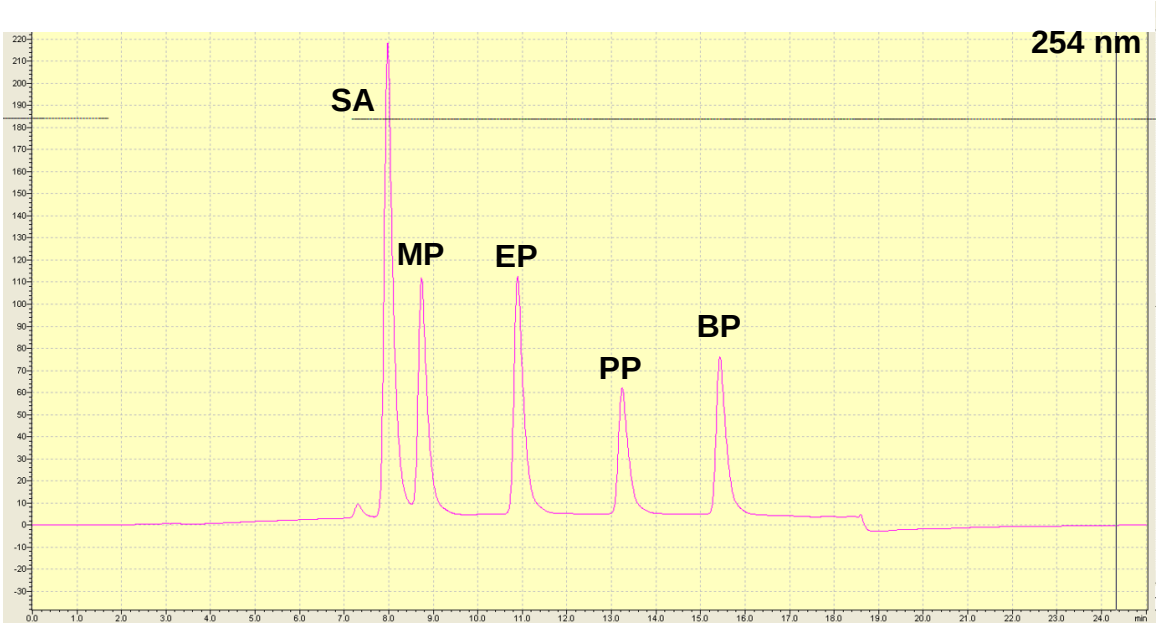

Figure 15: Chromatogram of Mix standard (10 µg/mL in 50:50 MeOH:H_2O), Elution Program 3 in 254 nm.

Although the elution times were reduced, the separation of the first three analytes is not satisfactory and BP does not elute in the analysis time. The last test carried out using MeOH as an organic modifier, was as follows:

- Elution Program 4: A: Ammonium Acetate 5mM (pH = 4.2) and B: MeOH in gradient elution (Table 6), 1 min/mL, 30 min.

Table 6: Gradient Elution Program 4.

Time (min)	Mobile Phase Composition
0-19 min	Gradual increase of 20% B to 80 % B
19-30 min	Return to initial conditions

Figure 16: Chromatogram of Mix standard (10 µg/mL in 50:50 MeOH:H_2O), Elution Program 4 in 230 nm.

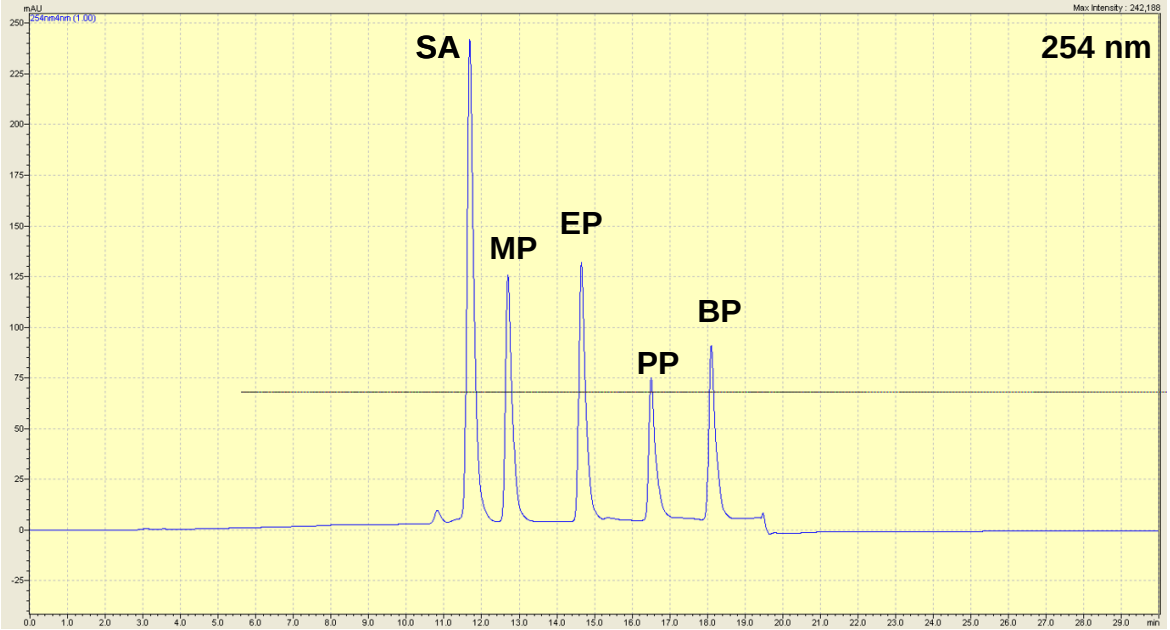

Figure 17: Chromatogram of Mix standard (10 μg/mL in 50:50 MeOH:H_2O), Elution Program 4 in 254 nm.

Best separation of BA, SA and MP was achieved, as well as all the analytes elute in the analysis time. Nevertheless, ACN has a higher dipole moment and is more predominantly acidic, compared with MeOH's lower dipole moment, more basic character and so lower elution strength. Also, it is worth mentioned that ACN has low UV cut-off, lower viscosity, higher boiling point, and in this way it was worth considering its use to compare the results between the two solvents.

Elution Programs with ACN

As before, ACN was tested in various elution programs, in order to choose the one with the best results. Stationary phase was 5 μm particle size, L × I.D. 150 mm × 4.6 mm (NUCLEOSIL Machery-Nagel).

Below, three elution programs, using ACN as organic modifier, are presented.

- Elution Program 5: A: Ammonium Acetate 5mM (pH = 4.2) and B: ACN in gradient elution (Table 7), 1 min/mL, 30 min.

Table 7: Gradient Elution Program 5.

Time (min)	Mobile Phase Composition
0-16 min	Gradual increase of 20% B to 80 % B
16-30 min	Return to initial conditions

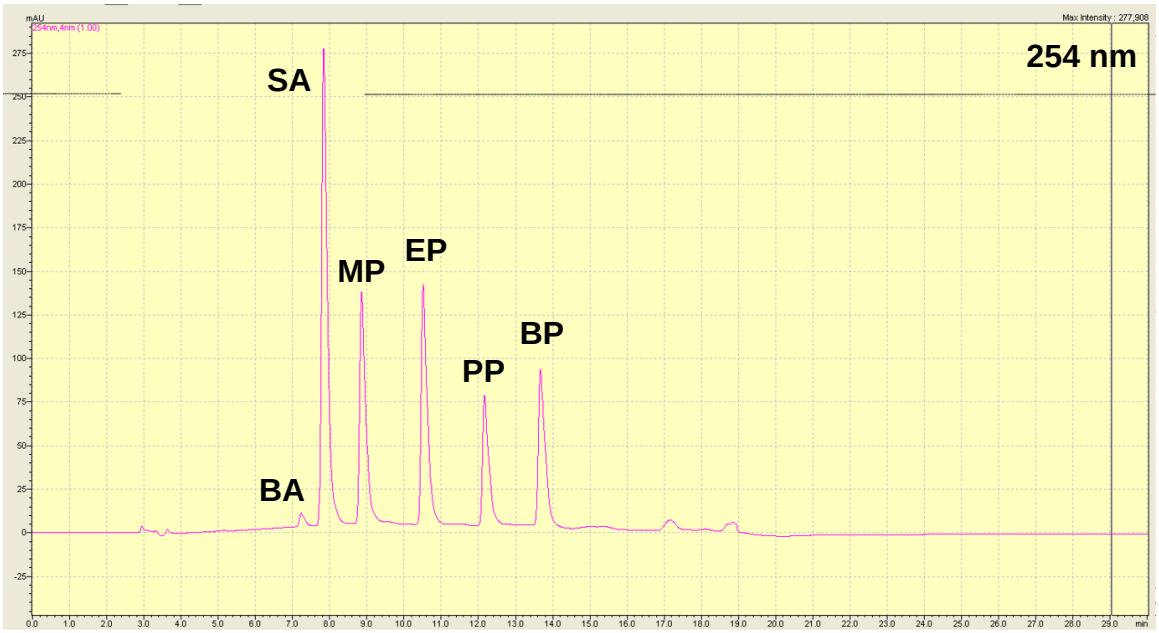

Figure 18: Chromatogram of Mix standard (10 μg/mL in 50:50 ACN:H_2O), Elution Program 5 in 254 nm.

The problem in the specific conditions was that when the percentage of the organic modifier was increased, an increase in tailing was observed in the last two peaks (PP, BP). Therefore, the next change involved a lower percentage of organic solvent.

- Elution Program 6: A: Ammonium Acetate 5mM (pH = 4.2) and B: ACN in gradient elution (Table 8), 0.8 min/mL, 30 min.

Table 8: Gradient Elution Program 6.

Time (min)	Mobile Phase Composition
0-19 min	Gradual increase of 20% B to 60 % B
19-30 min	Return to initial conditions

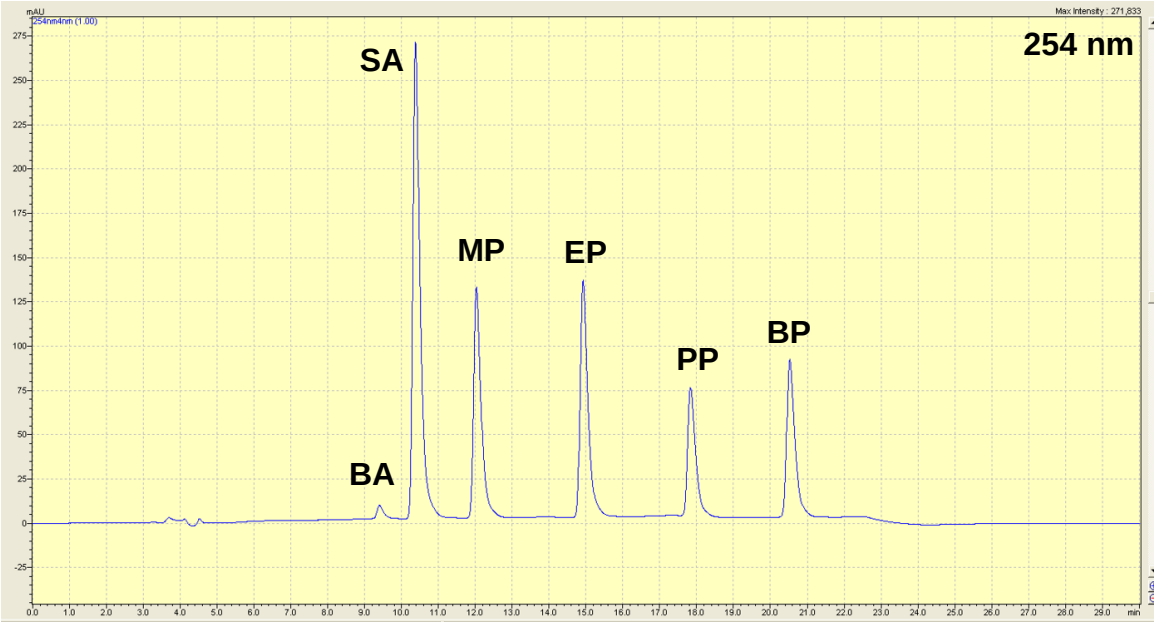

Figure 19: Chromatogram of Mix standard (10 µg/mL in 50:50 ACN:H_2O) Elution Program 6 in 254 nm.

Tailing was decreased, but BP was eluted after the changing in the initial conditions of the analysis, so the there was a last minimal change in the composition of the mobile phase.

- Elution Program 7: A: Ammonium Acetate 5mM (pH = 4.2) and B: ACN in gradient elution (Table 9), 0.8 min/mL, 30 min.

Table 9: Gradient Elution Program 7.

Time (min)	Mobile Phase Composition
0-19 min	Gradual increase of 20% B to 70 % B
19-30 min	Return to initial conditions

Figure 20: Chromatogram of Mix standard (10 µg/mL in 20:80 ACN:H_2O), Elution Program 7 in 230 nm.

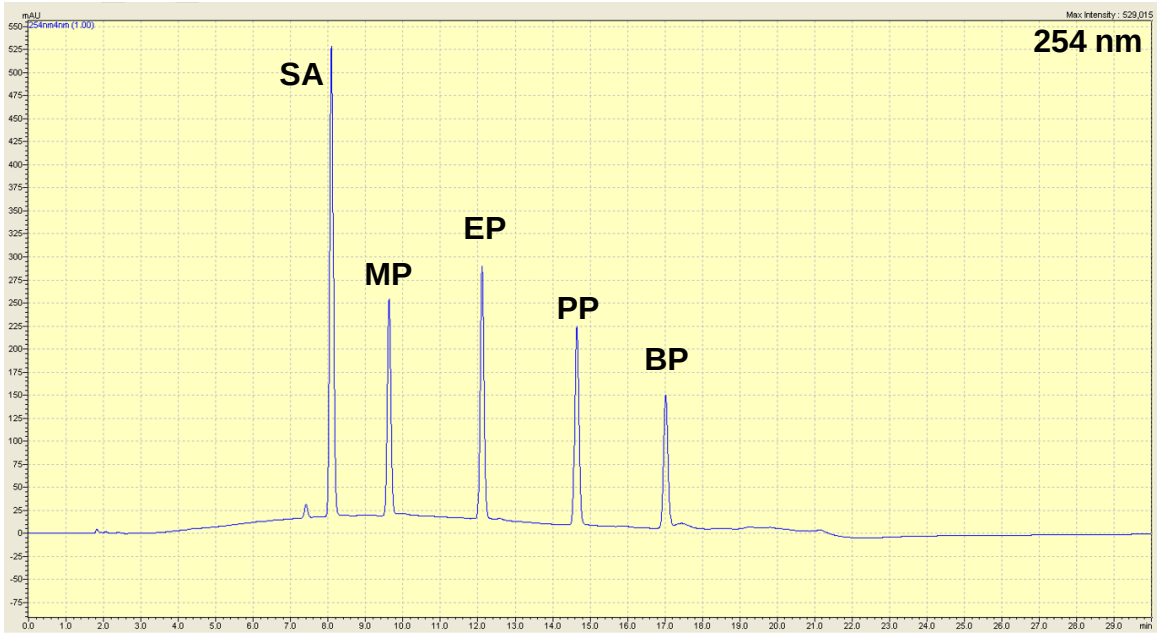

Figure 21: Chromatogram of Mix standard (10 µg/mL in 20:80 ACN:H_2O), Elution Program 7 in 254 nm.

These conditions offer sufficient and satisfactory separation between the six analytes within the analysis time, fast analysis time, sharp shape of peaks and high signal intensity, and for these reasons, comparing all the chromatograms and their

parameters, Elution Program 7 was what was decided to be implemented and validated for the rest of the course of this master's thesis.

4.3 Optimization of Standards Solvent

The mix standards (10 µg/mL) for the method development were tested, being dissolved in 50:50 MeOH:H_2O (Fig. 22), 50:50 ACN:H_2O (Fig. 23), and 20:80 ACN:H_2O, resulting that 20:80 ACN:H_2O was the best choice, as those were the initial conditions of the final, chromatographic method (Elution Program 7). This solvent resulted in sharper and more symmetrical peaks with no tailing.

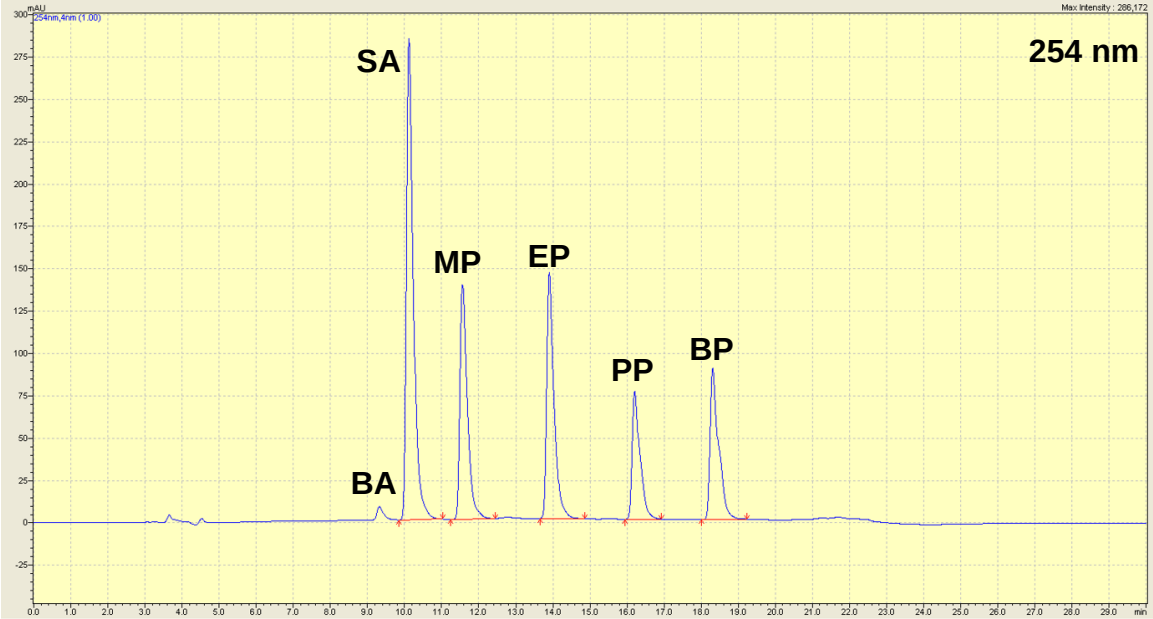

Figure 22: Chromatogram of Mix standard (10 µg/mL in 50:50 MeOH:H_2O), Elution Program 7 in 254 nm.

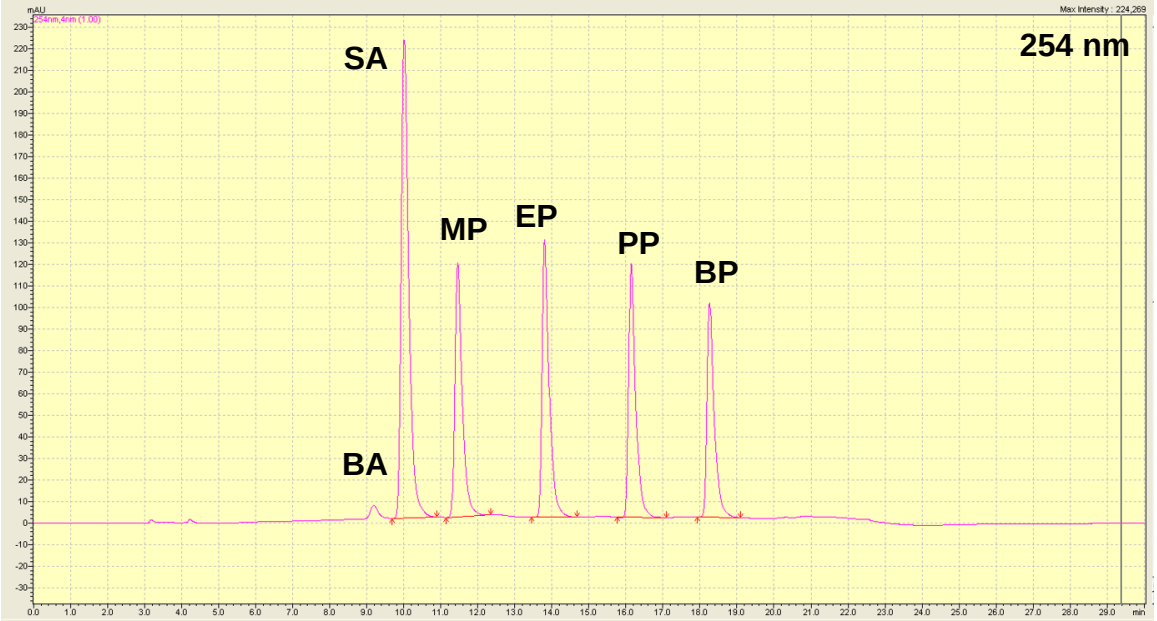

Figure 23: Chromatogram of Mix standard (10 µg/mL in 50:50 ACN:H_2O), Elution Program 7 in 254 nm.

4.4 Optimization of Flow rate

Another parameter that was checked during the development of the method is the flow rate. Three different rates (0.8, 1 and 1.2 mL/min) were tested and the corresponding chromatograms are presented below.

- Elution Program 7: Flow rate: 1 mL/min

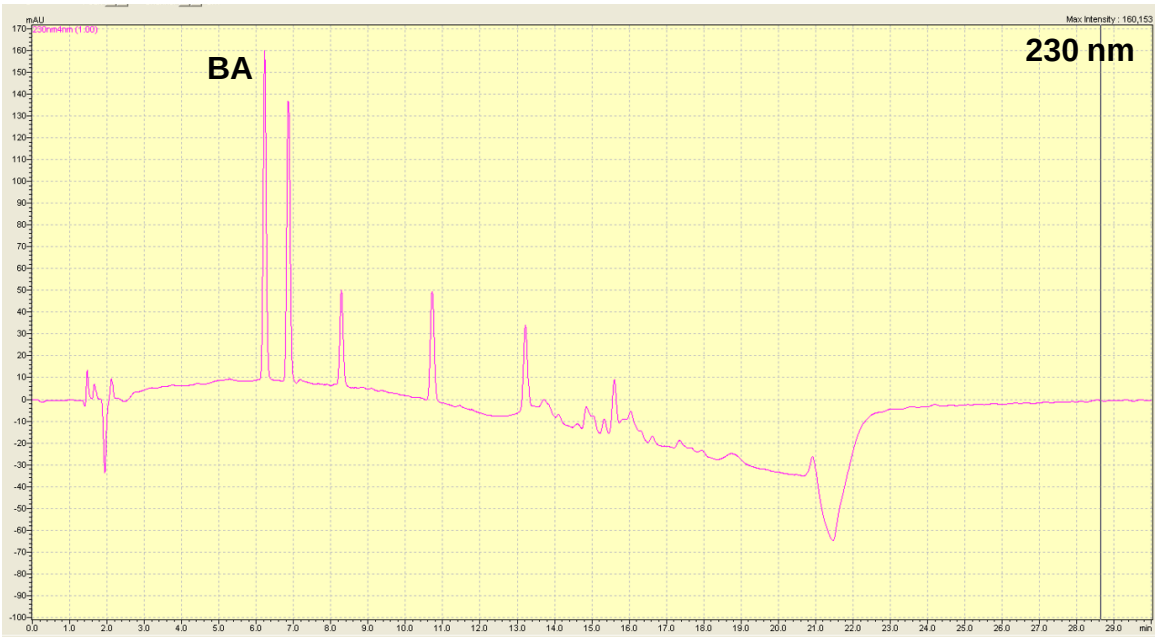

Figure 24: Chromatogram of Mix standard (10 µg/mL in 20:80 ACN:H_2O), Elution Program 7, Flow rate: 1 mL/min, 230 nm.

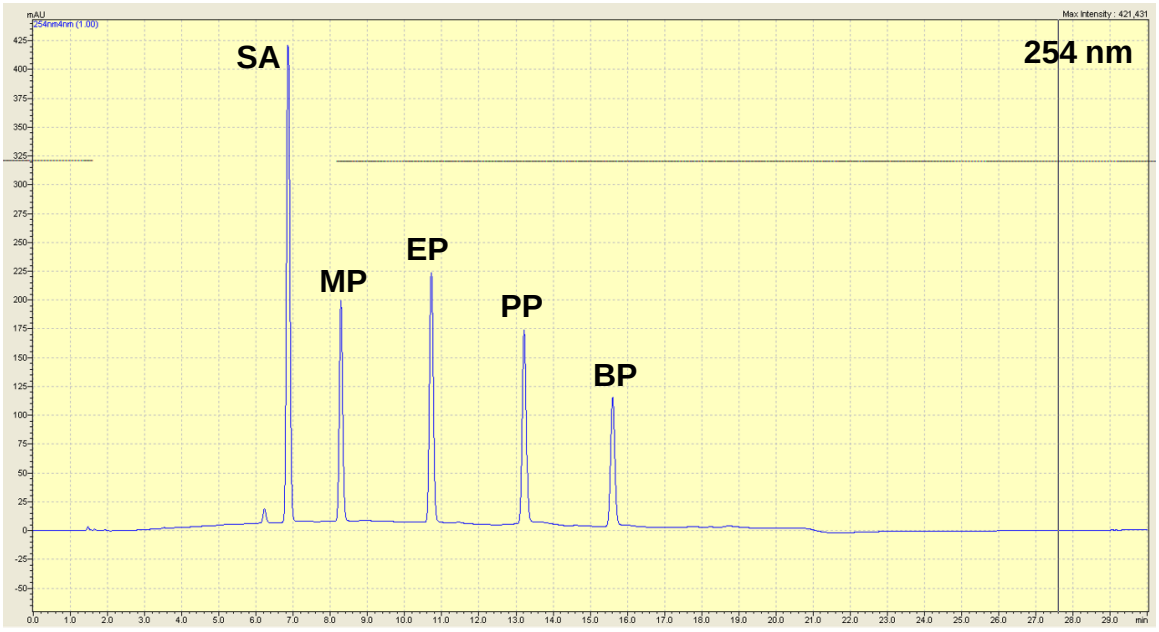

Figure 25: Chromatogram of Mix standard (10 µg/mL in 20:80 ACN:H_2O), Elution Program 7, Flow rate: 1 mL/min, 254 nm.

- Elution Program 7: Flow rate: 1.2 mL/min

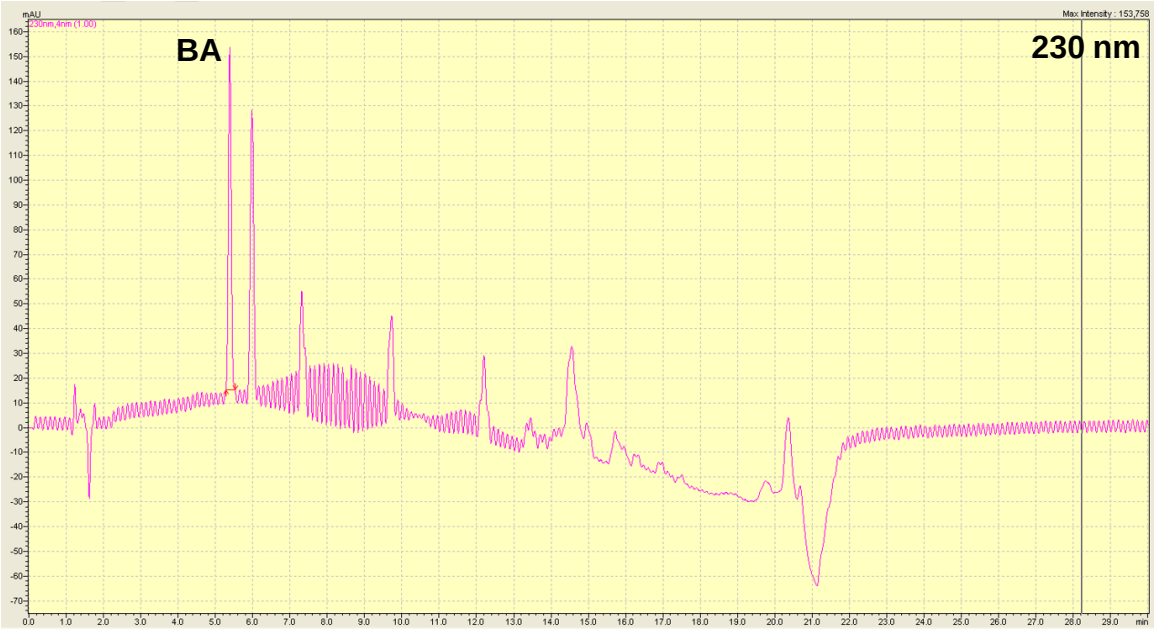

Figure 26: Chromatogram of Mix standard (10 µg/mL in 20:80 ACN:H_2O), Elution Program 7, Flow rate: 1.2 mL/min, 230 nm.

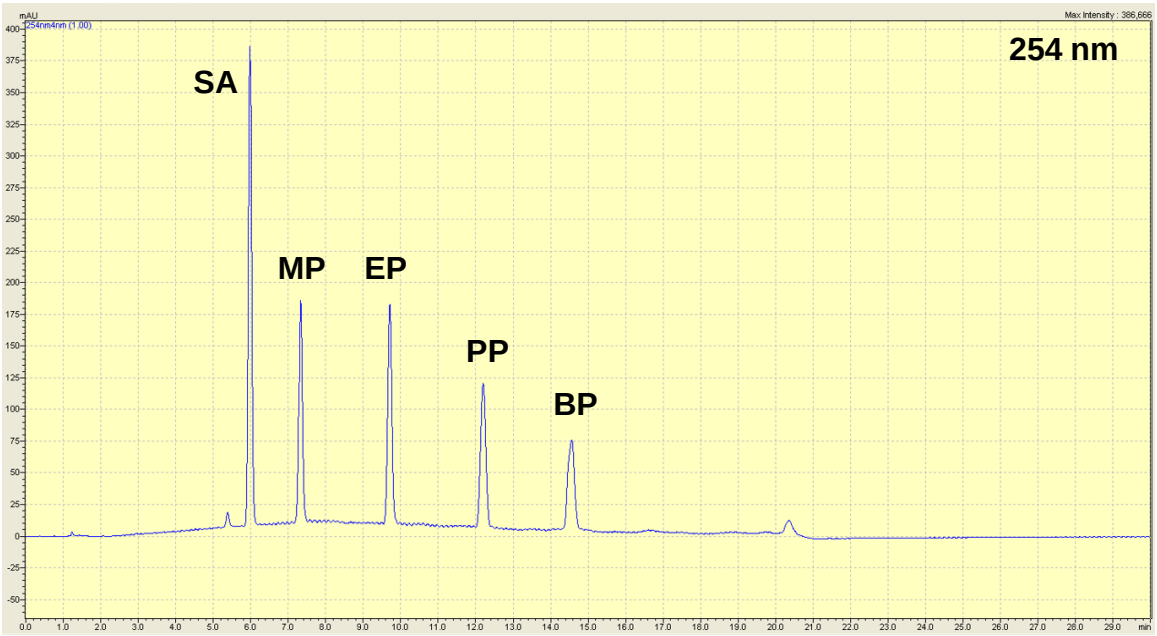

Figure 27: Chromatogram of Mix standard (10 µg/mL in 20:80 ACN:H_2O), Elution Program 7, Flow rate: 1.2 mL/min, 254 nm.

Comparing the six chromatograms (Fig. 20, 21, 24-27), the best choice for the parameter of flow rate is 0.8 mL/min, as in these conditions there is a stable baseline, symmetrical peaks and better signal intensity.

4.5 DAD conditions

As mentioned above, the photodiode array detector uses a visible lamp and a lamp that emits in the ultraviolet, and after the light passes through the cell with the sample, it falls on a barrier diffraction where the separation is done in the different wavelengths.

The analytes employed in this particular research absorbed radiation at the following wavelengths (Table 3):

- BA: 225 nm, 230 nm
- SA, Parabens: 254 nm, 260 nm

Testing the different wavelengths and comparing the relative sensitivity and absorbance of the corresponding compounds, it was decided to use 230 nm for the determination of BA and 254 nm for the determination of the remaining five compounds.

The identification of the target compounds was done both by comparing the retention time (RT) to the corresponding RT of standard solutions, as well as by matching UV spectrum of each compound with the UV spectrum of the standard solutions. Below, absorption spectra for each compound in standards solutions as well as in blank fruit juices and soft drink samples and spiked samples are attached.

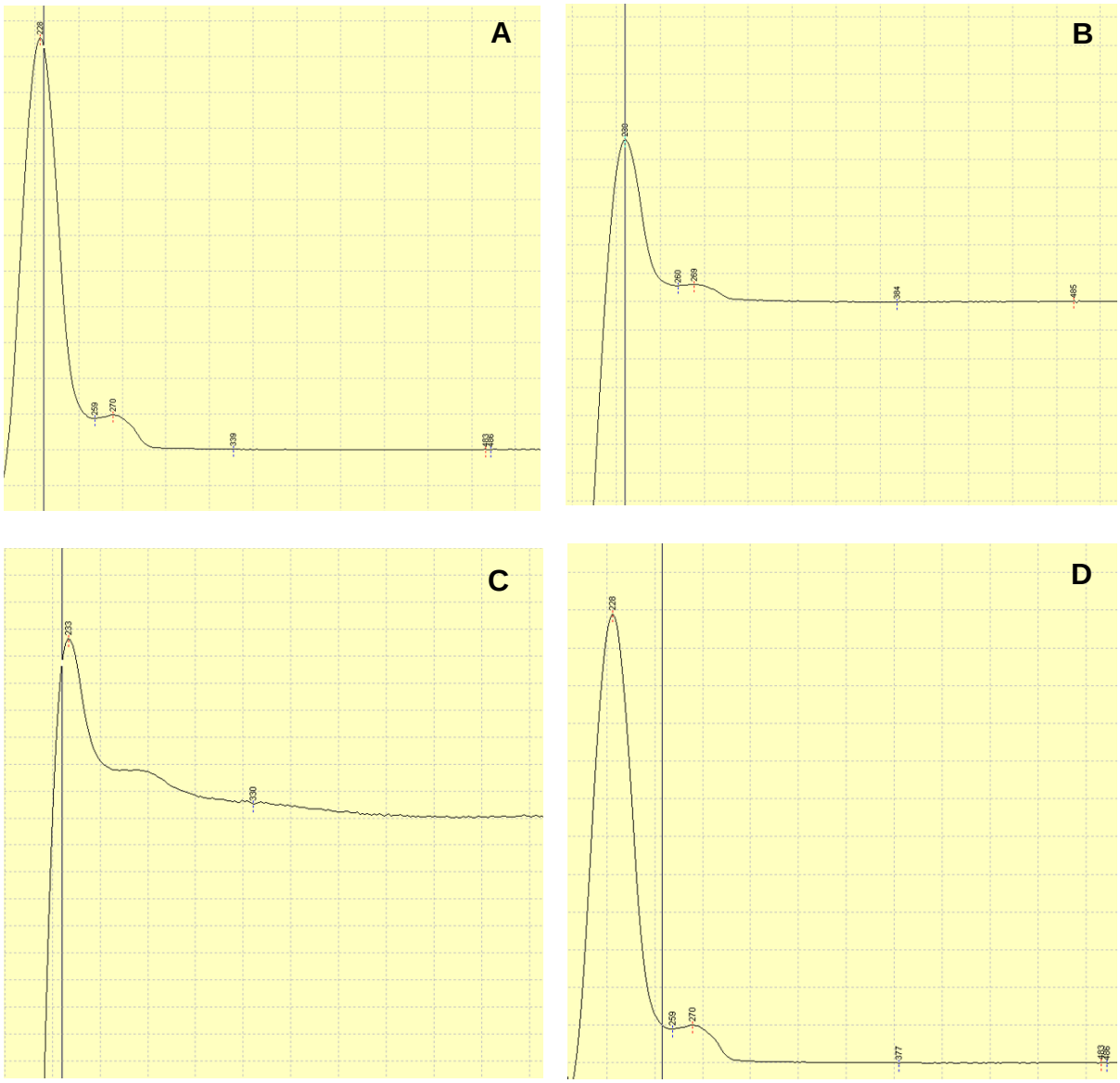

Figure 28: UV Spectrum of BA. A: in standard solution, B: in spiked fruit juice sample, C: in blank fruit juice sample, D: in blank soft drink sample.

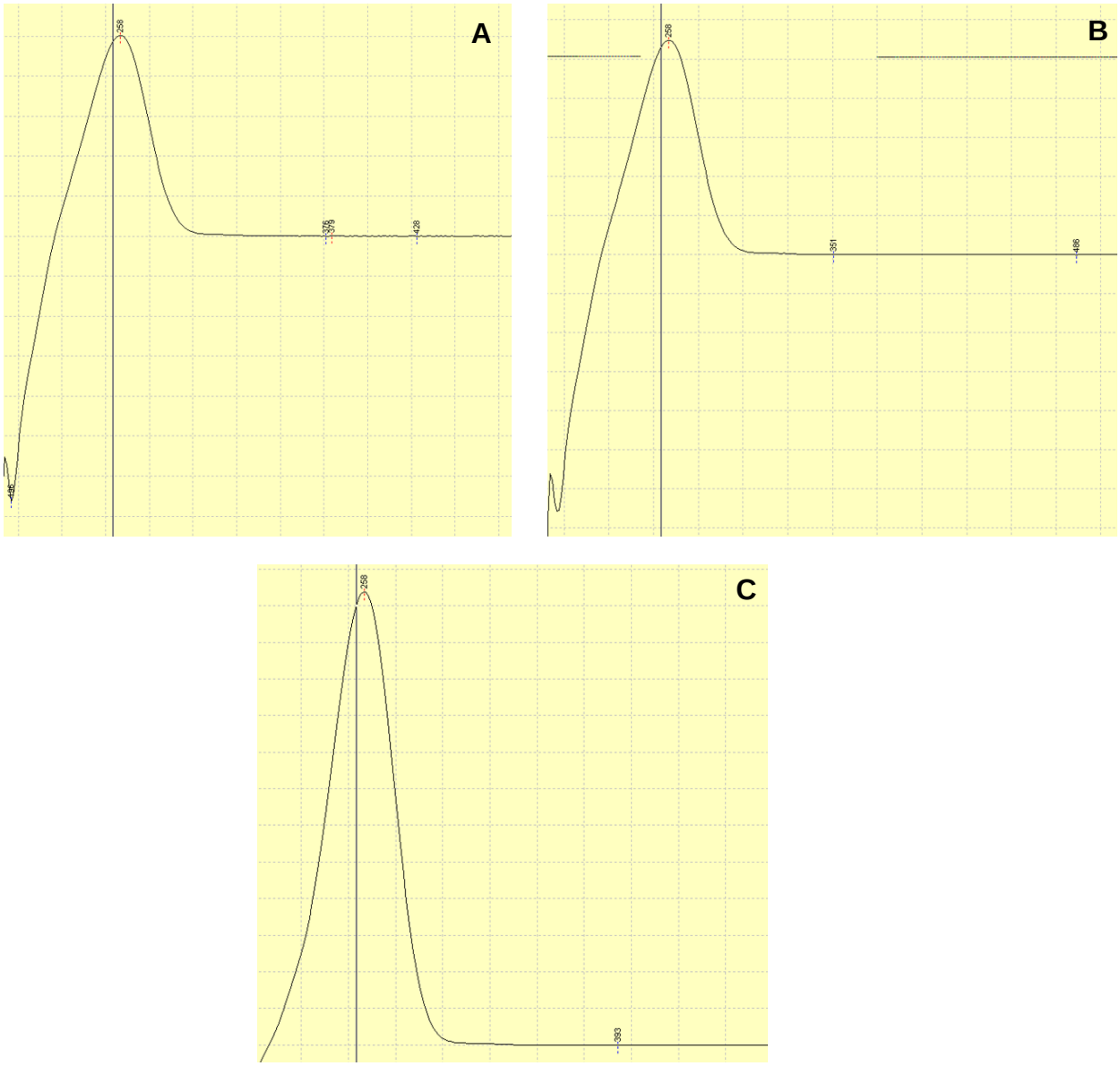

Figure 29: UV Spectrum of SA. A: in standard solution, B: in spiked soft drink sample, C: in blank soft drink sample.

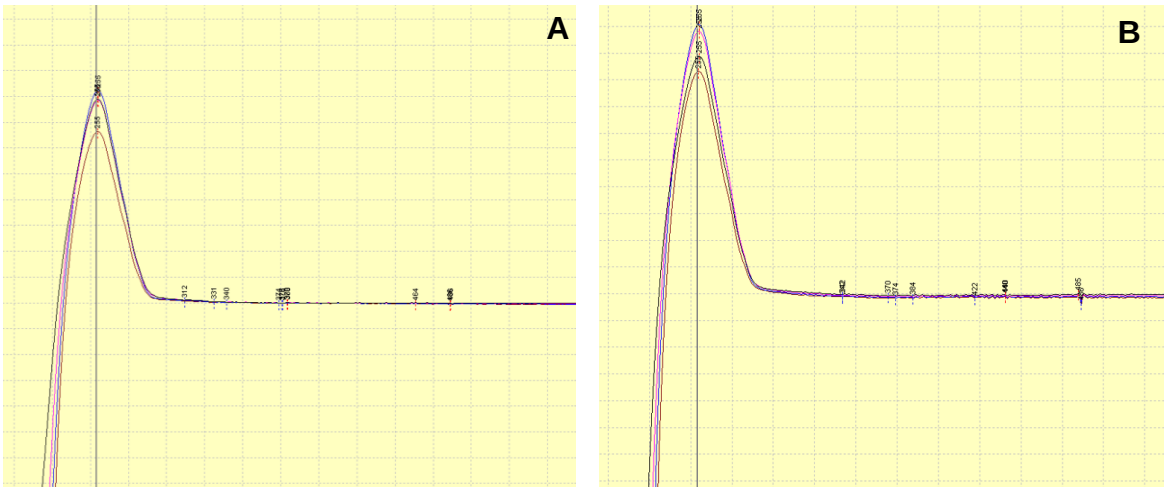

Figure 30: UV Spectrum of PHBs. A: in standard solution, B: in spiked sample.

4.6 Sample Preparation

Carrying out the literature review, it was found that several sample preparation methods were used for the determination of preservatives (benzoic acid, sorbic acid, methyl-, ethyl-, propyl- and butyl paraben) in fruit juices, soft drinks and energy drinks substrates (Table 3). Some of them are as follows: simple dissolution and filtration, DLLME-SFO, Quencher method, HA-LLME etc. The aim of this master thesis was to develop a method for the determination of these six preservatives, focusing at maximum efficiency and sensitivity, but also to be characterized by easiness, rapid analysis and low cost. In this way, the following two were chosen to be compared as sample preparation methods: simple dissolution and filtering (dilution factor 1:2) (Fig. 31) and the QuEChERS method (dilution factor 1:10) (Fig. 32).

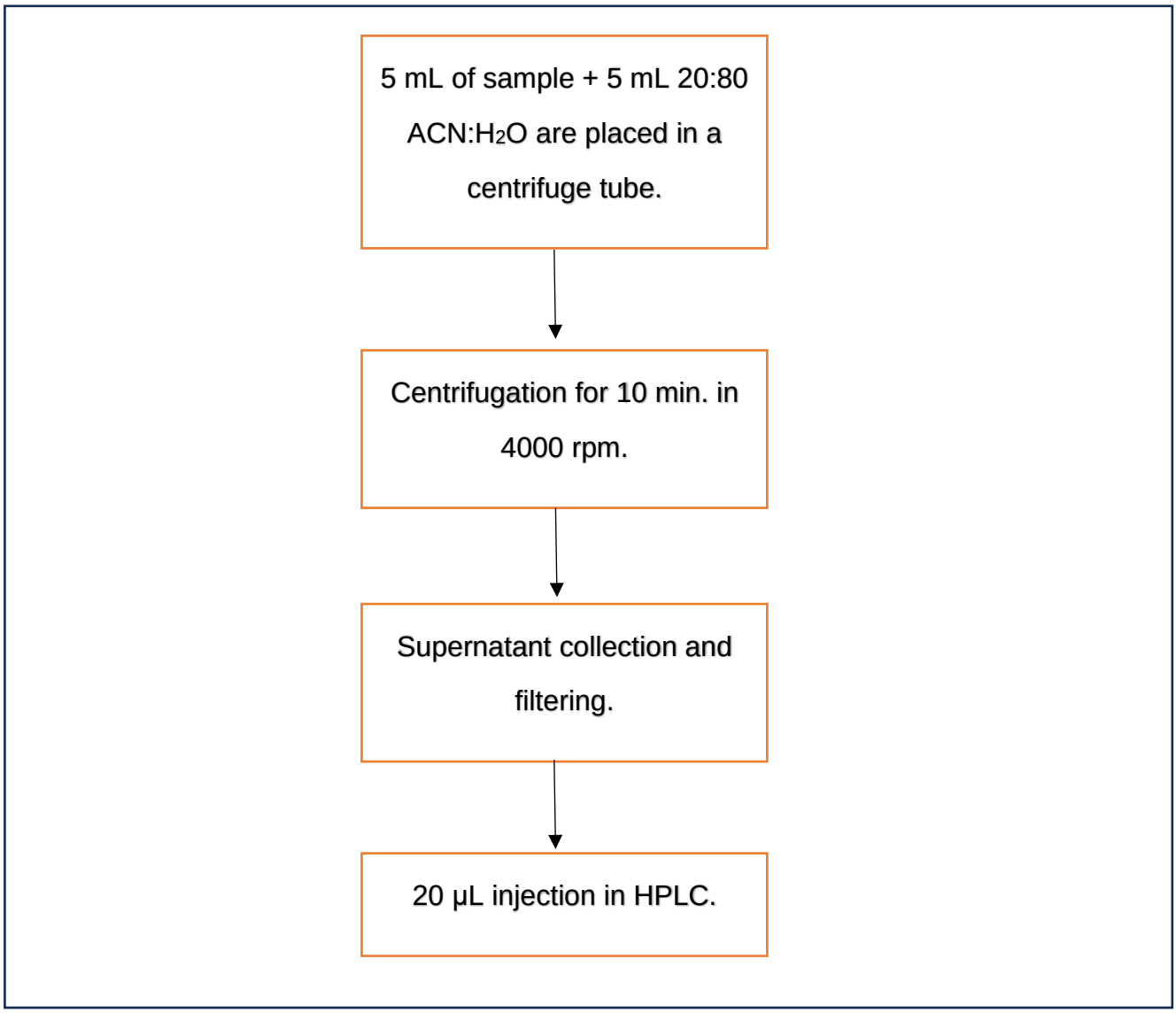

Figure 31: Simple Dilution and filtering methodology.

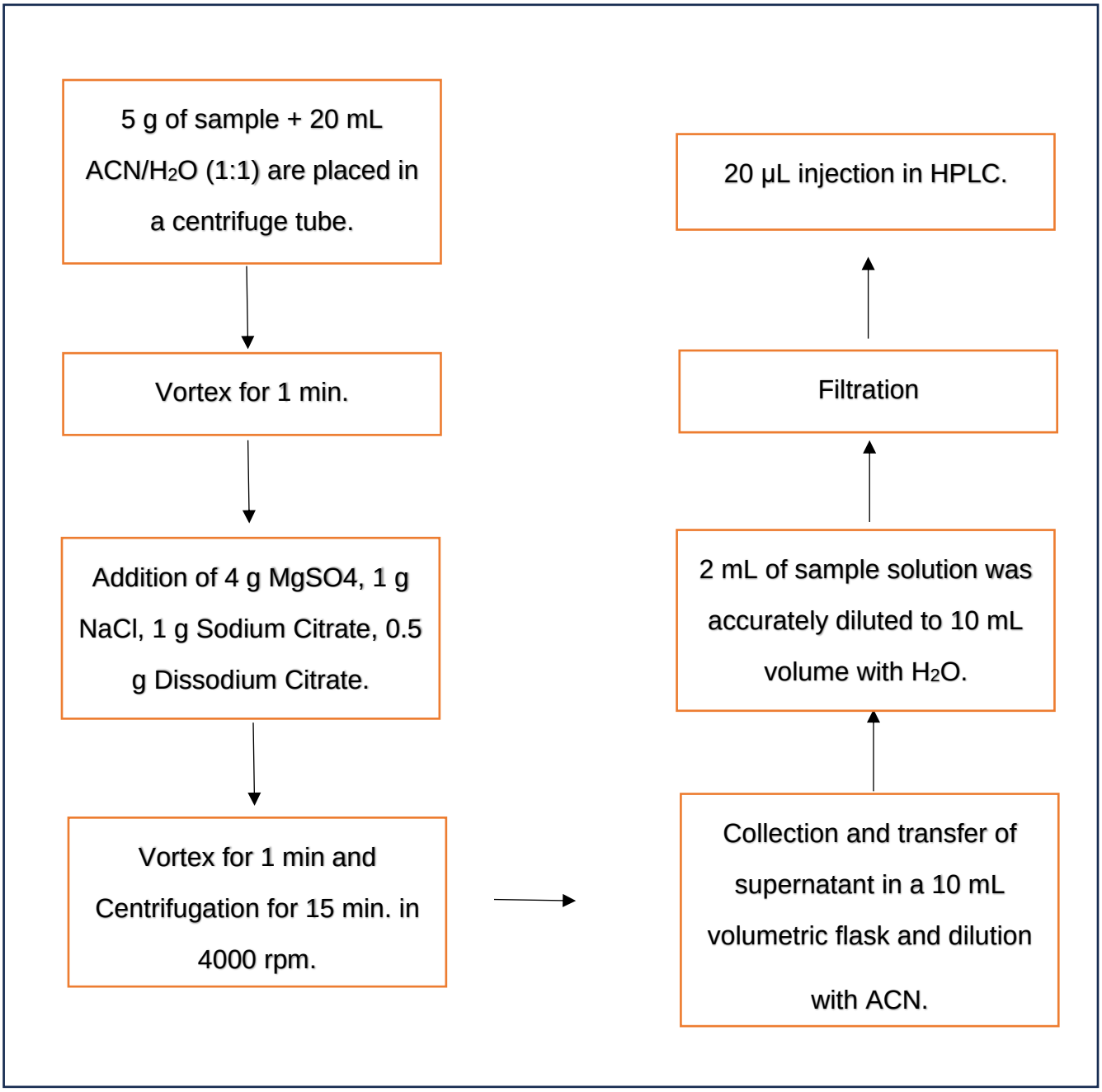

Figure 32: QuEChERS methodology.

In order to compare the two methods, the procedure was as follows: a sample of fruit juice was processed in both ways, obtaining the following chromatograms (Figure 33 and 34). Then a spiked juice sample was processed with both methods, to obtain the recovery rates (Table 10).

Table 10: Recovery Rates for Simple and QuEChERS Preparation Method.

Analytes	Simple Preparation Method (Dilution factor 1:2)			QuEChERS Method (Dilution factor 1:10)		
	Mix Standard Solution (25 µg/mL BA,SA + 12.5 µg/mL PHBs)	Spike Concentration (250 µg/10 mL BA, SA and 125 µg/10 mL PHBs)	Recovery Rates (%)	Mix Standard Solution (5 µg/mL BA,SA + 2.5 µg/mL PHBs)	Spike Concentration (250 µg/10 mL BA, SA and 125 µg/10 mL PHBs)	Recovery Rates (%)
	Time (min)	Time (min)		Time (min)	Time (min)	
BA(230 nm)	7.653	7.607	**84.5**	7.655	7.680	**92.5**
SA (254 nm)	8.248	8.219	**65.8**	8.226	8.240	**94.0**
MP (254 nm)	9.83	9.782	**68.8**	9.638	9.661	**94.1**
EP (254 nm)	12.287	12.246	**81.2**	12.129	12.150	**101**
PP (254 nm)	14.812	14.772	**102**	14.674	14.690	**98.6**
BP (254 nm)	17.192	17.134	**79.8**	17.064	17.070	**102**

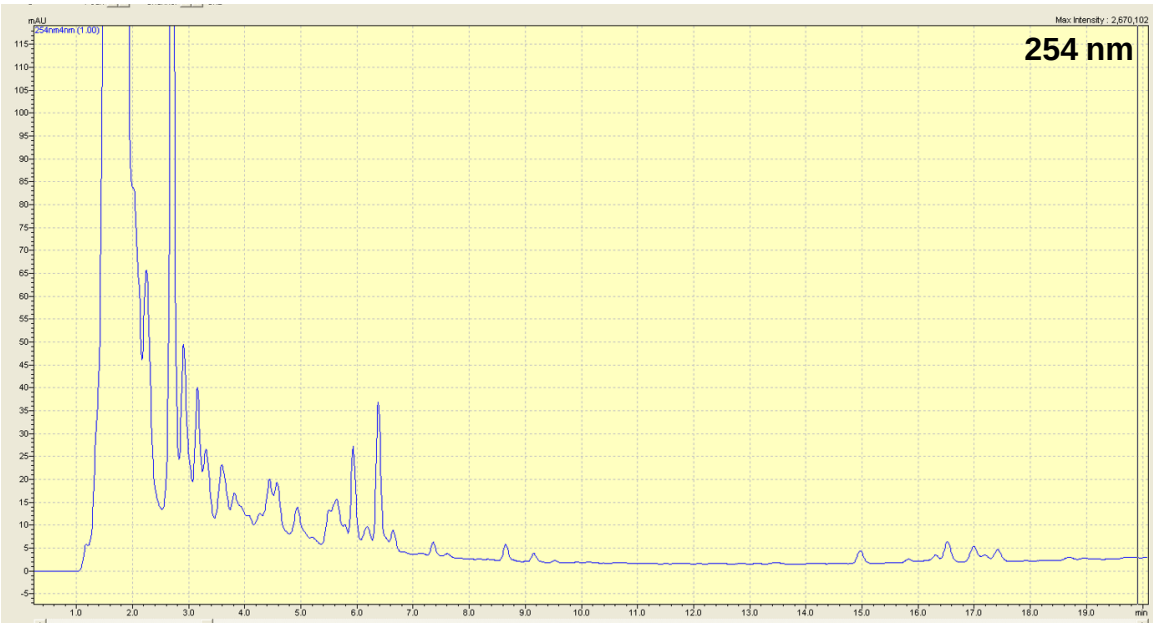

Figure 33: Chromatogram of the fruit juice sample, using simple dilution and filtering method, in 254 nm.

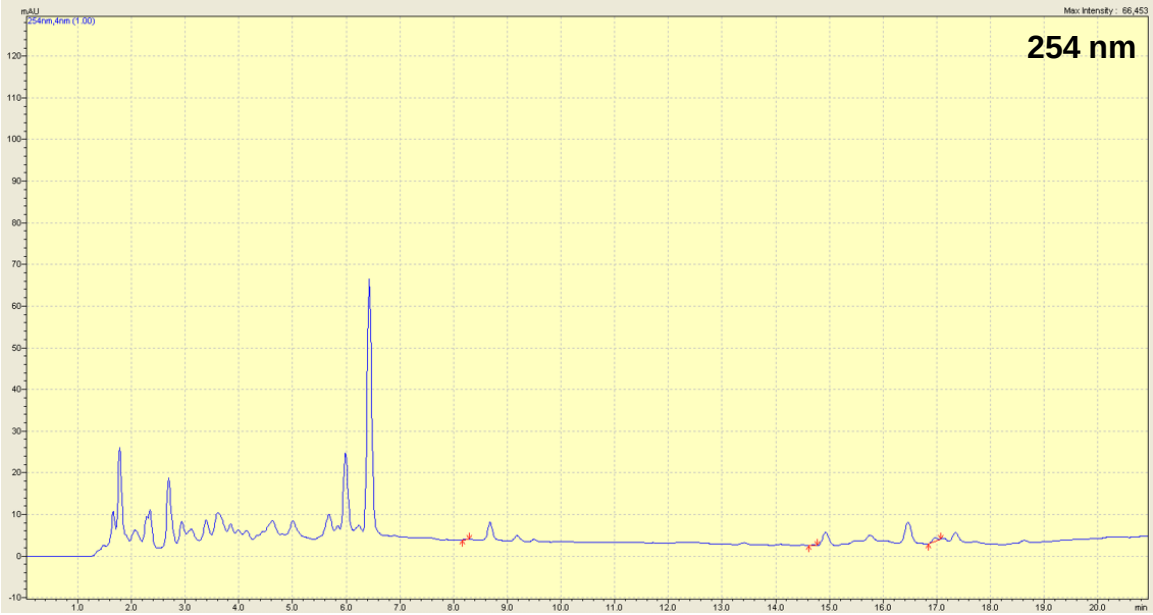

Figure 34: Chromatogram of the fruit juice sample, using QuEChERS method, in 254 nm.

As it is showed, the QuEChERS method offers higher recovery rates as well as chromatograms with less interferences and noise in the elution zone of the analytes. For these reasons, it was chosen as the method for preparation of all samples for the method validation and quantification of market samples.

4.7 Optimization of filtering procedure

To optimize the method, the type of filter applied before injecting the solution into the HPLC instrument was the last checked parameter. Initially, a RC (Regenerated cellulose) filter was used, as it is one of the most used filters, resistant to a very wide range of solvents and suitable for use with either aqueous solutions or organic solvents. Nonetheless, what was found during the experimental course is, as it could be seen in Table 11 and Fig. 35 and 36, that there was interference of the solution during the elution time of the BP. Therefore, the process was tested with a PVDF (Polyvinylidene difluoride) filter, which was proved to be suitable for the specific preparation of the sample.

Table 11: Optimization of filtering procedure.

		BP (254 nm)	
		Time (min)	Peak Area
Solution	**Filter**		
Standard (0.2 μg/mL BA, 0.1 μg/mL rest)	Nothing	17.109	20070
	RC	16.922	57798
	PVDF	17.027	24295

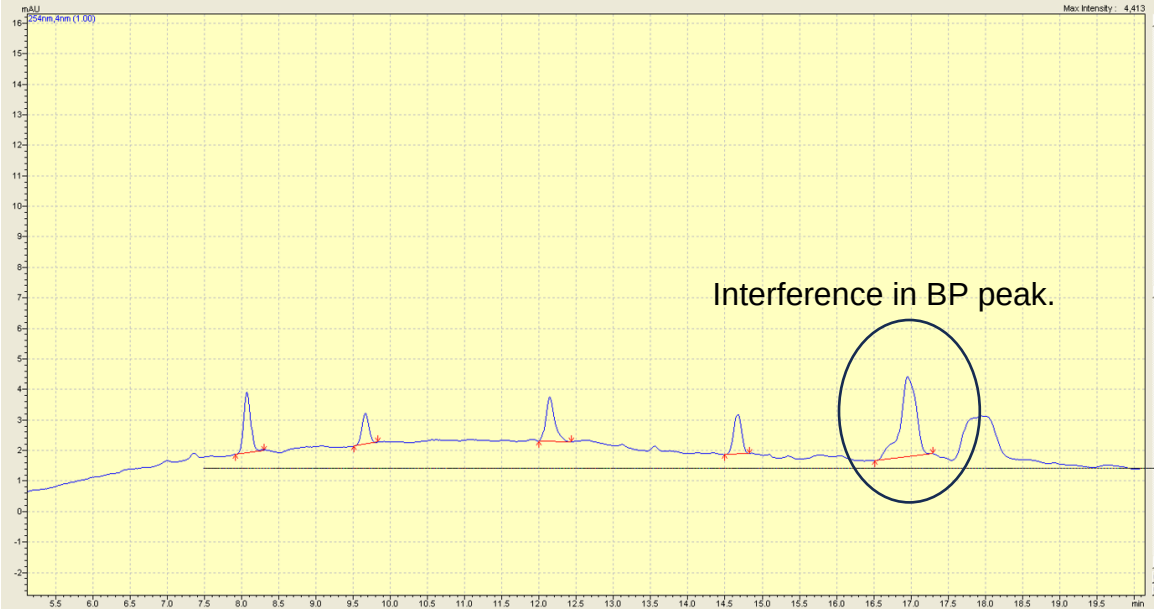

Figure 35: Chromatogram of mix standard, using RC filter.

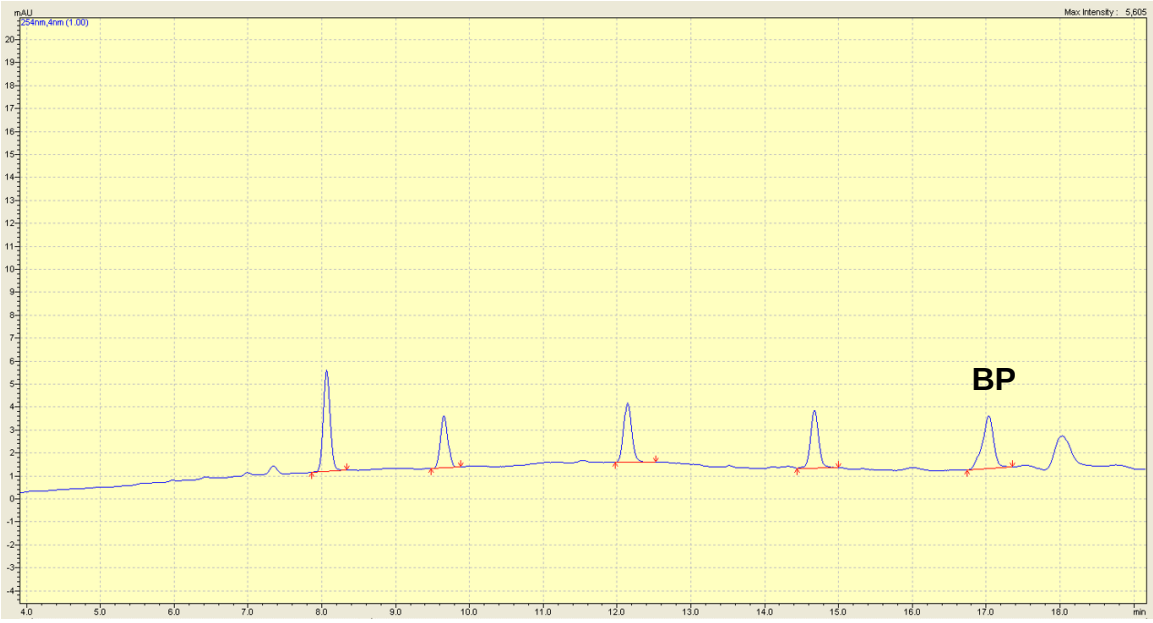

Figure 36: Chromatogram of mix standard, using PVDF filter.

CHAPTER 5
VALIDATION OF THE METHOD

The developed and optimized method has the following characteristics summarized below:

- Stationary Phase: 100-5 C_{18} column (150mm x 4.6 mm, 5μm)
- Mobile Phase/Elution Program: A: Ammonium Acetate 5mM (pH = 4.2) and B: ACN in gradient elution (Table 9), 0.8 min/mL, 30 min
- λ (nm): 230 nm for BA, 254 nm for SA, PHBs
- Sample Preparation: QuEChERS method (Fig. 32)
- Filtration: PVDF filter

Next step is the validation of the method. Validation of an analytical method refers to the evaluation of the method quality characteristics through experimental documentation and the examination of its response to specifications to proven to be fit for purpose. [74]

To validate the proposed method the following parameters were tested:

- Linearity
- Accuracy
- Precision
    - Repeatability
    - Reproducibility
- Detectability
    - Limit of Detection (LOD)
    - Limit of Quantitation (LOQ)

In this chapter the results for each quality parameter and for each analyte separately would be presented.

5.1 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The linearity of the method was assessed by preparing standards solutions of known concentration and the construction of a calibration curve for each analyte. In addition, standard addition method was applied in each substrate, in order to study the matrix effect of the method. [74]

5.1.1 Calibration curve technique

In the following Table, the concentration range (up to seven points), the regression equations, the slope, as well as correlation coefficient for each analyte are presented. The choice of concentrations used to construct the calibration curve, was made based on the ranges, that have been used in previous studies, and at much lower levels than the permissible legal framework.

Table 12: Regression equations for each analyte.

Analyte	Concentration Range (μg/mL)	Regression equations	Correlation coefficient (R^2)
BA	0.5-100	y = 104867(±879)x	0.9996
SA	0.5-100	y = 246023(±6416)x	0.9959
MP	0.25-50	y = 159019(±444)x	1.0000
EP	0.25-50	y = 170474(±464)x	1.0000
PP	0.25-50	y = 202933(±633)x	0.9999
BP	0.25-50	y = 167744(±422)x	1.000

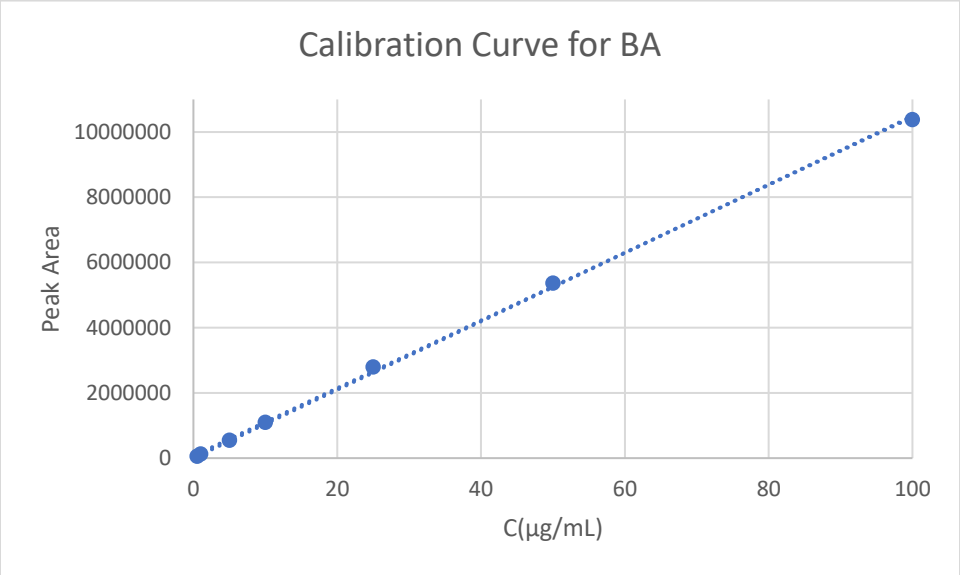

Figure 37: Calibration Curve for BA.

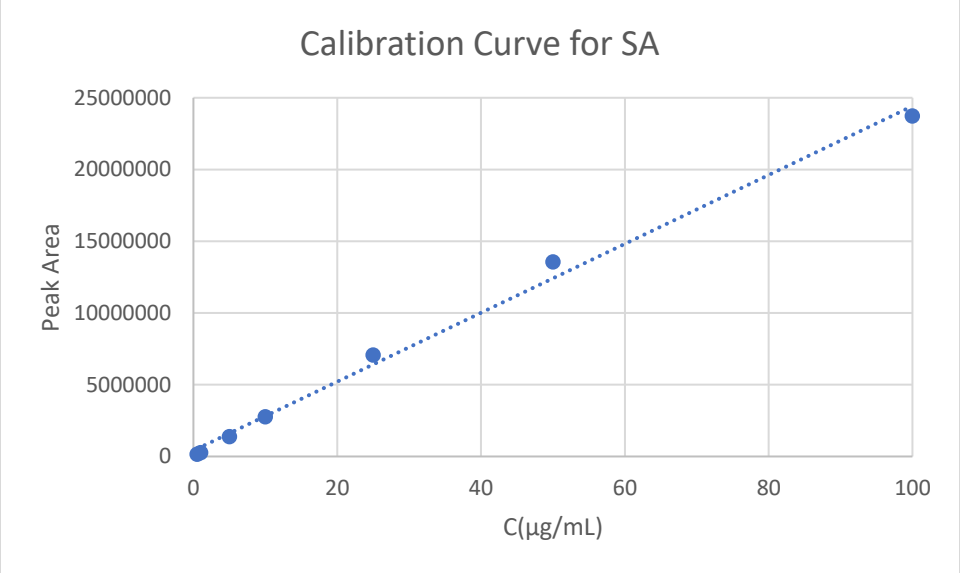

Figure 38: Calibration Curve for SA.

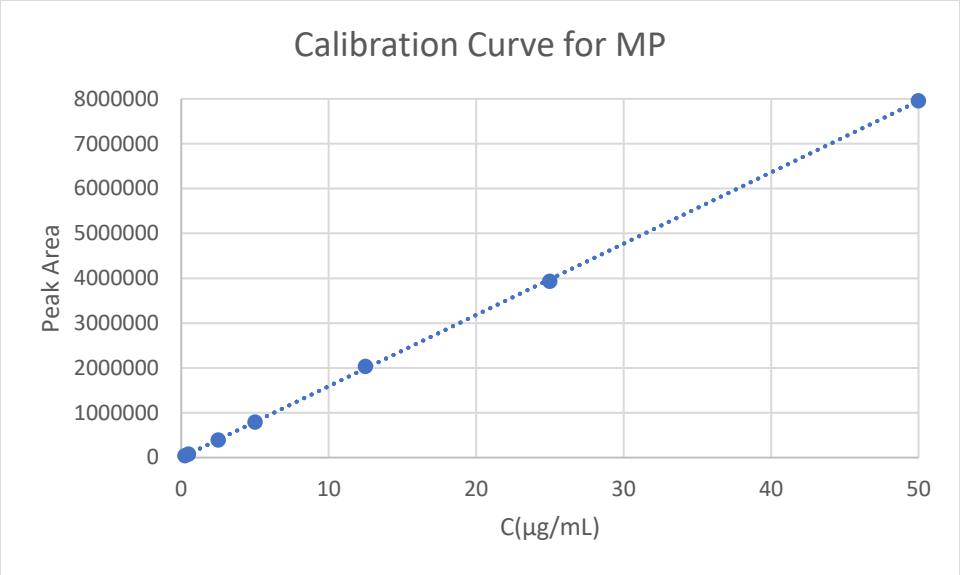

Figure 39: Calibration Curve for MP.

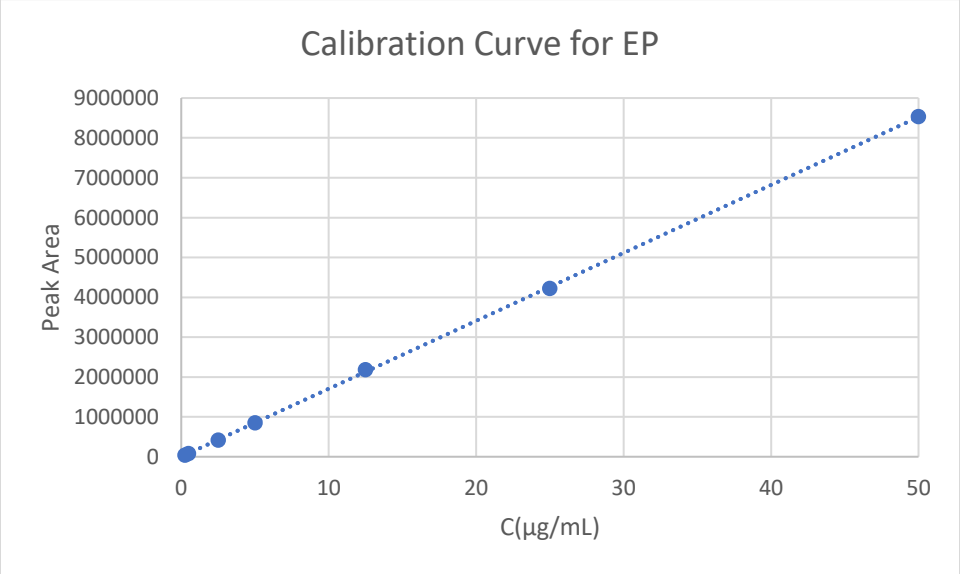

Figure 40: Calibration Curve for EP.

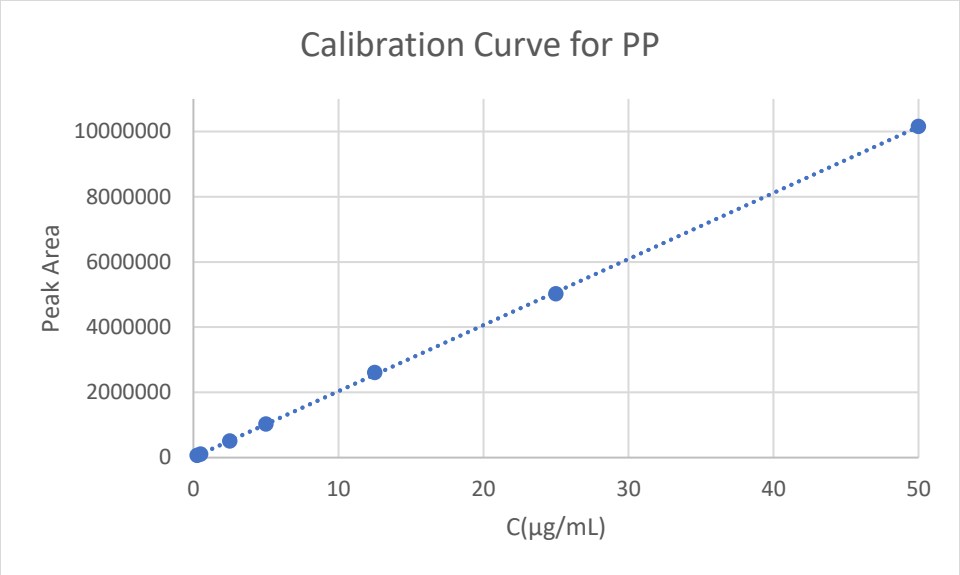

Figure 41: Calibration Curve for PP.

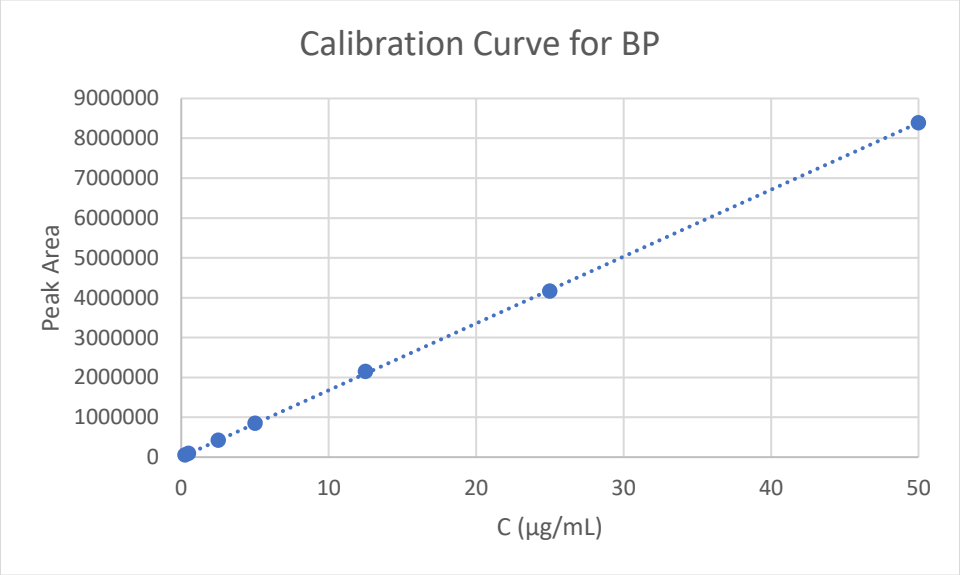

Figure 42: Calibration Curve for BP.

5.1.2 Standard addition method

A series of samples (fruit juices and soft drinks) spiked with a known increasing concentration (up to five points) of each analyte was prepared and was analyzed by the specific method. Then, the relative standard addition regression equations and curves were generated and are shown below.

Table 13: Regression equations for each analyte in Standard addition method.

Analytes	Concentration Spike Range (μg/mL)	Regression equations in fruit juices/soft drinks	Correlation coefficient (R^2) in fruit juices/soft drinks
BA	2.5-50	y = 11375(±115)x	0.9996
		y = 10480(±356)x	0.9954
SA	2.5-50	y = 25371(±684)x	0.9971
		y = 21766(±1336)x	0.9912
MP	1.25-25	y = 14707(±82)x	0.9999
		Y=13632(±194)x	0.9992
EP	1.25-25	y = 17659(±176)x	0.9996
		y = 15441(±137)x	0.9997
PP	1.25-25	y = 17772(±195)x	0.9995
		y = 15370(±193)x	0.9994
BP	1.25-25	y= 16814(±525)	0.9961
		y= 12341(±539)x	0.9943

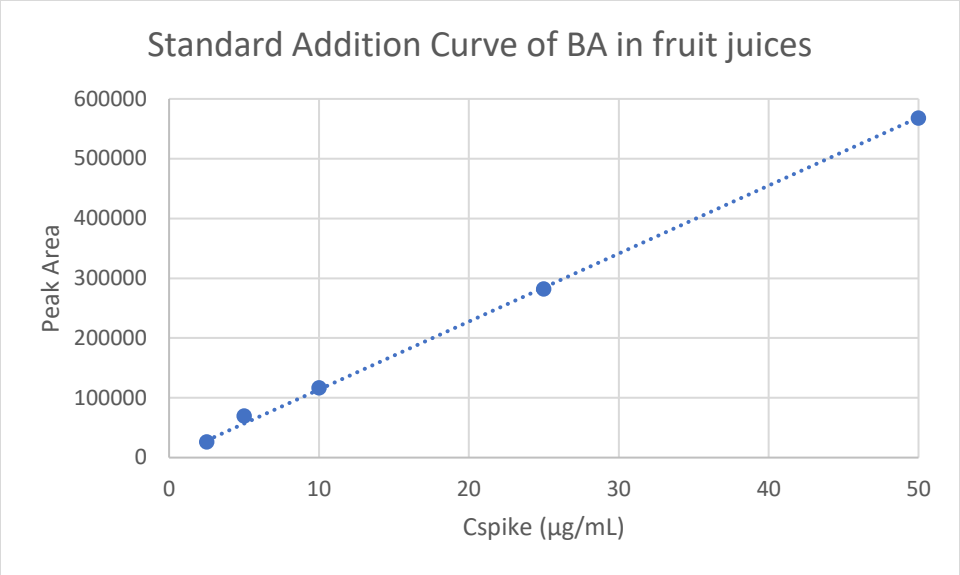

Figure 43: Standard Addition Curve of BA in fruit juices.

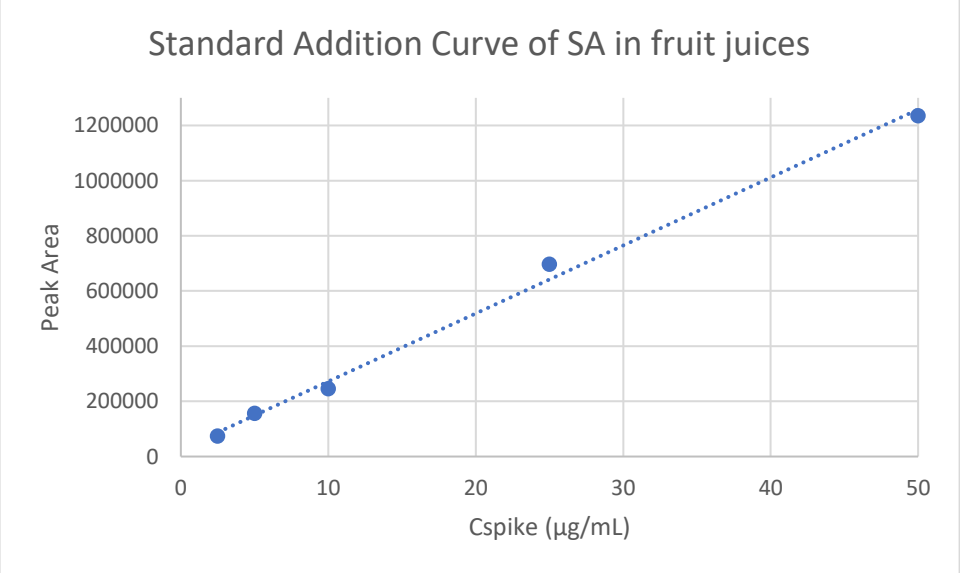

Figure 44: Standard Addition Curve of SA in fruit juices.

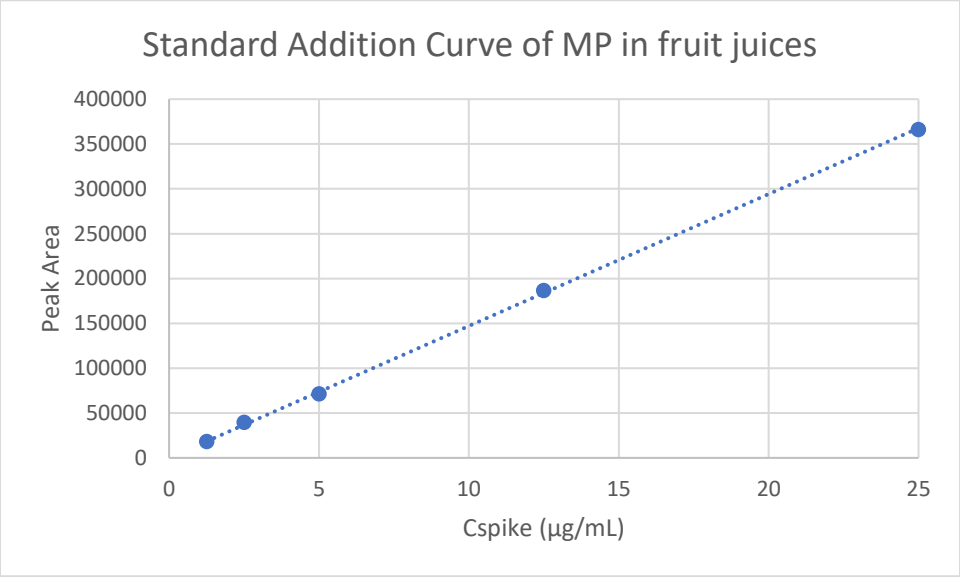

Figure 45: Standard Addition Curve of MP in fruit juices.

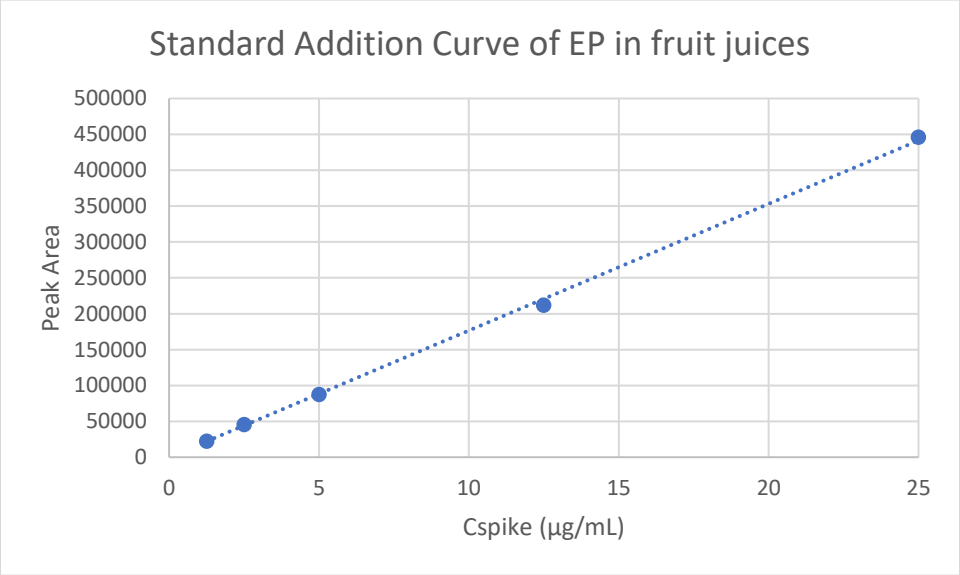

Figure 46: Standard Addition Curve of EP in fruit juices.

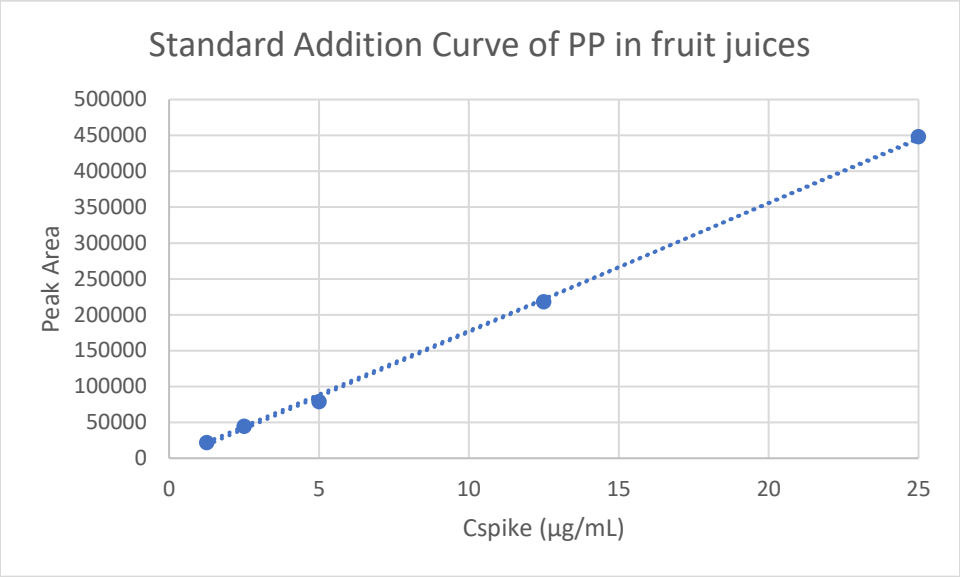

Figure 47: Standard Addition Curve of PP in fruit juices.

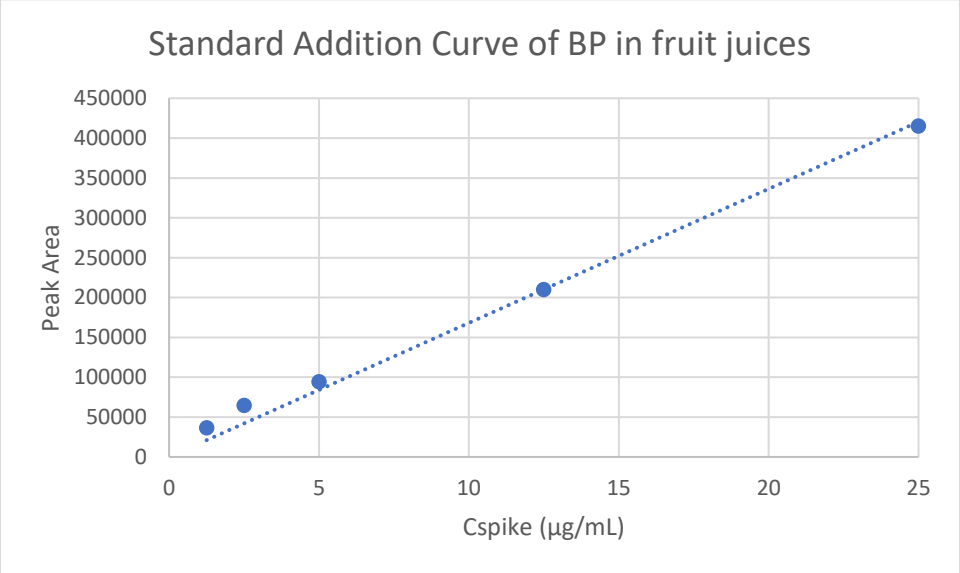

Figure 48: Standard Addition Curve of BP in fruit juices.

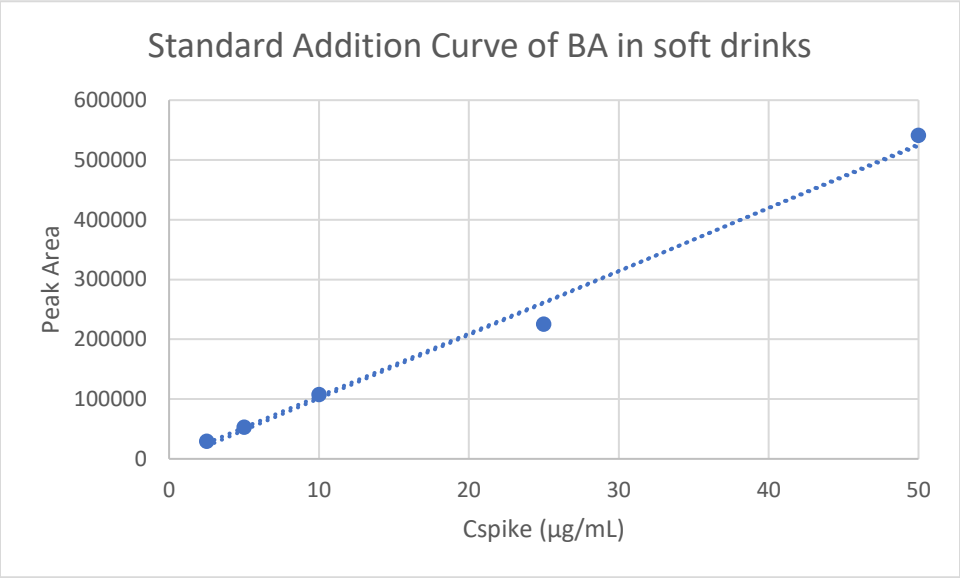

Figure 49: Standard Addition Curve of BA in soft drinks.

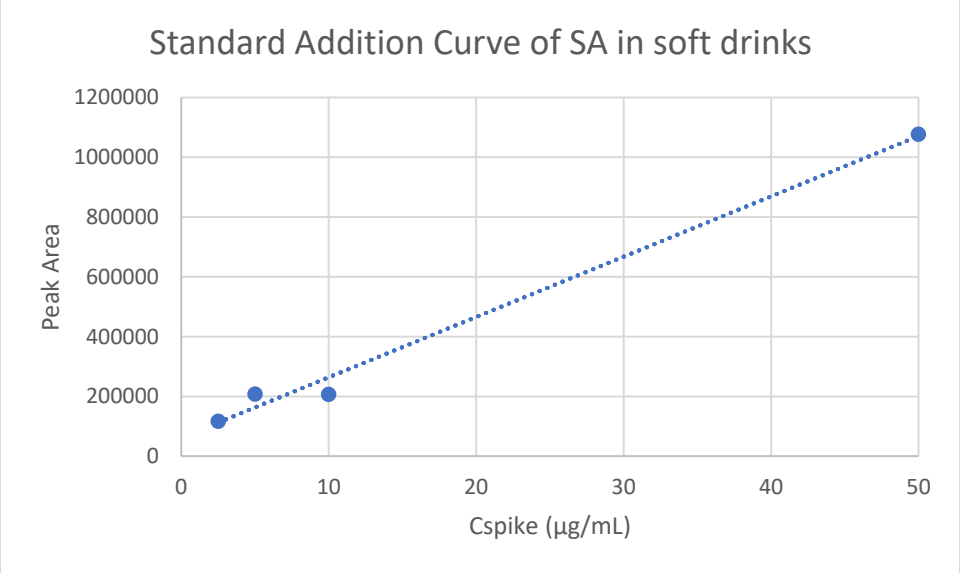

Figure 50: Standard Addition Curve of SA in soft drinks.

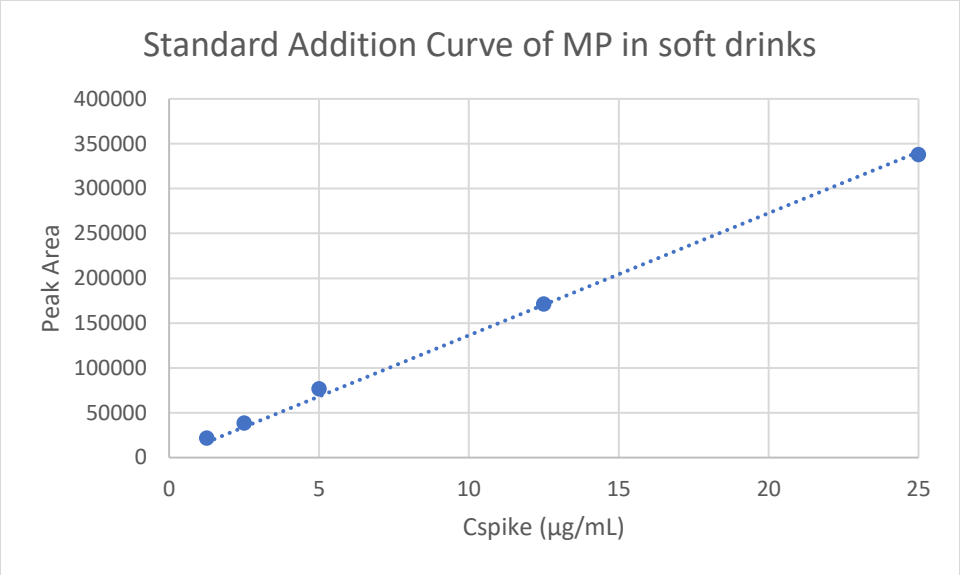

Figure 51: Standard Addition Curve of MP in soft drinks.

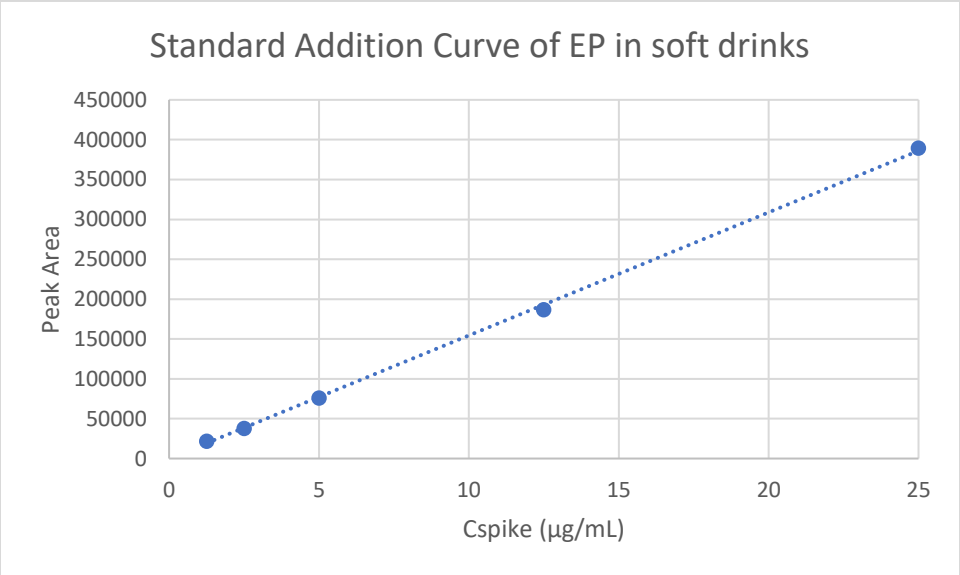

Figure 52: Standard Addition Curve of EP in soft drinks.

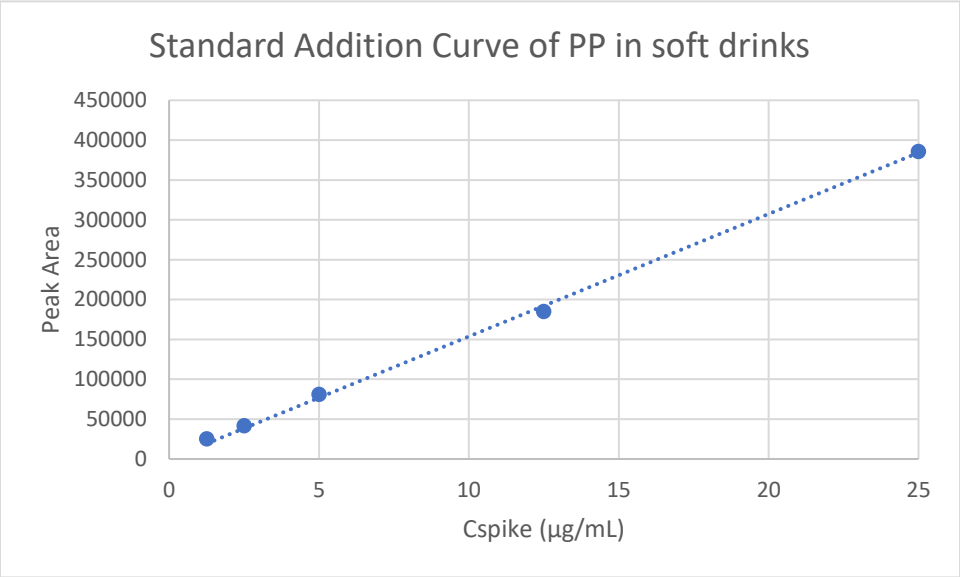

Figure 53: Standard Addition Curve of PP in soft drinks.

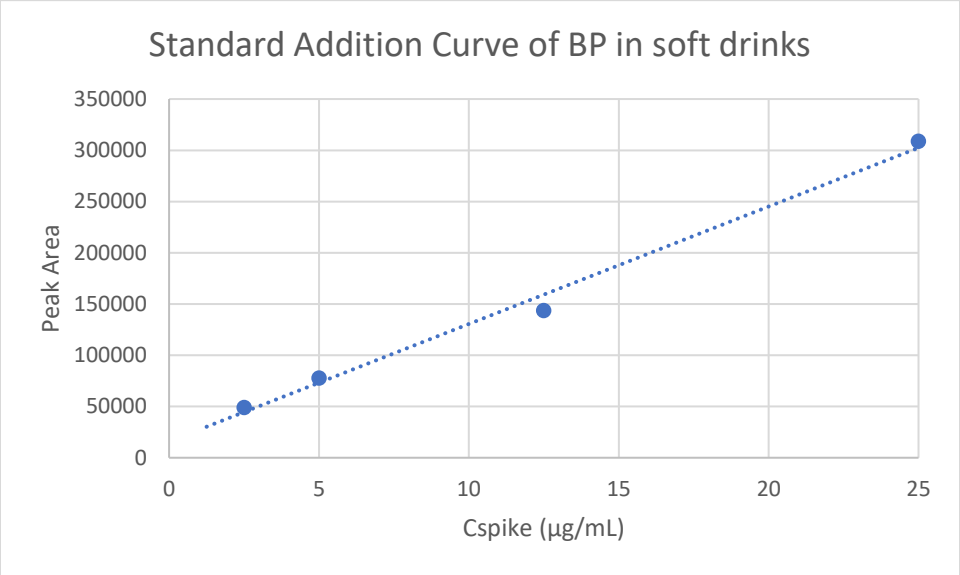

Figure 54: Standard Addition Curve of BP in soft drinks.

The proposed method follows the acceptable limits for the linearity parameter, as high correlation coefficient (>0.99, Table 12 and 13) is recommended as evidence of goodness of fit. [74]

5.2 Accuracy and Precision

5.2.1 Instrumental Repeatability

To check the instrumental validity of the experimental procedure, the repeatability of the instrument was examined. This was done by injecting into HPLC instrument standard mix solutions containing the six analytes in a concentration of 10 μg/mL in ACN/H_2O (20:80%, v/v), six times during the same laboratory day. Below, the mean value of Peak Area, the standard deviation (SD) and %RSD are presented.

Table 14: Instrumental Repeatability.

Analyte	Mean Value of Peak Area	SD	%RSD
BA	1.170.510	6415	0.55
SA	3.315.787	10925	0.33
MP	1.640.066	14852	0.90
EP	1.955.386	18352	0.94
PP	1.649.032	13145	0.80
BP	1.181.627	11165	0.94

RSD(%) for each day was found below 2%, meaning that the repeatability of the instrument is stable and without variation between each analysis.

5.2.2 Method Accuracy and Repeatability

Accuracy is one of the most critical method validation parameters, as it expresses the closeness of agreement between the value which is accepted as a conventional true value and the value found. The accuracy of the method was assessed by adding the required amount of each analyte (2 spike levels: low and high level) into the sample and analyzing this spiked sample to calculate the recoveries (Recovery Rate, R%). The two spike levels for each substrate, as well as how much quantity of each preservative was added in blank sample are presented:

- Fruit Juices

Low Level

BA: 2 μg/mL, 10 μg (for 5 g sample), 10 μL from 1000 μg/mL stock solution

SA, MP, EP, PP: 1 μg/mL, 5 μg (for 5 g sample), 500 μL from 10 μg/mL mix solution

BP: 2.6 μg/mL, 13 μg (for 5 g sample), 13 μL from 1000 μg/mL stock solution

High Level

BA, SA: 25 μg/mL, 125 μg (for 5 g sample), 500 μL from 250 μg/mL mix solution

Parabens: 12.5 μg/mL, 62.5 μg (for 5 g sample), 500 μL from 125 μg/mL mix solution

- Soft Drinks

Low Level

BA: 2 μg/mL, 10 μg (for 5 g sample), 10 μL from 1000 μg/mL stock solution

SA, Parabens: 1 μg/mL, 5 μg (for 5 g sample), 50 μL from 100 μg/mL mix solution

High Level

BA, SA: 25 μg/mL, 125 μg (for 5 g sample), 500 μL from 250 μg/mL mix solution

Parabens: 12.5 μg/mL, 62.5 μg (for 5 g sample), 500 μL from 125 μg/mL mix solution

Every level of spiking concentration of each analyte was examined 4-10 times. For the calculation of the recovery, Equation 1 was used and the recovery results for each analyte in fruit juices and soft drinks are presented below. Regarding the precision of the method, the repeatability and reproducibility parameters were checked. Repeatability is defined as the precision under the same operating conditions over a short interval of time and was measured through RSD% (Relative Standard Deviation) of the total of 4-10 repetitions of each spike level at the same day. [74]

$$R(\%) = \frac{\text{Peak Area in Spiked Sample} - \text{Peak Area in Blank Sample}}{\textit{Peak Area in Standard Solution}} \times 100\ (\%) \qquad \textbf{(Equation 1)}$$

Table 15: Accuracy and Repeatability of BA in fruit juices and soft drinks.

Analyte (BA)	Low Spike level (2 µg/mL)		High Spike Level (25 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=5)	Soft Drinks (n=7)	Fruit Juices (n=5)	Soft Drinks (n=10)
Number of replicates	105	76.9	78.0	83.9
		84.2		114
	97.2	93.7	81.4	115
		92.0		101
	125	81.8	81.5	95.0
				111
	99.8	79.6	80.9	106
				93.7
	105	80.8	87.7	116
				106
Mean Value (MV)	**106**	**84.2**	**81.9**	**104**
SD	**11.0**	**6.36**	**3.53**	**10.7**
RSD(%)	**10.3**	**7.55**	**4.31**	**10.3**

Table 16: Accuracy and Repeatability of SA in fruit juices and soft drinks.

Analyte (SA)	Low Spike level (1 µg/mL)		High Spike Level (25 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=5)	Soft Drinks (n=7)	Fruit Juices (n=5)	Soft Drinks (n=7)
Number of replicates	86.4	74.3	121	95.6
		78.6		118
	94.3	75.4	116	112
		82.3		118
	91.9	78.4	111	111
	86.5	79.6	119	100
	79.6	72.5	120	90.8
Mean Value (MV)	**87.7**	**77.3**	**118**	**107**
SD	**5.69**	**3.40**	**4.32**	**11.0**
RSD(%)	**6.48**	**4.69**	**3.68**	**10.3**

Table 17: Accuracy and Repeatability of MP in fruit juices and soft drinks.

Analyte (MP)	Low Spike level (1 µg/mL)		High Spike Level (12.5 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=4)	Soft Drinks (n=7)	Fruit Juices (n=6)	Soft Drinks (n=7)
Number of replicates	96.7	81.0	78.6	93.8
		75.0		
	88.1	81.2	74.2	118
		77.6		112
	88.7	78.7	71.1	117
	82.0	74.1	76.8	109
		75.6	78.1	115
			85.8	101
Mean Value (MV)	**88.9**	**77.6**	**77.4**	**109**
SD	**6.00**	**2.85**	**4.97**	**8.94**
RSD(%)	**6.75**	**3.67**	**6.42**	**8.17**

Table 18: Accuracy and Repeatability of EP in fruit juices and soft drinks.

Analyte (EP)	Low Spike level (1 µg/mL)		High Spike Level (12.5 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=4)	Soft Drinks (n=8)	Fruit Juices (n=6)	Soft Drinks (n=6)
Number of replicates	93.1	70.7	84.9	97.9
		69.9		91.5
	87.4	78.1	80.0	120
		67.3		114
	87.1	74.1	76.5	
	72.8	74.5	82.7	113
		67.4	85.1	106
		70.4	92.1	
Mean Value (MV)	**85.1**	**71.6**	**83.5**	**107**
SD	**8.67**	**3.74**	**5.31**	**10.8**
RSD(%)	**10.2**	**5.23**	**6.35**	**10.1**

Table 19: Accuracy and Repeatability of PP in fruit juices and soft drinks.

Analyte (PP)	Low Spike level (1 µg/mL)		High Spike Level (12.5 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=4)	Soft Drinks (n=8)	Fruit Juices (n=5)	Soft Drinks (n=8)
Number of replicates	79.4	76.5	83.7	92.3
		75.6		110
	70.8	84.9	78.4	110
		71.4		105
	75.1	77.9	82.6	118
	62.1	77.5	81.2	115
		71.4	91.2	104
		72.4		96.6
Mean Value (MV)	**71.8**	**76.0**	**83.4**	**106**
SD	**7.38**	**4.49**	**5.39**	**8.67**
RSD(%)	**10.3**	**5.91**	**6.46**	**8.15**

Table 20: Accuracy and Repeatability of BP in fruit juices and soft drinks.

Analyte (BP)	Low Spike level (2.6 µg/mL)	Low Spike level (1 µg/mL)	High Spike Level (12.5 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=5)	Soft Drinks (n=7)	Fruit Juices (n=6)	Soft Drinks (n=8)
Number of replicates	76.9	67.7	98.5	87.8
		69.8		95.8
	88.2	72.2	89.9	94.7
		84.4		89.5
	87.6	71.5	89.3	99.2
	71.3			
	74.7	70.5	99.0	106
		66.1	97.6	89.0
			88.8	86.7
Mean Value (MV)	**79.7**	**71.7**	**93.9**	**93.6**
SD	**7.71**	**5.97**	**4.97**	**6.74**
RSD(%)	**9.67**	**8.32**	**5.96**	**7.20**

From the above experimental data, the Relative Standard Deviations (RSD%) for repeatability, are considered satisfactory since they are less than 15%. Also, all calculated recoveries for accuracy were between 70-120% (ICH Q2(R2)). Thus, the repeatability and accuracy of the method are rated as good and satisfactory. Compared to previous studies (Table 3), the proposed method shows better recovery rates than the corresponding methods, where the QuEChERS methodology was implemented for the analysis of soft drinks (Rocha et al.,2019) but also of juices (Iwakoshi et al.,2019). Compared with other sample methodologies, such as Liquid-Liquid Extraction (LLE), equivalent recoveries were observed with the developed method, for the determination of benzoic and sorbic acid in juices (Javanmardi et al.,2015 and Pinto et al., 2019) but reduced in others (Javanmardi et al., 2014 and Timofeeva et al., 2018). Specifically, lower recoveries were obtained for the analysis of benzoic acid and sorbic acid (98.8%-111.1%) and (95.6%-105.3%) respectfully at the corresponding spike level. To our best knowledge, there is no published LLE method for the determination of all 6 target compounds, because it is a time consuming, laborious, less robust procedure, where a large amount of toxic solvents are being used. Finally, the simple filtering method presented similar recoveries (Saad et al., 2005) but also reduced (Islam et al., 2016) in relation to the proposed method. This is happening, because when the one-step sample preparation (filtering) is used, the simultaneous determination of six target compounds is not possible in complex matrices and there is a risk of column destruction and high noisy chromatograms. In this way, the proposed sample preparation and elution program offer good recoveries, characterized by simplicity, fast analysis, high efficiency, using a small amount of sample and solvents.

5.2.3 Reproducibility

Reproducibility expresses the variation of the measurement results when the measurement is carried out at same laboratory under conditions of higher variation than the repeatability (different analysts, different instruments, different experiment days). [74] In this master thesis, reproducibility was measured in different experiment days. Reproducibility was checked by spiking a known amount of

analytes (low and high level spike, Subsection 5.2.2) in the blank sample (n= 3-10 each day) following the experimental course mentioned above, with the difference that the samples were analyzed on two different laboratory days. The average recovery, the standard deviation (SD) and the relative standard deviation (RSD%), were then calculated. The data are presented in the tables below.

Table 21: Reproducibility of BA in fruit juices and soft drinks.

Analyte (BA)	Low Spike Level (2 µg/mL)		High Spike Level (25 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=7)	Soft Drinks (n=10)	Fruit Juices (n=8)	Soft Drinks (n=15)
Number of replicates in two days	105	76.9	76.4	83.9
		80.8	79.8	114
	112	93.7	79.7	115
	97.2	84.2	79.2	101
	97.7	92.0	86.0	95.0
	125	81.8	90.4	111
		74.1	92.3	106
	99.8	79.6	91.9	93.7
	105	93.3		116
		71.6		106
				116
				113
				124
				125
				95.7
Mean Value (MV)	**106**	**82.8**	**84.5**	**108**
SD	**9.87**	**7.95**	**6.46**	**11.9**
RSD (%)	**9.32**	**9.60**	**7.64**	**11.0**

Table 22: Reproducibility of SA in fruit juices and soft drinks.

Analyte (SA)	Low Spike Level (1 µg/mL)		High Spike Level (25 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=9)	Soft Drinks (n=12)	Fruit Juices (n=8)	Soft Drinks (n=13)
Number of replicates in two days	86.4	74.3	121	95.6
	75.9	78.6	109	118
	94.3	75.4	116	112
	91.0	82.3	115	118
	91.9	78.4	111	111
	90.3	61.0	110	100
	86.5	79.6	119	100
	79.6	72.5	120	85.0
	71.4	62.8		105
		64.1		113
				109
		75.1		103
				90.8
		60.0		
Mean Value (MV)	**85.3**	**72.0**	**115**	**105**
SD	**7.89**	**6.08**	**5.00**	**10.2**
RSD (%)	**9.25**	**8.44**	**4.34**	**9.72**

Table 23: Reproducibility of MP in fruit juices and soft drinks.

Analyte (MP)	Low Spike Level (1 µg/mL)		High Spike Level (12.5 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=7)	Soft Drinks (n=11)	Fruit Juices (n=10)	Soft Drinks (n=12)
Number of replicates in two days	96.7	81.0	78.6	93.8
	89.7	75.0	77.4	
	88.7	81.2	74.2	118
	63.6	77.6	65.9	112
	88.1	78.7	71.1	117
	70.9	76.9	69.5	109
		74.1	76.8	115
	82.0	74.3	85.8	111
		75.6		101
		96.7	78.1	92.2
				117
		75.2	66.4	120
				113
Mean Value (MV)	**82.8**	**78.8**	**74.4**	**110**
SD	**11.6**	**6.58**	**6.21**	**9.37**
RSD (%)	**14.0**	**8.36**	**8.35**	**8.52**

Table 24: Reproducibility of EP in fruit juices and soft drinks.

Analyte (EP)	Low Spike Level (1 µg/mL)		High Spike Level (12.5 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=9)	Soft Drinks (n=12)	Fruit Juices (n=8)	Soft Drinks (n=10)
Number of replicates in two days	93.1	70.7	84.9	97.9
	87.3	69.9	80.0	
	87.4	78.1	76.5	120
	66.8	67.3	82.7	114
	87.1	70.4	85.1	
	64.6	74.1	92.1	113
	72.0	67.4	81.5	104
	72.8	74.5		106
		77.2		111
		77.5	75.8	119
	64.5			114
		88.9		106
		72.6		
Mean Value (MV)	**77.3**	**74.1**	**82.3**	**111**
SD	**11.4**	**5.98**	**5.24**	**6.97**
RSD (%)	**14.7**	**8.07**	**6.37**	**6.31**

Table 25: Reproducibility of PP in fruit juices and soft drinks.

Analyte (PP)	Low Spike Level (1 µg/mL)		High Spike Level (12.5 µg/mL)	
	Recovery Rates (%)			
	Fruit Juices (n=6)	Soft Drinks (n=12)	Fruit Juices (n=7)	Soft Drinks (n=13)
Number of replicates in two days	79.4	76.5	83.7	92.3
	77.7	75.6	78.4	110
	70.8	84.9	81.2	110
		71.4	82.6	105
	75.0	77.9	91.2	118
		77.5	79.4	115
	65.3	71.4		104
	62.1	72.4	70.2	96.6
		76.4		97.3
		79.4		105
				111
		89.1		107
		73.4		99.9
Mean Value (MV)	**71.7**	**76.9**	**81.0**	**105**
SD	**6.94**	**5.23**	**6.30**	**7.44**
RSD (%)	**9.7**	**6.80**	**7.78**	**7.05**

Table 26: Reproducibility of BP in fruit juices and soft drinks.

<table>
<tr><th>Analyte (BP)</th><th colspan="2">Low Spike Level (2.6 µg/mL)</th><th colspan="2">Low Spike Level (1 µg/mL)</th><th colspan="2">High Spike Level (12.5 µg/mL)</th></tr>
<tr><td></td><td colspan="6">Recovery Rates (%)</td></tr>
<tr><td></td><td colspan="2">Fruit Juices (n=11)</td><td colspan="2">Soft Drinks (n=10)</td><td>Fruit Juices (n=9)</td><td>Soft Drinks (n=13)</td></tr>
<tr><td rowspan="15">Number of replicates in two days</td><td colspan="2">76.3</td><td colspan="2">84.1</td><td rowspan="2">98.5</td><td>87.8</td></tr>
<tr><td colspan="2">95.8</td><td colspan="2">86.2</td><td>95.8</td></tr>
<tr><td colspan="2">87.6</td><td colspan="2">88.6</td><td>89.9</td><td>94.7</td></tr>
<tr><td colspan="2">96.9</td><td colspan="2">101</td><td>89.3</td><td>89.5</td></tr>
<tr><td colspan="2" rowspan="2">88.2</td><td colspan="2" rowspan="2">87.9</td><td rowspan="2">99.0</td><td>86.1</td></tr>
<tr><td>89.0</td></tr>
<tr><td colspan="2">69.8</td><td colspan="2">86.9</td><td>97.6</td><td>99.2</td></tr>
<tr><td colspan="2" rowspan="4">73.8</td><td colspan="2" rowspan="4">82.6</td><td rowspan="4">88.8</td><td>86.7</td></tr>
<tr><td>94.6</td></tr>
<tr><td>98.0</td></tr>
<tr><td>96.2</td></tr>
<tr><td colspan="2" rowspan="3">73.9</td><td colspan="2">75.8</td><td>88.9</td><td>106</td></tr>
<tr><td colspan="2">83.5</td><td>81.3</td><td rowspan="5">89.0</td></tr>
<tr><td colspan="2" rowspan="4">69.3</td><td rowspan="4">78.8</td></tr>
<tr><td colspan="2">71.3</td></tr>
<tr><td colspan="2">74.7</td></tr>
<tr><td colspan="2">76.9</td></tr>
<tr><td>Mean Value (MV)</td><td colspan="2">80.5</td><td colspan="2">84.6</td><td>90.2</td><td>93.3</td></tr>
<tr><td>SD</td><td colspan="2">9.82</td><td colspan="2">5.97</td><td>7.20</td><td>5.91</td></tr>
<tr><td>RSD (%)</td><td colspan="2">12.2</td><td colspan="2">9.77</td><td>7.98</td><td>6.34</td></tr>
</table>

As it is shown, the recoveries are satisfactory and between the range of 70-120% for both spike levels in every substrate. RSD% are below 20%, which is the criteria for the reproducibility (ICH Q2(R2)). Therefore, the method satisfies the precision criterion, in terms of repeatability and reproducibility. Any violation of the criteria limits is due to errors during the experimental process.

5.2.4 Horwitz equation

As another criterion for the evaluation of the repeatability and the reproducibility of the method, the Horwitz equation was used. As Horwitz ratio (HorRat) has been named a normalized performance parameter indicating the acceptability of methods of analysis with respect to within- (repeatability) and among-laboratory precision (reproducibility). More specifically, it is the ratio of the observed relative standard deviation within and among laboratories calculated from the actual performance data, $RSD_{R/r}$ (%), to the corresponding predicted relative standard deviation calculated from the Horwitz equation $PRSD_{R/r}$ (%) = $2C^{-0.15}$ (when C is betwwen $1.2 \times 10^{-7} \leq C \leq 0.138$), where C is the concentration added, expressed as a mass fraction. The equation is independent of analyte, matrix, and method used. The index R or r refers to reproducibility (R) and repeatability (r) respectively and $PRSD_r = 0.66 * PRSD_R$. The HorRat formula is shown below and must be between 0.5-2 for the method to meet the precision criteria. [75]

$$\text{HorRat} = \frac{\text{RSDR/r (\%)}}{PRSDR/r\ (\%)} \qquad \textbf{(Equation 2)}$$

The Horwitz equation was used to calculate $PRSD_{R/r}$ for each analyte, at each spike level and the results were compared with those obtained from the experimental data. The results are presented in the tables below.

Table 27: HorRat in Fruit Juices.

Analyte	Spike Level	$PRSD_R$ (%)	RSD_R (%)	$HorRat_R$	$PRSD_r$ (%)	RSD_r (%)	$HorRat_r$
BA	2 µg/mL	14.3	9.32	**0.65**	9.44	10.3	**1.09**
	25 µg/mL	9.8	7.64	**0.78**	6.47	4.31	**0.67**
SA	1 µg/mL	15.9	9.25	**0.58**	10.5	6.48	**0.62**
	25 µg/mL	9.8	4.34	**0.44**	6.47	3.68	**0.57**
MP	1 µg/mL	15.9	14.0	**0.88**	10.5	6.75	**0.64**
	12.5 µg/mL	10.9	8.35	**0.77**	7.19	6.42	**0.89**
EP	1 µg/mL	15.9	14.7	**0.92**	10.5	10.2	**0.97**
	12.5 µg/mL	10.9	6.37	**0.58**	7.19	6.35	**0.88**
PP	1 µg/mL	15.9	9.7	**0.61**	10.5	10.3	**0.98**
	12.5 µg/mL	10.9	7.78	**0.71**	7.19	6.46	**0.90**
BP	2.6 µg/mL	13.8	12.2	**0.88**	9.11	9.67	**1.06**
	12.5 µg/mL	10.9	7.98	**0.73**	7.19	5.96	**0.83**

Table 28: HorRat in Soft Drinks.

Analyte	Spike Level	$PRSD_R$ (%)	RSD_R (%)	$HorRat_R$	$PRSD_r$ (%)	RSD_r (%)	$HorRat_r$
BA	2 µg/mL	14.3	9.60	**0.67**	9.44	7.55	**0.80**
	25 µg/mL	9.8	11.0	**1.12**	6.47	10.3	**1.59**
SA	1 µg/mL	15.9	8.44	**0.53**	10.5	4.69	**0.45**
	25 µg/mL	9.8	9.72	**0.99**	6.47	10.3	**1.59**
MP	1 µg/mL	15.9	8.36	**0.53**	10.5	3.67	**0.35**
	12.5 µg/mL	10.9	8.52	**0.78**	7.19	8.17	**1.14**
EP	1 µg/mL	15.9	8.07	**0.51**	10.5	5.23	**0.50**
	12.5 µg/mL	10.9	6.31	**0.58**	7.19	10.1	**1.40**
PP	1 µg/mL	15.9	6.80	**0.43**	10.5	5.91	**0.56**
	12.5 µg/mL	10.9	7.05	**0.65**	7.19	8.15	**1.13**
BP	1 µg/mL	15.9	9.77	**0.61**	10.5	8.32	**0.79**
	12.5 µg/mL	10.9	6.34	**0.58**	7.19	7.20	**1.00**

Observing the HorRat values for repeatability and reproducibility ($HorRat_r$ and $HorRat_R$ respectively), the values are between the permitted limit of 0.5 and 2, with a very few exceptions, due to experimental errors. Therefore, the method meets the precision criteria.

5.3 Detectability

Detectability is the ability of the method to detect and quantify low concentrations of the analyte and it has two expressions [74]:

• Limit of Detection (LOD): the lowest signal that can be observed with a sufficient degree of confidence, but it cannot be quantified.

• Limit of Quantification (LOQ): the lowest concentration of a substance that could be reliably measured using standard tests.

There are three cases:

- When the signal is lower than LOD, the detection of the analyte is not possible (noise).
- When the signal is between LOD-LOQ, the detection of the analyte is reliable and its signal is distinguished from noise.
- When the signal is above LOQ, the quantification of the analyte is reliable.

To calculate the detection limits, the following equations were used:

$$LOD = \frac{3.3\ x\ SD}{b} \qquad \textbf{(Equation 3)}$$

$$LOQ = \frac{10\ x\ SD}{b} \qquad \textbf{(Equation 4)}$$

Where b is the slope of the standard addition method curve of each analyte and SD is standard deviation of the signal response of a set of replicate measurements

of a sample containing small but known concentrations (2 μg/mL BA, 2.6 μg/mL BP and 1 μg/mL SA, MP, EP, PP for fruit juices and 2 μg/mL BA and 1 μg/mL SA, MP, EP, PP, BP for soft drinks) of the substance of interest.

Results for the limits of detection and quantification of the six preservatives are presented in the following tables.

Table 29: LOD and LOQ of the analytes in fruit juices.

Analyte	Slope	LOD (μg/g)	LOQ (μg/g)
BA	11375	0.720	2.161
SA	25371	0.185	0.554
MP	14707	0.226	0.677
EP	17659	0.317	0.950
PP	17772	0.347	1.050
BP	16814	0.737	2.210

Table 30: LOD and LOQ of the analytes in soft drinks.

Analyte	Slope (b)	LOD (μg/g)	LOQ (μg/g)
BA	10480	0.654	1.963
SA	21766	0.149	0.447
MP	13632	0.131	0.392
EP	15441	0.175	0.525
PP	15370	0.219	0.658
BP	12341	0.278	0.833

Referring to Table 3, it is observed that the detection limits and quantification of the six analytes are much lower than those of previous surveys, proving the strong method detectability. [43, 44, 45, 51, 53] More specifically, Sun and his team (Sun et al., 2015), calculated a value LOD=3-10 μg/g for the six target compounds in both matrices, using the same aqueous buffer, but different organic modifier (MeOH). Another example is the Islam's group research (Islam et al., 2019), where the LOD values ranged between 1.08-5.46 μg/g, when the simple sample preparation of dissolution, centrifugation and filtration was followed. In both cases, higher LOD values were observed with respect to the developed method (0.185-0.737 μg/g in

fruit juices and 0.131-0.654 μg/g in soft drinks). Moreover, the limits of detection and quantification are very low in relation to the permitted legislation (Table 1 and 2), concluding that the specific method that has been developed is suitable for detection and quantification of very low amounts of the six preservatives simply, easily and without high cost.

5.4 Evaluation of method applicability in energy drinks

The last stage for the validation of the method was to check whether it could be applied for the analysis of energy drinks. To test the applicability on the specific substrate, six replicates of an energy drink sample were spiked with an amount of each analyte (25 μg/mL BA, SA and 12.5 μg/mL PHBs). Finding the Mean Value of Recovery, Standard Deviation (SD) and RSD%, a t-test was performed with the corresponding results obtained by the analysis of soft drinks in order to evaluate whether there is a statistically significant difference between the results of the matrices. The chromatograms of a blank sample and a spiked sample are also shown.

Table 31: Recovery of analytes in energy drinks.

Analyte	Recovery Rate (%)	MV±SD, %RSD	P value
BA	104	**95.3±5.30, %5.57**	**0.57**
	92.8		
	98.5		
	95.8		
	89.4		
	91.3		
SA	104	**99.7±4.66, %4.68**	**0.53**
	96.8		
	106		
	99.5		
	93.7		
	97.84		
MP	126	**119.6±7.95, %6.65**	**0.31**
	124		
	128		
	116		
	107		
	116		
EP	113	**106±5.94, %5.61**	**0.84**

	103		
	113		
	105		
	98.8		
	102		
PP	99.4	**93.7±5.88, %6.28**	**0.15**
	91.4		
	102		
	92.6		
	87.2		
	89.2		
BP	97.3	**92.6±5.85, %6.32**	**0.92**
	99.6		
	90.5		
	87.7		
	95.8		
	84.7		

Comparing the recoveries for the specific spike level between the soft drink (High Spike Level in Table 15-20) and the energy drink substrate (Table 31), a t-test was performed. The value was $p>0.05$ for all six analytes, thus there was not a significant difference between the two substrates, leading to the conclusion that the method can be reliably used in energy drinks as well.

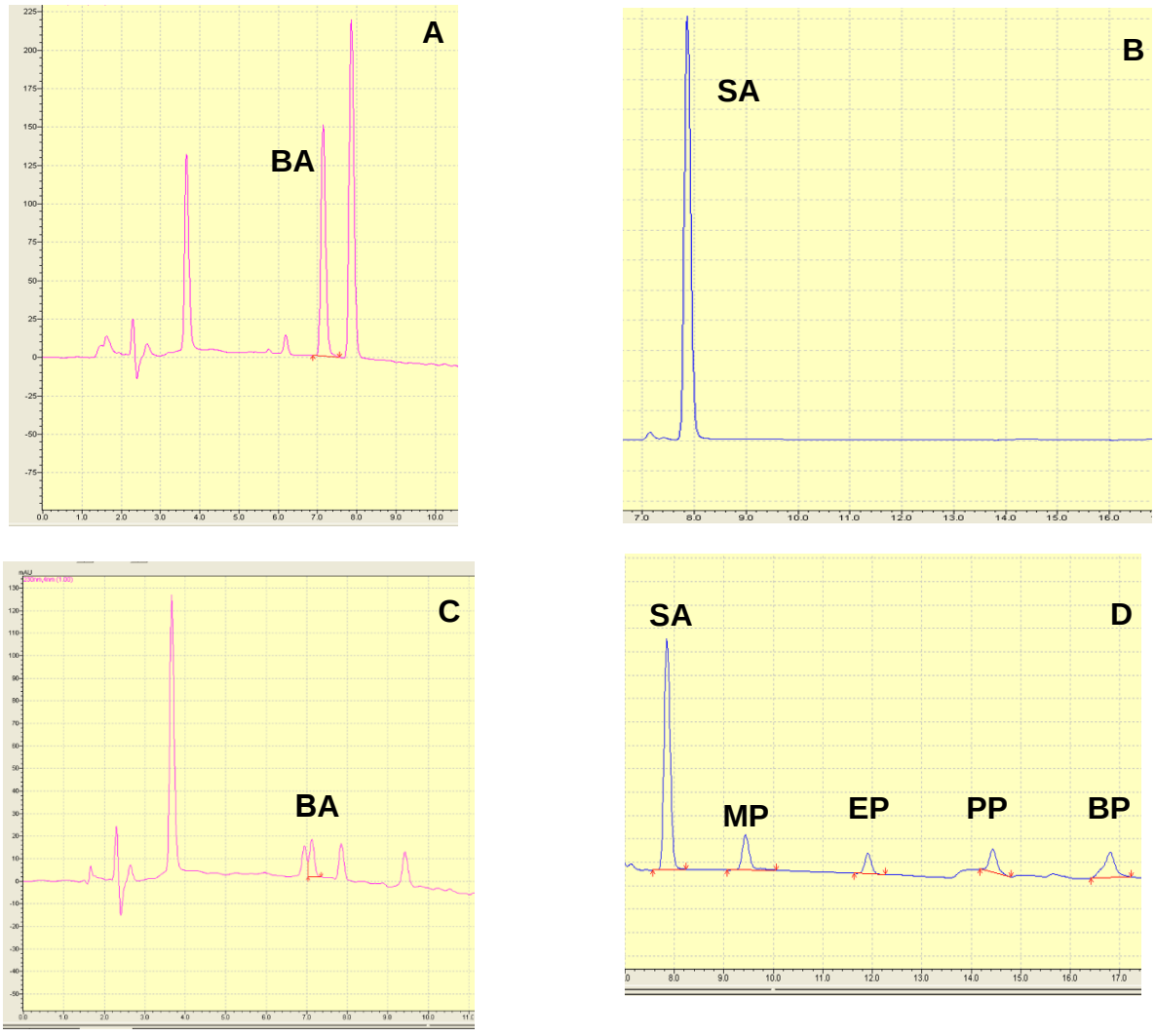

Figure 55: Chromatogram of A: blank energy drink sample in 230 nm, B: blank energy drink sample in 254 nm, C: spiked energy drink sample in 230 nm, D: spiked energy drink sample in 254 nm.

CHAPTER 6
Analysis of market samples

The last part of the thesis was the quantification of real market samples, in order to check the applicability of the developed method, the existence and the quantity of six preservatives: benzoic acid, sorbic acid and four parabens in Greek market. To our best knowledge, there is no similar study, analysing greek market samples for the determination of preservatives, using HPLC-DAD. The method above was tested on a range of fruit juices and soft drink samples of Greek origin and energy drinks which were subjected to a minimal sample pretreatment prior to injection into the chromatographic apparatus, which is described in Fig. 32. In this way, compliance with the European legislative framework will be checked and any kind of violation will be noted.

6.1 Sampling

In total, 44 market samples were purchased and collected from local Greek market stores or from Greek supermarket chains. Of these, 22 samples were fruit juices, 15 soft drinks and 7 energy drinks. The samples analyzed belonged to the following companies:

Fruit Juices-Greek Companies

- Mr Grand
- Chris family
- Kampos Chiou
- Koukaki
- Olympos
- Apo ton topo mas
- Agroktima
- Viva fresh
- Economy
- Lakonia juices

- AB think nutri
- AB Vasilopoulos

Soft drinks-Greek Companies

- Green Cola Hellas
- Mr Grand
- Epsa
- Apo ton topo mas
- Vikos
- Economy
- Nektar
- Loux
- Kliafa

Energy drinks-Non Greek companies

- Bang
- Monster
- Red Bull
- Hell
- 365 essential

For the quantification of the samples, a sample was used in every analysis, in which all six analytes (10 μg/mL BA, SA and 5 μg/mL PHBs) were added in the blank sample, to calculate the amount of spike in relation to the matrix.

6.2 Analysis of samples and results

The samples mentioned above were analyzed, using the method for the determination of preservatives developed in this master thesis. In this subsection, the results from this study will be presented, numerically and diagrammatically.

6.2.1 Quantitative Results

Quantification of samples was performed, as mentioned above, by spiking a sample with known quantity of all analytes (10 μg/mL BA, SA and 5 μg/mL PHBs), to calculate the amount of each compound on each substrate. In addition, beyond identification of retention time, the use of identification of UV spectrum was used for confirmation. The formula used for quantification in every sample is as follows:

$$C\ (\mu g/g) = \frac{Peak\ Area\ of\ blank\ sample * Cspike}{Peak\ Area\ of\ Spiked\ Sample - Peak\ Area\ of\ blank\ sample} \quad \textbf{(Equation 5)}$$

Where C_{spike}= 10 μg/mL for BA, SA and 5 μg/mL for PHBs.

6.2.2 Presentation of results

The presentation of the samples was carried out using pie charts for each preservative in every substrate (fruit juices, soft drinks, energy drinks), as well as chromatograms and tables showing the amounts of preservatives in each food matrix. In each chart, the number of samples that are positive for that analyte and contain an amount above the limit of quantification, suspect samples, samples with amount between LOD-LOQ and negative samples are shown. As suspect samples are those that may contain preservatives, (>LOQ), but the UV spectrum of the peak at the specific time does not match 100% with that of the preservative, so identification cannot be completed and further mass spectrometry analysis would be needed, in order to confirm the detection of this preservative in these samples.

6.2.2.1 Fruit Juices Samples

The identification was carried out, using the elution time of the method, as well as the UV spectrum (Subsection 4.5). In Fig. 57, it is shown the chromatogram of the positive fruit juice sample in BA.

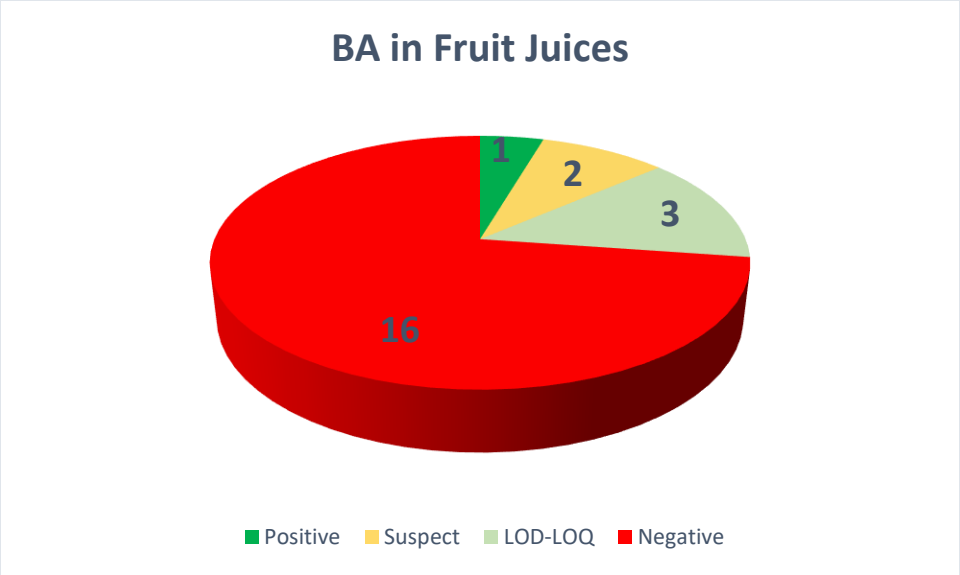

Figure 56: BA in Fruit Juices Samples.

Figure 57: Chromatogram of fruit juice market sample in 230 nm, positive in BA and UV spectrum of this peak, confirming the presence of BA.

As it can be seen, the greater percentage of the fruit juice samples do not contain benzoic acid and the positive fruit juices sample contained 5.45 µg/g of BA, amount much lower than the legal limit (250 µg/g).

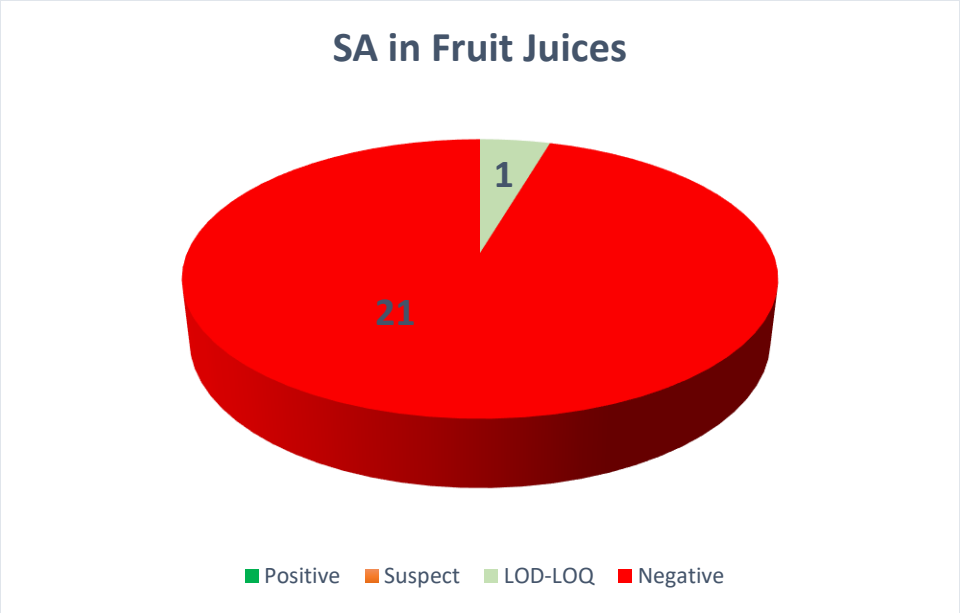

Figure 58: SA in Fruit Juices Samples.

Looking at the diagram, only one sample contains sorbic acid and specifically in an amount that is between the detection and the quantification limit.

Regarding the addition of parabens as preservatives, they were not detected in any of the fruit juice samples.

In summary, the results of the analysis for the fruit juices are presented below:

Table 32: Fruit Juices Results.(*N.D: Not Detected)

Samples	BA (μg/g)	SA (μg/g)	Parabens (μg/g)
No 1	Peak (LOD-LOQ)	*N.D	*N.D
No 2	Peak (LOD-LOQ)	*N.D	*N.D
No 3	*N.D	*N.D	*N.D
No 4	*N.D	*N.D	*N.D
No 5	*N.D	*N.D	*N.D
No 6	*N.D	*N.D	*N.D
No 7	2.80 (not UV-matched)	*N.D	*N.D
No 8	*N.D	*N.D	*N.D
No 9	*N.D	*N.D	*N.D
No 10	*N.D	*N.D	*N.D
No 11	*N.D	*N.D	*N.D
No 12	*N.D	*N.D	*N.D
No 13	*N.D	*N.D	*N.D

Samples	BA (µg/g)	SA (µg/g)	Parabens (µg/g)
No 14	5.45 (positive sample, >LOQ)	*N.D	*N.D
No 15	*N.D	*N.D	*N.D
No 16	*N.D	*N.D	*N.D
No 17	*N.D	*N.D	*N.D
No 18	*N.D	Peak (LOD-LOQ)	*N.D
No 19	*N.D	*N.D	*N.D
No 20	Peak (LOD-LOQ)	*N.D	*N.D
No 21	3.64 (not UV-matched)	*N.D	*N.D
No 22	*N.D	*N.D	*N.D

6.2.2.2 Soft Drinks Samples

According to the elution time and the UV spectrum (Subsection 4.5), the preservatives were identified (Fig. 59 and 60).

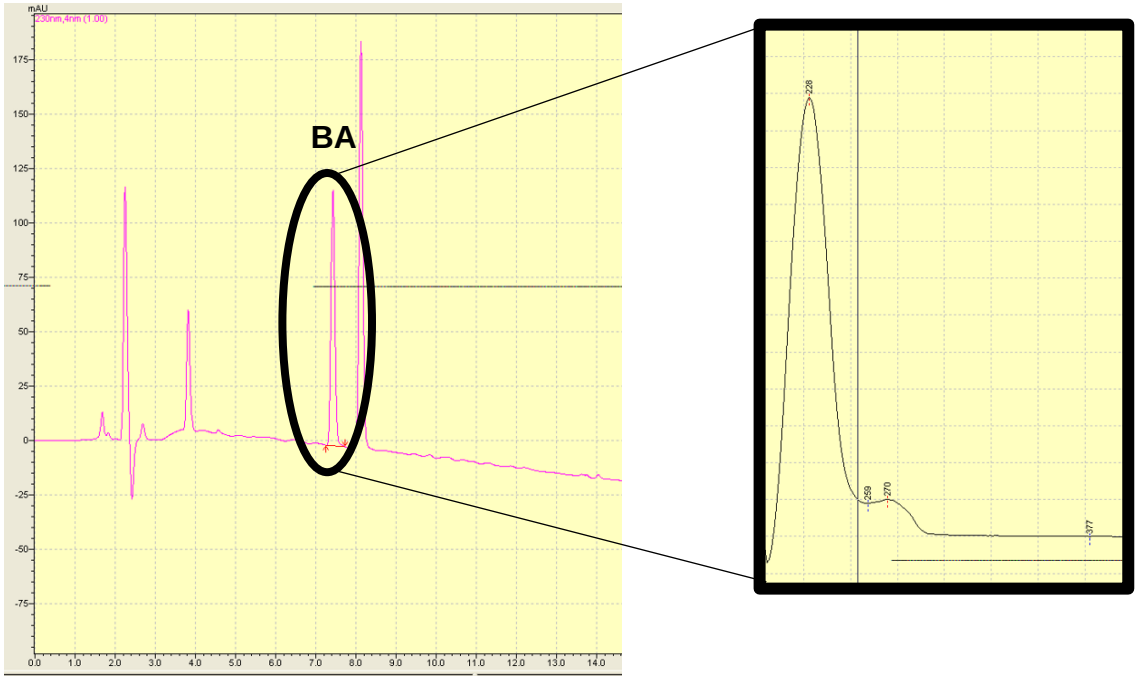

Figure 59: Chromatogram of soft drink market sample in 230 nm, positive in BA and UV spectrum of this peak, confirming the presence of BA.

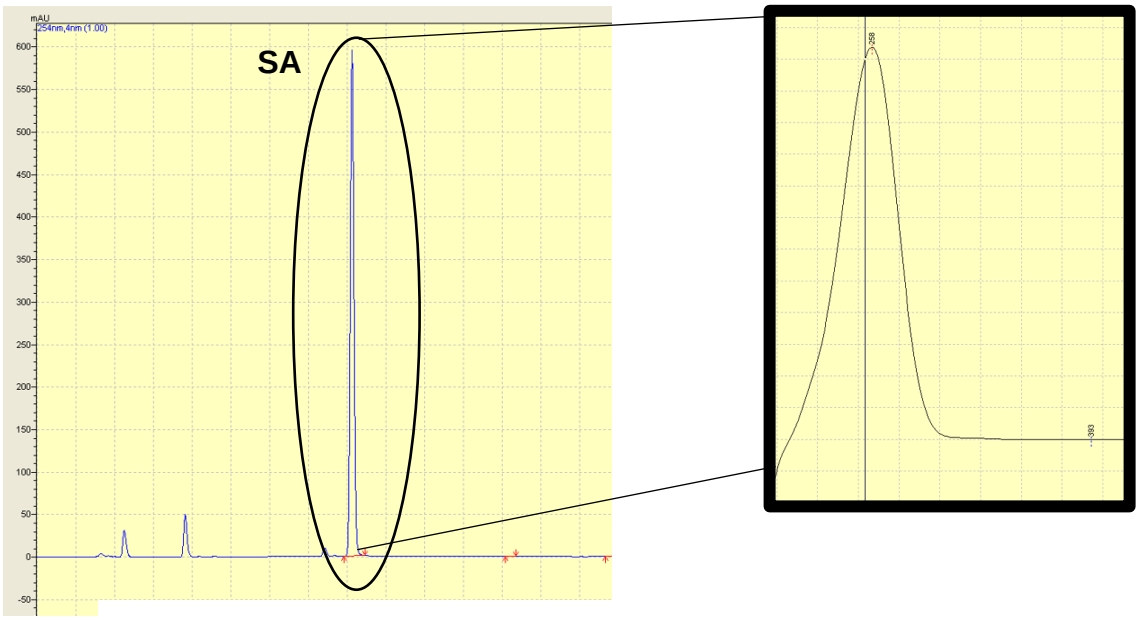

Figure 60: Chromatogram of soft drink market sample in 254 nm, positive in SA and UV spectrum of this peak, confirming the presence of SA.

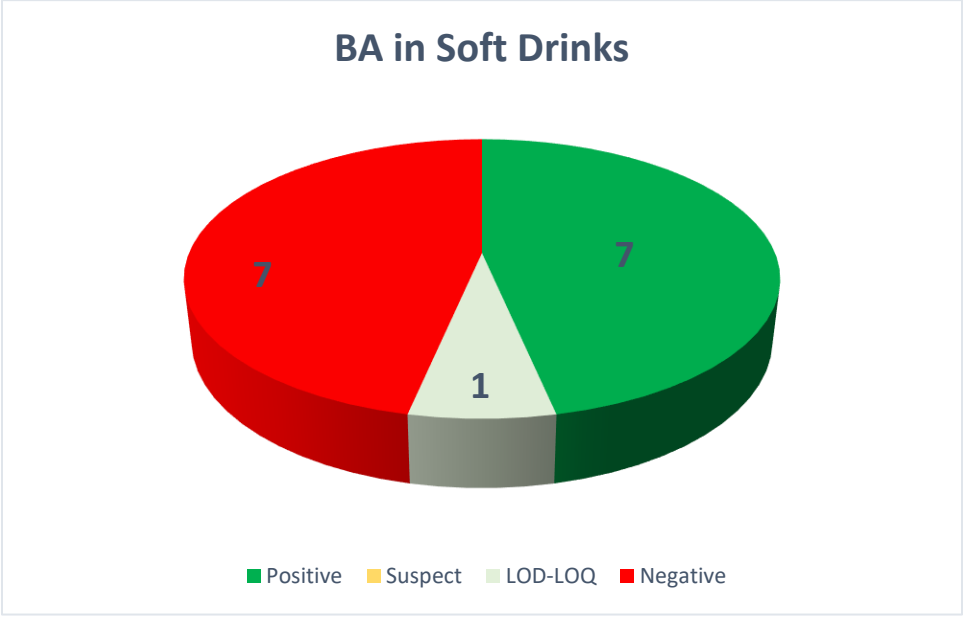

Figure 61: BA in Soft Drinks Samples.

Almost half of the soft drink samples contain benzoic acid and these amounts are above the LOQ of BA in soft drinks (Table 30) and below the legal limit (150 μg/g).

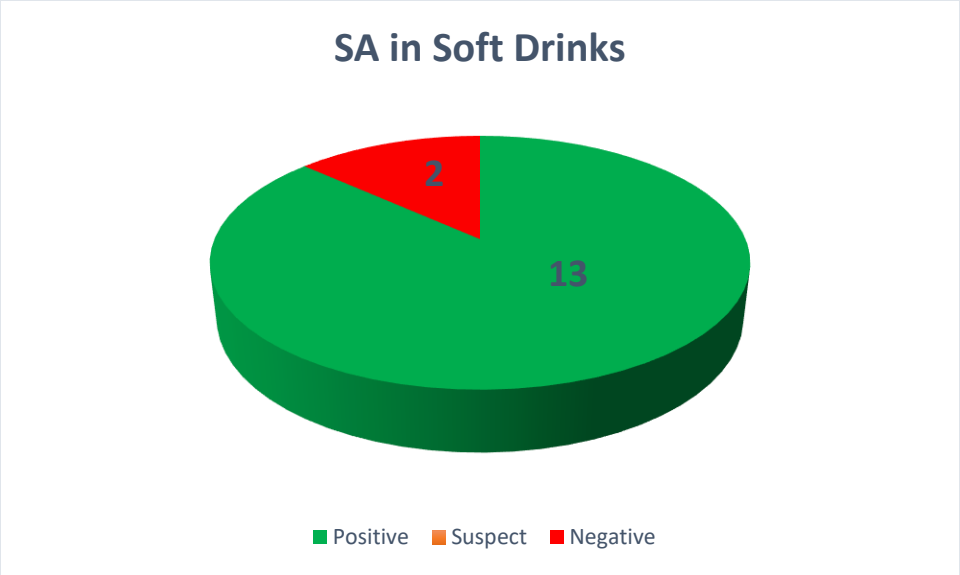

Figure 62: SA in Soft Drinks Samples.

Almost all samples contain amounts of sorbic acid and these quantities exceed the limit of quantification (Table 30), but do not exceed the limit of the legislative framework [300 μg/g, 250 μg/g (if benzoic acid is present)].

No parabens were detected.

Specifically, the results are shown below:

Table 33: Soft Drinks Results. (*N.D: Not Detected)

Samples	BA (μg/g)	SA (μg/g)	Parabens (μg/g)
No 1	*N.D	65.3 (Positive sample, >LOQ)	*N.D
No 2	67.4 (Positive sample, >LOQ)	46.8 (Positive sample, >LOQ)	*N.D
No 3	Peak (LOD-LOQ)	74.7 (Positive sample, >LOQ)	*N.D
No 4	*N.D	74.0 (Positive sample, >LOQ)	*N.D
No 5	*N.D	78.2 (Positive sample, >LOQ)	*N.D
No 6	80.8 (Positive sample, >LOQ)	45.2 (Positive sample, >LOQ)	*N.D
No 7	*N.D	49.5 (Positive sample, >LOQ)	*N.D

Samples	BA (µg/g)	SA (µg/g)	Parabens (µg/g)
No 8	67.4 (Positive sample, >LOQ)	0.89 (Positive sample, >LOQ)	*N.D
No 9	*N.D	41.6(Positive sample, >LOQ)	*N.D
No 10	*N.D	*N.D	*N.D
No 11	41.8 (Positive sample, >LOQ)	41.5 (Positive sample, >LOQ)	*N.D
No 12	102 (Positive sample, >LOQ)	128 (Positive sample, >LOQ)	*N.D
No 13	140 (Positive sample, >LOQ)	*N.D	*N.D
No 14	13.9 (Positive sample, >LOQ)	191 (Positive sample, >LOQ)	*N.D
No 15	*N.D	0.99 (Positive sample, >LOQ)	*N.D

6.2.2.3 Energy Drinks Samples

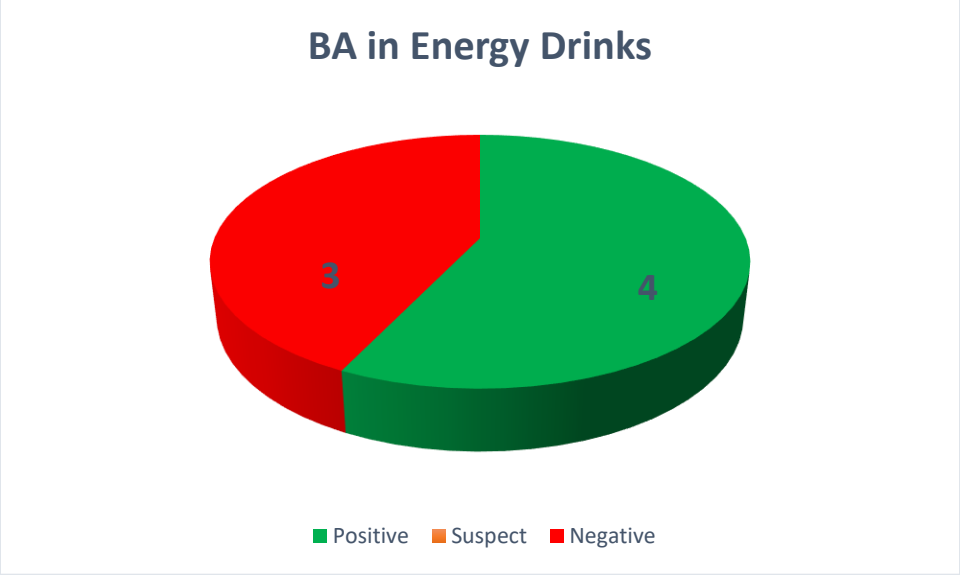

Figure 63: BA in Energy Drinks Samples.

The majority of energy drinks contain benzoic acid as a preservative above the LOQ limit.

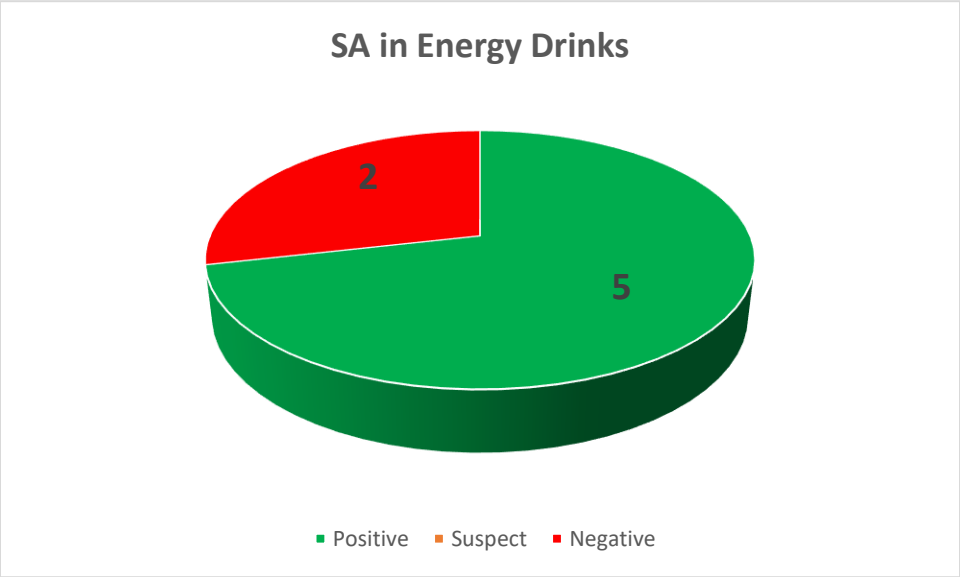

Figure 64: SA in Energy Drinks Samples.

The most used preservative is sorbic acid, which is used synergistically with benzoic acid, in energy drinks. The measured values are above the limit LOQ, but do not exceed the legal limits (Table 2).

No parabens were detected.

Taking into account the legislative framework for BA [250 μg/g and 450 μg/g if pH>3.5)] (Table 1), in the first three samples, pH measurement was performed, in order to check if there is exceeding of the permitted limit of addition of the specific preservative. As it could be seen in Table 34 below, samples No. 1, No. 2 and No.3 contain amount of benzoic acid above the legal limit, as their pH was measured below 3.5 and thus the permitted limit of addition of this preservative is 250 μg/g. The chromatogram of energy drink sample No.1, where benzoic acid has been added in an amount above the permitted legislative limit is presented. Below, the measured quantities for each sample are listed.

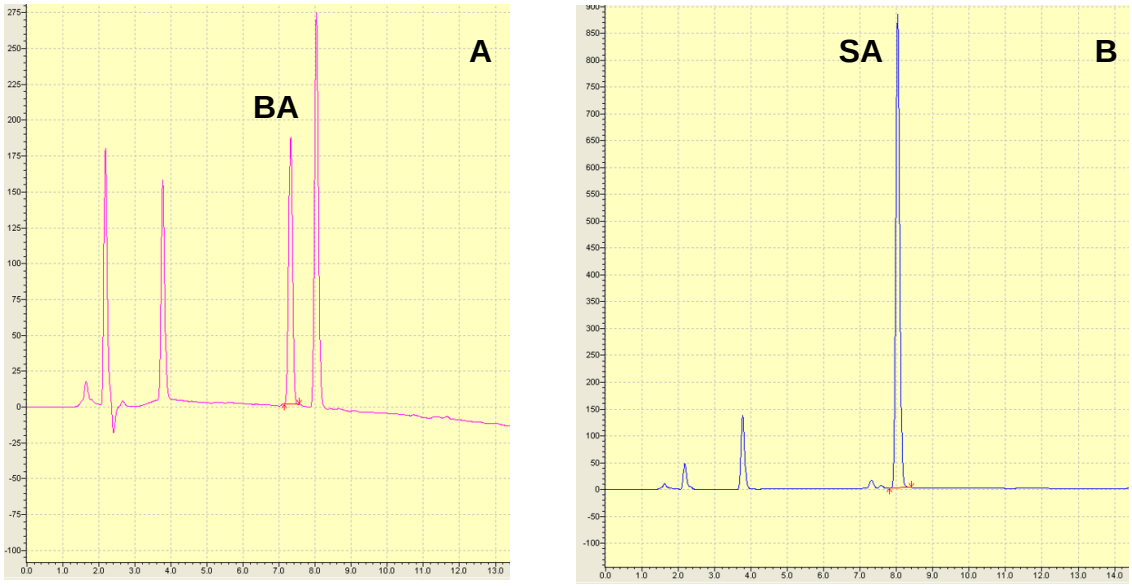

Figure 65: Chromatogram of energy drink market sample, positive in BA and SA in A: 230 nm and B: 254 nm. The quantity of BA is exceeding the permissible limit.

Table 34: Energy drinks Results.(*N.D: Not Detected

***N.M: Not Measured)**

Samples	pH	BA (µg/g)	SA (µg/g)	Parabens (µg/g)
No 1	3.18	341 (Positive sample, >LOQ, >Legal Limit)	357 (Positive sample, >LOQ)	*N.D.
No 2	3.40	316 (Positive sample, >LOQ, >Legal Limit)	380 (Positive sample, >LOQ)	*N.D.
No 3	3.07	317 (Positive sample, >LOQ, >Legal Limit)	320 (Positive sample, >LOQ)	*N.D.
No 4	*N.M.	*N.D.	2.23 (Positive sample, >LOQ)	*N.D.
No 5	*N.M.	*N.D.	*N.D.	*N.D.
No 6	*N.M.	24.3 (Positive sample, >LOQ)	12.4 (Positive sample, >LOQ)	*N.D.
No 7	*N.M.	*N.D.	*N.D.	*N.D.

CHAPTER 7
CONCLUSIONS

In this master thesis, a new, rapid and accurate method for the simultaneous analysis of six preservatives in fruit juices, soft drinks and energy drinks, using HPLC-PDA, was developed, optimized and validated. The six preservatives: benzoic acid, sorbic acid and four parabens are known for their continuous use, and for this reason they were chosen for this study.

Compared to previous methods, the developed methodology accelerated the sample preparation and improved the efficiency using modified QuEChERS extraction without clean-up. In terms of instrumental analysis, parameters such as mobile phase composition, standards' solvent, flow rate, and type of filtration, were investigated in order to select the best conditions for the fast, sensitive and inexpensive detection and quantification of the six preservatives.

A gradient elution system was applied, using ammonium acetate 5mM (pH = 4.2) and acetonitrile and was capable of qualitative and quantitative detection of these additives. The method for the six preservatives in three types of samples was then validated in terms of linearity (R^2>0.99), accuracy (R(%)= 71.8-118% and 71.6-109% for fruit juices and soft drinks respectively), precision (RSD_r < 15% and RSD_R < 20% with HorRat ratio between acceptable limits for all analytes in every substrate) and detectability (LOD: 0.185-0.737 and 0.131-0.654 μg/g for fruit juices and soft drinks respectively).

After the development and the validation of the method, this method was applied to the analysis of commercially available Greek products: 22 fruit juices and 15 soft drinks of Greek origin and 7 energy drinks of foreign companies. As for the fruit juices, only one sample contained amount of benzoic acid, but in a significantly smaller quantity than the permitted limit. In soft drinks, the presence of sorbic acid was observed in 13 samples and benzoate in 7 in the total of 15. None of the positive samples exceeded the limits specified in EU Regulation. Finally, regarding the energy drinks, 4 were positive for the presence of benzoate, of which 3 had

violated the legal framework. Also 5 out of 7 energy drinks contain sorbic acid. In none of the 44 samples was the presence of parabens detected.

As it could be understood, the sample pre-treatment procedure, in combination with the HPLC developed method was found to be suitable for the routine determination of these preservatives in food products, like fruit juices, soft drinks and energy drinks. The application of the method in products of Greek origin showed that the Greek market has products that respect and follow the legislative framework, using preservatives as little as possible.

ABBREVIATIONS – ARCHIVES – ACRONYMS

BA	Benzoic Acid
SB	Sorbic Benzoate
SA	Sorbic Acid
PS	Potassium Sorbate
MP	Methyl Paraben
EP	Ethyl Paraben
PP	Propyl Paraben
BP	Butyl Paraben
RP	Reversed Phase
HPLC	High Performance Liquid Chromatography
DAD	Diode Array Detector
PDA	Photodiode Array Detector
PHBs	*p*-hydroxybenzoates
ACN	Acetonitrile
MeOH	Methanol
Tetrahydrofuran	THF
Isopropanol	IPA
RT	Retention time
RC	Regenerated cellulose
PVDF	Polyvinylidene difluoride
R(%)	Recovery
MV	Mean Value
SD	Standard Deviation
RSD (%)	Relative Standard Deviation
RSD_r (%)	Relative Standard Deviation for repeatability
RSD_R (%)	Relative Standard Deviation for repeatability for reproducibility
LLE	Liquid-Liquid Extraction

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