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UNIVERSITAT DE BARCELONA

UNIVERSITY OF BERGEN

Department of Chemistry

EVALUATION OF ANALYTICAL STRATEGIES FOR QUANTIFYING SELECTED POLYPHENOLS IN FEED PRODUCTION: FROM INGREDIENT TO FISH

Supervisor: Prof. Pedro Araujo

ERASMUS MUNDUS MASTER IN QUALITY IN ANALYTICAL LABORATORIES - EMQAL

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I can still remember how much I wanted to be part of EMQAL!... It was a dream… a dream which has come true!

Words cannot express how grateful I am for this invaluable experience. Firstly, I thank God for opening all the doors that have brought me here. He has been with me throughout this journey; thus, this work is for him.

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My loved parents and brother have also been of major support during this stage. I could not have undertaken this journey without their unconditional love and company. Thank you for loving me so much, for making me the person I am and accepting me just as I am, for always being with me and for encouraging me to pursue my dreams even when you do not completely agree with them.

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To all of you, my angels and everyone who knows is special for me… Thank you!

LIST OF ABREVIATIONS

12-O-MECA	12-O-methyl carnosic acid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CA	Carnosic acid
CAT	Catechin
CAV	Cell accelerator voltage
CE	Collision energy
COH	Carnosol
CV	Cofficient of variation
DT	Dwell time
EIC	Extracted ion chromatogram
FCR	Feed conversion rate
GA	Gallic acid
HOH	Hydroxytyrosol
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NA	Not applicable
NAOX	Natural antioxidants
NC	Not calculated
OLE	Oleuropein
PI	Product Ion
PPHs	Polyphenols
PUFAs	Polyunsaturated fatty acids
RA	Rosmarinic acid
RME	Relative matrix effect
ROS	Rosmanol
SAM	Standard addition method
SIM	Single ion monitoring
TBHQ	Tert-Butylhydroquinone or tertiary butylhydroquinone

ABSTRACT

Polyphenols are an extensive group of compounds with important antioxidant properties, whose industrial use has shown a continuous increase in the last years. Their utilization in aquaculture is becoming progressively frequent, mainly for ameliorating the detrimental effects of the oxidative stress on fish. Their application for the partial or total replacement of the synthetic antioxidants is limited, despite numerous claims of similar, and in some cases, better performance than commonly used synthetic compounds as BHT and BHA. For the safe and efficient use of polyphenols in aquaculture, adequate analytical methods are needed, a task that is challenging due to their diversity in nature, instability, and the variety of matrices involved in the production process of the fish.

This work aims at analyzing quantitatively six selected polyphenols in various stages of a fish farming process for salmon. Simple extraction methods for the selected polyphenols were developed for different matrices: antioxidant ingredient, fish feed and fish tissue, and further quantification by LC-MS/MS was conducted, allowing the monitoring of the content of polyphenols from the antioxidant ingredient to the fish tissue.

The main polyphenols quantified in fish feed ingredients, fish diets at different production stages, and fish organs were hydroxytyrosol, carnosol, gallic acid and carnosic acid. Additionally, other related components, presumably 12-O-methyl carnosic acid, rosmanol and rosmarinic acid, were detected and semi-quantified by using the standards that closely resemble their chemical structure.

This is the first study monitoring the levels of natural polyphenol antioxidants from fish ingredients and diet production to fish organs.

1. INTRODUCTION

1.1. Relevance of the study

Aquaculture is of paramount importance for food security assurance worldwide and has shown a continuous growth and development in the last years. As the sector has been flourishing, many challenges have arisen in the attempt to reach the environmental and economical sustainability while meeting the elevated demand with high quality products that meet expectations from consumers and market trends. Some important challenges are:

- Substitution or reduction of synthetic antioxidants used in fish feed for safer and more natural alternatives (e.g., polyphenols).
- Amelioration of the effects on fish caused by oxidative stress, originated for the intensive production practices and other causes (pollution of water, climate change, seasons, among others).
- Development of analytical methods to establish appropriate/safe levels of the potential natural alternatives to synthetic compounds.

Natural antioxidants (NAOX) are considered a promising alternative to cope with the mentioned challenges, due to their well-known antioxidant and antimicrobial properties. There has been extensive research in the field of NAOX, mainly focused on extraction, identification, and characterization of polyphenols from diverse natural sources, an also on the evaluation of the antioxidant capacity of polyphenol extracts and the determination of their dietary supplementation in different organisms. Although there are many studies evaluating the effect of NAOX in animals (poultry and livestock in a greater extent than fish), the limited research on their inclusion in fish diets is surprising, especially considering that the few studies evaluating the total or partial replacement of synthetic antioxidants by NAOX in fish feed have observed that NAOX have equivalent or better performance than the synthetic ones. These positive findings highlight the significance of integrating animal feed production, impact on animals and analytical methods in the context of NAOX. Integrated studies are characterized by their complexity; however, they will allow the regulatory organizations to propose appropriate dietary levels for the successful utilization of NAOX in aquaculture.

For successful application in aquaculture, it is vital to have fish feed with a controlled content of NAOX to have reproducible results and to assure that the desired dosage is given to the fish. Additionally, this content must be known with the best possible accuracy, as the existence of an optimum dose of some NAOX, have been reported. Some authors have found that exceeding a specific threshold, the effects of NAOX could become detrimental for the species. Quantitative analytical methods allow monitoring the NAOX content during all the stages of the process.

When it comes to regulation, the most popular synthetic antioxidant, ethoxyquin, was banned in 2017 in EU, and the other synthetic antioxidant seems to be also in their way out from the market due to safety concerns and demands from the consumers, who are favoring products without artificial additives.

The present study aims at exploring the levels of selected NAOX in the whole chain of the fish production using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Different matrices were analyzed: commercial NAOX ingredients, diets samples at different stages of the industrial production,

and different fish tissues. The correlation of the various samples will contribute to understand better the performance of the compounds for a better evaluation and future implementation at industrial level.

1.2. Objectives

1.2.1. General

Analysis of selected polyphenols at different stages of the fish production: antioxidant ingredients, fish feed, and fish tissue by means of LC-MS/MS.

1.2.2. Specific

1.2.2.1. Optimization of the instrumental LC-MS/MS parameters for the analysis of selected polyphenols in commercial fish ingredients.

1.2.2.2. Analysis of selected polyphenols in commercial ingredients for fish feed containing natural antioxidants by using LC-MS/MS at the optimal instrumental conditions.

1.2.2.3. Analyze by LC-MS/MS the selected polyphenols in:

- Three different stages of a manufacturing process of fish feed, (before extrusion, before drying and final feed), for three diets containing different proportions of an antioxidant ingredient.
- Fish tissue (liver and muscle) from fish fed with diets containing different proportions of an antioxidant ingredient.

2. THEORY

2.1. Polyphenols and their classification

The polyphenols are an important and very diverse group of compounds that have in common the presence of one or more aromatic rings with at least one hydroxyl substituent. They are naturally present in plants and algae, and act in defense against reactive nitrogen and oxygen species, UV light, pathogens, parasites, and predators. Moreover, in many cases, they give special colors, flavors and smells to plants and herbs. They have been used from ancient times due to their important antioxidant and organoleptic properties and nowadays the research and development of application of these bioactive compounds is continuously increasing.[1]

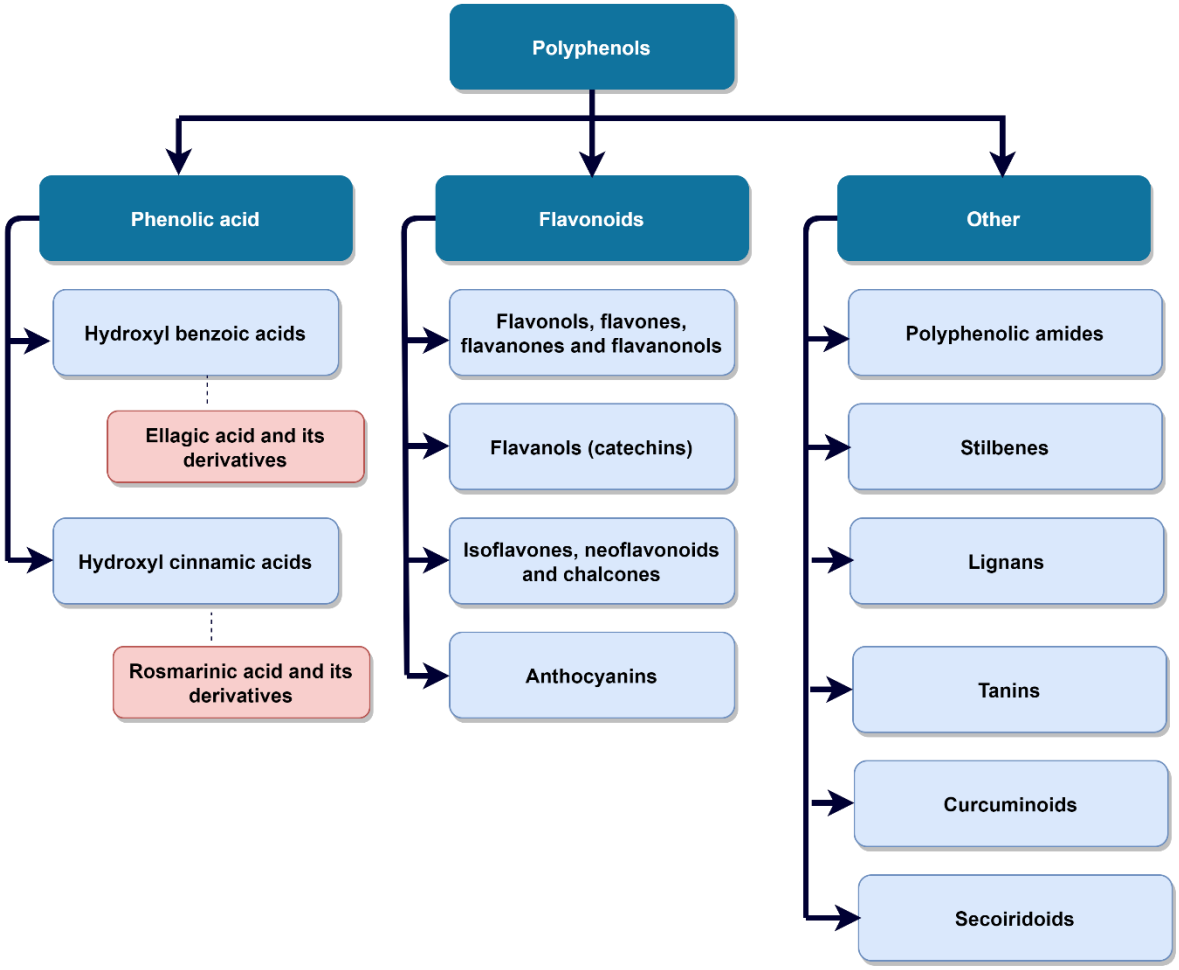


Figure 2.1. Polyphenols classification according to their chemical structure

Several studies have associated the consumption of polyphenols with the prevention of illnesses and/or the improvement of health by means of increasing plasma antioxidant capacity and reducing the occurrence or the effects of health problems caused by oxidative stress. Evidence has shown beneficial effects of polyphenols on the cardiovascular system, cancer prevention, improvement of diabetes effects, aging process, neurodegenerative diseases, and chronic inflammation, in animals, microorganisms, and humans. They act synergically with antioxidant vitamins and enzymes for contributing to the defense against excessive presence of reactive oxygen species (ROS). Polyphenols can neutralize free radicals

as donators of an electron or hydrogen atom, reducing both, the production of free radicals and the number of active species that can produce them.[2,3]

This category of phytochemicals has more than 10.000 compounds[1] which have been classified according to different criteria, as the origin, the biological action and, the chemical structure. [3] There are different classifications for PPHs and the described in the present work is summarized in Figure 2.1. In this document, the PPHs and other compounds with antioxidant properties are referred as NAOX.

2.1.1. Phenolic acids

This category, largely found in all plants and especially in acid fruits, comprises approximately one third of the polyphenol in human consumption. They divided into derivatives of benzoic and cinnamic acids.[2] Some of the most common phenolics acids are illustrated in Figure 2.2. The most common benzoic acids are gallic and ellagic acid (a dimer of gallic acid) and for cinnamic acids, the most frequent are caffeic and ferulic acid. [1,3]

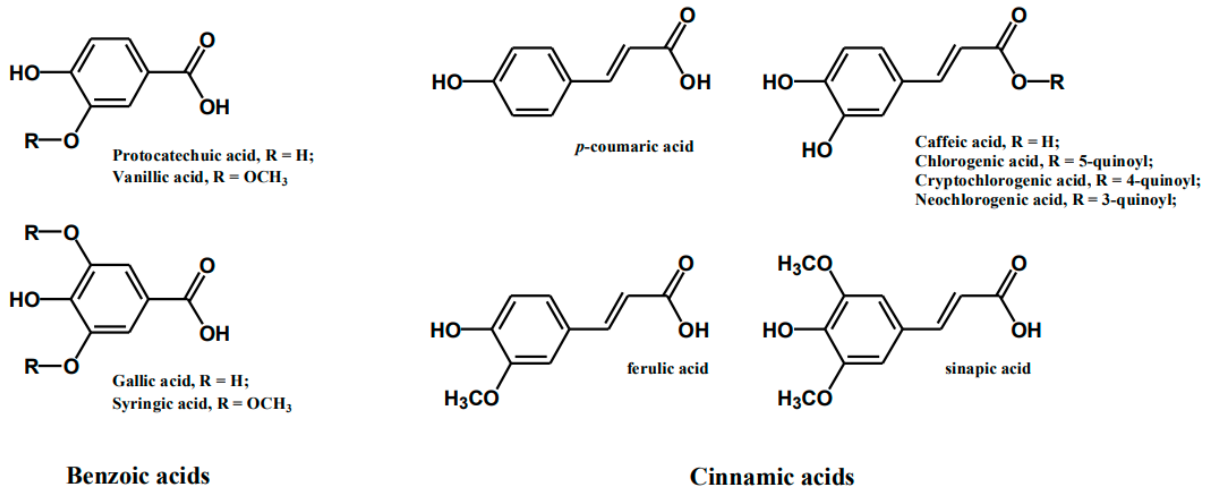


Figure 2.2. Different structures and examples for phenolic acids. From Tsao et al. [3]

2.1.2. Flavonoids

PPHs belonging to this group are the most abundant in human diet and the most studied. [2] Their basic structure, depicted in Figure 2.3., corresponds to diphenyl propanes (C6-C3-C6), the two C6 components (A and B) being phenolic rings linked by a heterocyclic ring, commonly a closed pyran.[4]

The wide variety of flavonoids and the differences in the presence of the hydroxyl groups and in the characteristics of the ring C, cause a further division within this group. Generally, rings B and C are bonded at position 2, except in isoflavones and neoflavonoids, in which the joint is by the positions 3 and 4 respectively (Ring C). [3]

2.1.2.1. Flavonols, flavones, flavanones and flavanonols:

PPHs in this category are the most frequent and almost ever-present in plants.[3] In flavones, a carbonyl group is found in the position 4 and carbons 2 and 3 are joint by a double bound. Flavonols have also and hydroxyl substituent in position 3.[4]

Flavanones differ from flavones in the double bound in positions 3-4. Flavanonols, follow this structure but with and hydroxyl group in position 3. The structures and some examples of this class are shown in Figure 2.4.

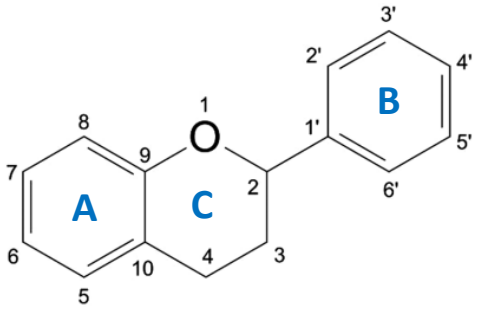


Figure 2.3. General chemical structure of flavonoids. Position of oxygen is defined as 1 and positions 2 to 10 are assigned to the other carbon atoms in rings C and A. Positions in ring B are numbered from 1' to 6'. From Singla et al. [4]

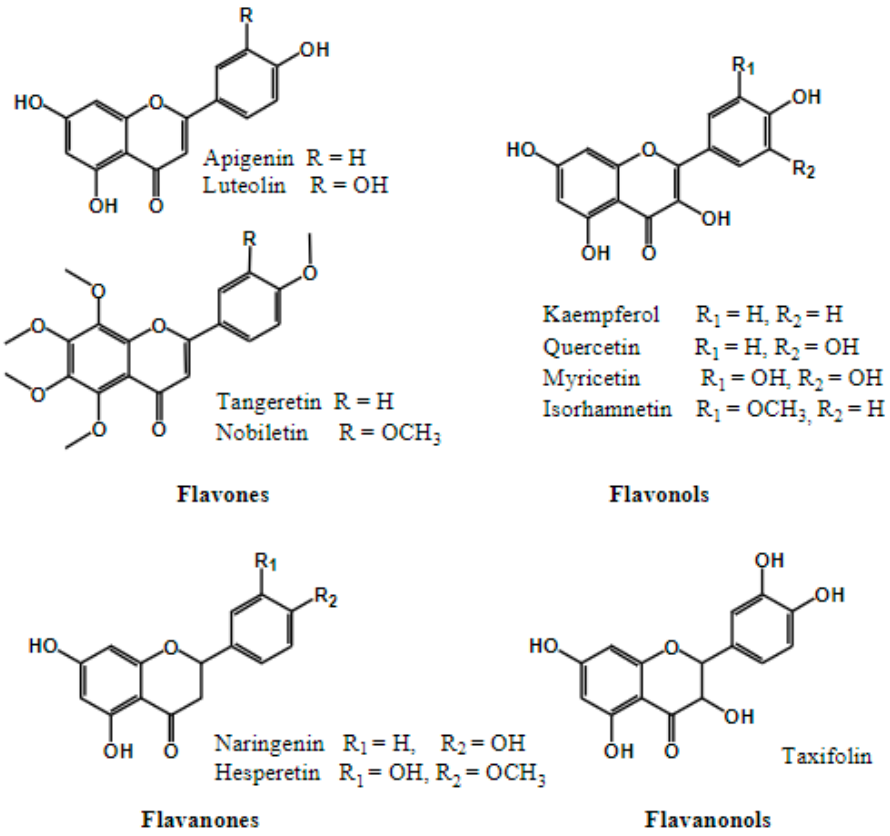


Figure 2.4. Structure and examples of the first division of flavonoids. From Tsao et al.[3]

2.1.2.2. Flavanols (also flavan-3-ols commonly known as catechins):

The general structure of flavanols and procyanidins are presented in Figure 2.5. Ring C in this group is saturated, there is no double bound in 2-3 neither carbonyl group in 4. This, and the presence of a hydroxyl group in position 3, causes flavanols to have two chiral centers and thus, four possible diastereoisomers. Trans isomer corresponds to catechin, and cis configuration to epicatechin, each one with two stereoisomers, originating (+)catechin, (-)epicatechin, usually found in plants, and (-) catechin, and (+)epicatechin.

Flavanols can be monomers (catechins and epicatechins), or polymers (considered as condensed tannins). Monomeric flavanols and their derivatives are the main flavonoids in tea and cacao. A subgroup of the polymeric flavanols, condensed tannins, are also known as proanthocyanidins due to their capacity of converting to anthocyanidins under oxidative conditions. [3,4] Figure 2.5.

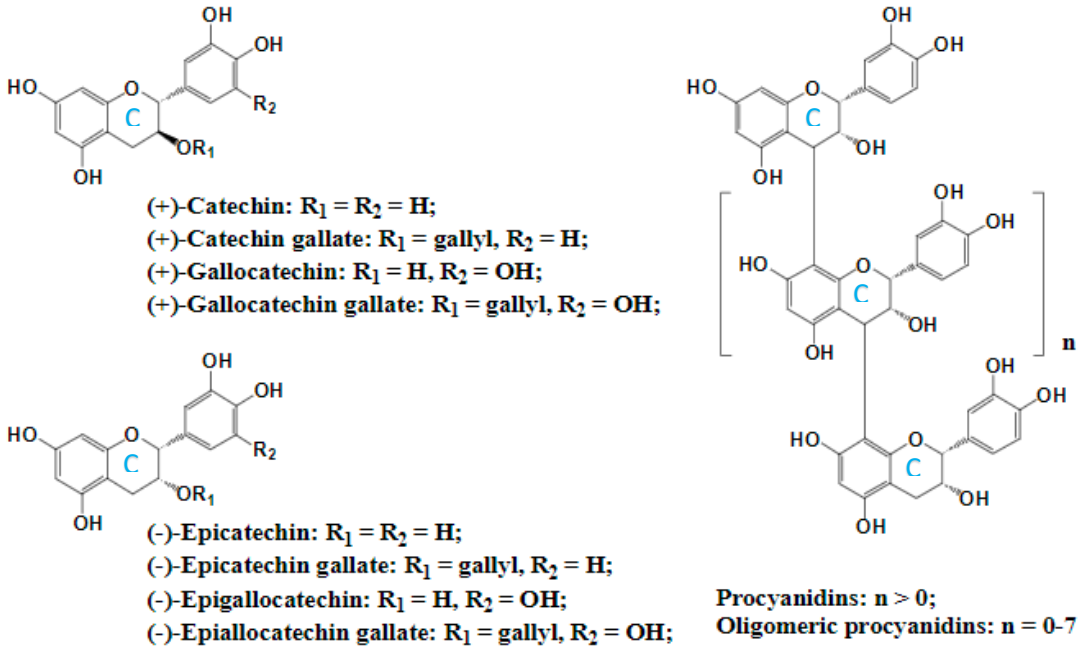


Figure 2.5. Some examples of flavanols and procyanidins. From Tsao et al.[3]

2.1.2.3. Isoflavones, neoflavonoids and chalcones

Isoflavones and neoflavoniods are particular because they have the union with ring C in the position 3 and 4 respectively instead of position 2 as the other flavonoids. Chalcones do not have the heterocyclic structure for ring C but are still included in the flavonoid category.[3] (Figure 2.6.)

Isoflavones are mainly found in soy products and are called phytoestrogens as they have similar effects to the estrogens. Chalcones can be found mainly in hops and beer and also in some plants and species. [1] Sources of neoflavonoids in the plant kingdom are scarce, those compounds are mainly known from the Dalbergia genus, which has been used in traditional Chinese medicine. Some of the neoflavonoids identified in Dalbergia are dalbergin, melanettin, stevenin, 4-methoxydalbergione and ceraoin,[5]

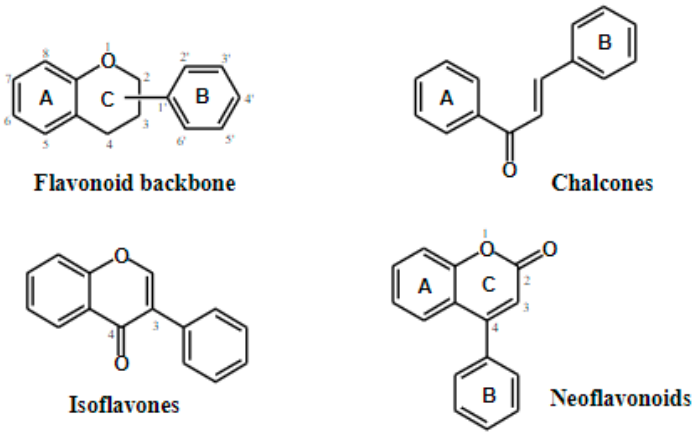


Figure 2.6. Structure of isoflavones, chalcones and neoflavonoids. From Tsao et al.[3]

2.1.2.4. Anthocyanins

The particular characteristic of the compounds belonging to this group is that they have two double bonds in ring C. The anthocyanidins are generally bounded to a sugar molecule, usually monosaccharides as glucose, galactose and arabinose, and are known as anthocyanins. These compounds also have different hydroxylation and methoxylation patterns on ring B, as well as different nature and number of sugar units attached. These kinds of flavonoids are the main responsible of the red, blue and purple pigments in flowers and fruits, are pH-dependent and stable in acidic solutions. (Figure 2.7.) [3,4]

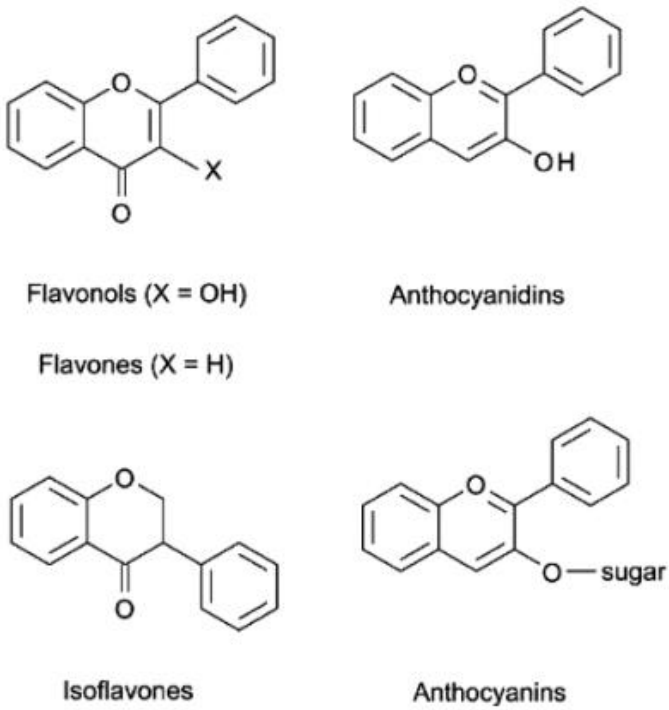


Figure 2.7. Structure of anthocianins and anthocianidins compared to flavonols and isoflavones. From Singla et al. [4]

2.1.3. Other PPHs

2.1.3.1. Polyphenolic amides

PPHs with nitrogen substituents are classified in this group. Particularly important are the capsaicinoids, responsible for the spiciness of chili peppers and the avenanthramides in oats, which have been pointed as compounds with strong antioxidant activity.[3] (Figure 2.8)

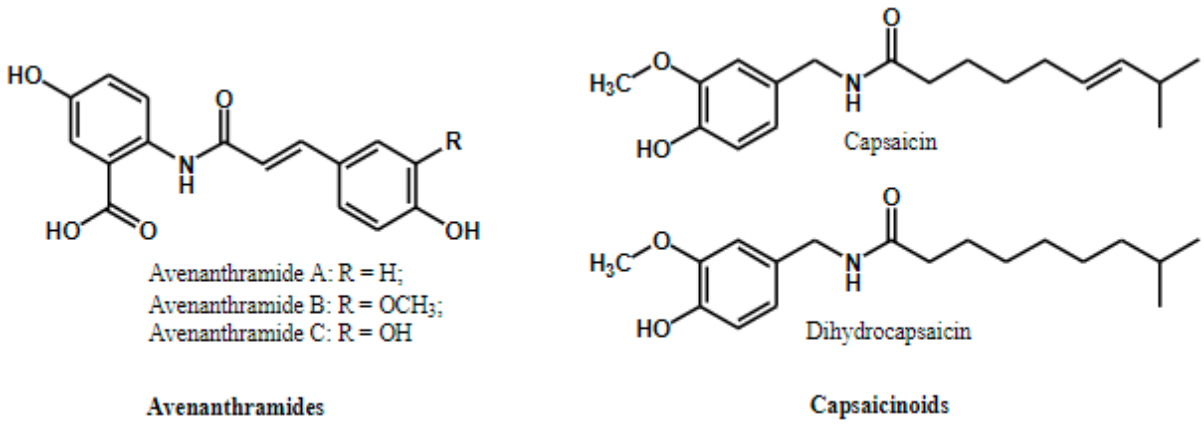


Figure 2.8. Structure of polyphenolic amides. From Tsao et al.[3]

2.1.3.2. Stilbenes

The basic structure for this group corresponds to a two-carbon methylene group which links two phenyl moieties. Stilbenes are found in cis and trans configuration, as well as in free and glycosylated forms.[4] Resveratrol, pterostilbene and piceatannol are the most important compounds in this class, being the first one the most studied and targeted for anticancer research, but it has shown potential against other health conditions as diabetes, inflammatory and neurological diseases. The high availability of these compounds in wines and grapes is well known, but they are also quickly metabolized and excreted, decreasing their bioavailability. Moreover, they are highly photosensitive and present low chemical stability which hinders its therapeutic use.[1] Extensive research has been done to overcome these drawbacks for their use as antioxidants. (Figure 2.9.)

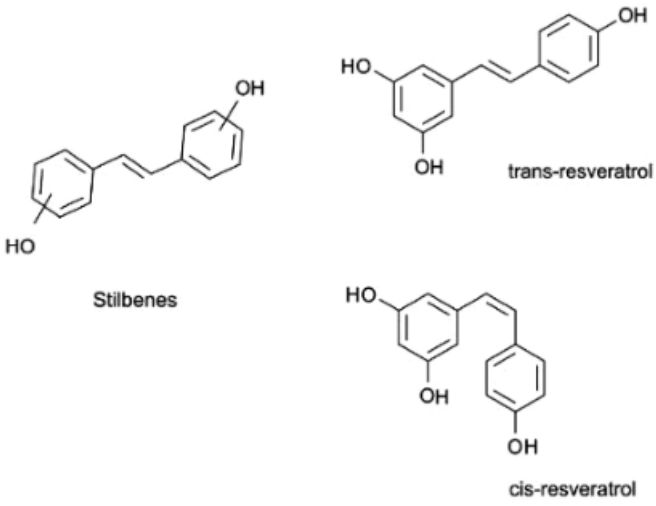


Figure 2.9. *Stilbenes and resveratrol isomers. From Singla et al.*[4]

2.1.3.3. Lignans

Lignans are dimeric compounds with a 2,3-dibenzylbutane structure which are found in small quantities in plants and play a role in the plant cell wall development. They are mainly found in flaxseed meal and flour, but also in soybeans, whole grains, fruits and vegetables.[1] They are classified in 8 groups due to their various substitution patterns which creates different structural forms[4] and are known for their antioxidant properties. (Figure 2.10)

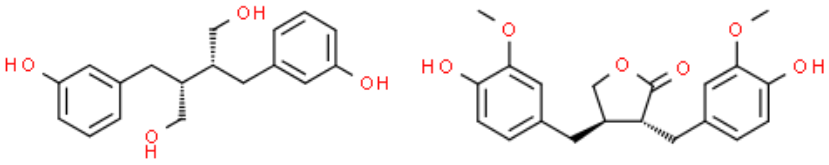


Figure 2.10 Examples from lignans: enterodiol (left) and matairesinol (right). Structures obtained from www.chemspider.com

2.1.3.4. Tannins

Compounds included in this category are oligo- and polymers of PPHs, having from medium to high molecular weight. They exhibit an important interaction with carbohydrates and proteins because they are highly hydroxylated molecules, property which is the cause of their name, as their plant extracts can transform animal hides into leather and cause the precipitation of proteins when in contact with saliva. They are further classified in hydrolyzable tannins, as its name implies, easily hydrolyzed by acid, alkali, hot water or the use of enzymes, and condensed tannins, from flavan-3-ol or flavan-3-4-diol, referred to as proanthocyanidins discussed earlier. They exhibit the antioxidant properties generally known for PPHs, but they have been also classified as anti-nutrients due to the complex formation with nutrients as protein, starch and enzymes and their negative effect in the use of micronutrients.[1]

2.1.3.5. Curcuminoids

Curcumin is a dimer derivative of the ferulic acid that could be classified within the phenolic acids but, due to its major importance and biological effects, is usually classified in a different category, the curcuminoids, which also includes its derivatives. This yellow pigment is the major bioactive component in turmeric and is also found in mustards. It has been widely used in the food, pharmaceutical and cosmetic industries.[1]

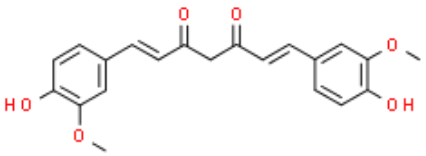


Figure 2.11. Curcumin. Structure obtained from www.chemspider.com

2.1.3.6. Phenolic compounds from Oleaceae: Secoiridoids

Although these compounds do not appear in the general categories for PPHs, they have phenolic moieties, have shown a major importance for their antioxidant properties and are referred usually as well

as PPHs, thus they are included in the present classification. The category of secoiridoids and their derivatives includes most of the bioactive phenolic compounds found solely in plants from the family *Oleaceae*, including *Olea europaea L.*, the olive tree from Europe. Oleuropein and ligstroside are the main secoiridoids and the precursors of the majority of phenolic derivatives in these plants.[6] That is the case of the important phenolic alcohols tyrosol and hydroxytyrosol, simple molecules produced by the hydrolysis of more complex compounds, both during the growth and ripening of the olive and during its processing. Highest content of oleuropein has been determined in the early stages of the olive with a trend to decrease during maturation, reaching zero for black fruits. However, even low content of oleuropein for green picked cultivars has been considered of great importance due to its attractive properties. Content of hydroxytyrosol, tyrosol, and tyrosol glycoside, has been reported to vary widely in the different stages of the fruit, but their increased was found to be correlated with the hydrolysis of their more complex precursors.[7] Other important compounds belonging to the secoiridoids and derivatives family, are the oleocanthal, oleacein and ligstroside aglycone, which have also shown interesting antioxidant properties.[6]

2.1.4. Phenolic compounds from rosemary species*:*

The species rosemary has traditionally been well known for its antioxidant properties. It has volatile and non- volatile bioactive compounds. The compounds in the non-volatile fraction are less hydrophobic than those found in the volatile fraction, as PPHs and phenolic terpenes, from which rosmarinic acid and carnosic acid are the most important. These acids and their related compounds are also found in herbs usually used as spices like oregano, thyme, sage, peppermint, and lemon balm.[1]

Rosmarinic acid, has four hydroxyl substituents, divided in the two peripheral aromatic rings in its structure, and it's a derivative of caffeic acid, chemically an ester of that polyphenol classified in the phenolic group [8] and thus, rosmarinic acid and its derivatives could be classified in the same category, as cinnamic acids and their derivatives.

Carnosic acid is a phenolic diterpene from which many other compounds are derived, for instance, carnosol is produced by oxidation of the carboxylic part of the carnosic acid, rosmanol and epirosmanol are produced by the later hydroxylation of carnosol, methyl carnosate is formed by the methylation of its carboxylic group[8] and 12-O-methyl carnosic acid corresponds to the methylation in one of its two hydroxyl groups. Carnosic acid is unstable and affected by temperature and light, its degradation in extracts can occur even at room temperature. However, a lower content of the carnosic acid practically does not affect the properties of the extract as its derivatives show similar antioxidant capacity.[8]

2.2. Contextualization of NAOX in aquaculture

The aquaculture sector has a major importance for humankind, among others, for meeting the increasing demand for global food. There are several reasons for considering the marine species farming an outstanding option for food supply, such as, the high nutritional quality of the feed provided, the efficiency of the fish production given by their low feed conversion rates (FCR, feed necessary for 1-kg weight gain for an animal), the greater values of harvest yield (percentage of edible or useful parts of the animal) in comparison with poultry, pigs and livestock[9], and the extensive areas available for farming, including offshore aquaculture.

Between 1987 and 2017, the production of farmed fish (live- weight) showed an increase of about 800%, from 10 million tons (Mt) of fish and shellfish in 1987, to 80 Mt by 2017, including additionally 32 Mt of

seaweeds for the later.[10] Additionally, the contribution of aquaculture to the total fish production in comparison with capture fisheries has also shown an important increase in the last 20 years. (Figure 2.12) The global production of aquatic animals was 178 Mt in 2020, from which aquaculture contributed with 88 Mt, almost half of the total production. Almost 90% of the total production was used for human consumption and the main use for the remaining quantity was the manufacturing of fish meal and fish oil. From 1961 to 2019, the consumption of aquatic foods worldwide showed a steady average increase of 3% per year, almost doubling the growth in the world population in the same period. Important contents of proteins and micronutrients are obtained from a diet including aquatic species, and besides providing a high-quality food, the aquaculture sector generates different products for food, cosmetics, nutraceuticals, fuels, pharmaceuticals, among others industrial products.[11] The aquaculture sector is also important socially, as it provides employment for a huge population worldwide in all the stages of fish farming.

In response to the important growth in aquaculture, there has been an increase in the use of feed additives and the husbandry practices on farms have been more stressful. When it comes to the fish feed, levels of marine proteins had decreased in favor of plant proteins, this accompanied by the reduction/replacement of the fish oil for vegetable oils in the fish diet, both changes for reducing the environmental impact of aquaculture on wild fish stocks. These conditions cause stress and oxidation in the fish, affecting its welfare and hence the productivity of the farms. For the successful contribution of aquaculture to the global food security, its development must be based on environmental and economic sustainability, thus efforts must be made to attempt the needed increasing in the production while protecting the fish of the detrimental effects of intensive production practices.

PPHs and natural antioxidants (NAOX) arise as a promising alternative for contributing to solve the described challenges in the fish farming industry. Due to their well-known antioxidant properties, they could be an excellent alternative not only for the fish feed but also for the fish well-being.

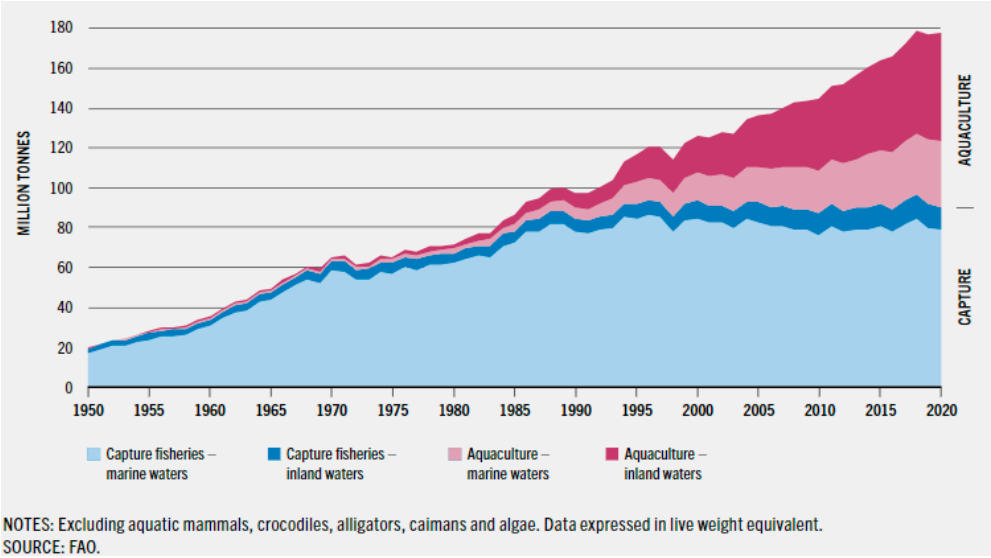


Figure 2.12. World capture fisheries and aquaculture production. Taken from [11]

2.2.1. NAOX and fish feed:

In animal farming, nutrition is a critical factor as it has a major influence on the growing rate and on the welfare of the animals. In aquaculture, the feed is a complex mixture of protein, carbohydrates, lipids,

vitamins, and minerals which could represent about 50% of the variable costs of the fish production.[12] The nutrients come from different sources as fish meal, fish oil, which are the most common marine ingredients, but also vegetable-based meals and oils, by-products from farmed animals and wild-capture fish, meal and gluten from various cereals and plants, innovative ingredients from insects and algae, mixes of micronutrients and antioxidants.[13]

The manufacturing process of the feed consists mainly in the following stages[14,15] (Figure 2.13):

Grinding: Reduction of the particle size of the raw materials for getting adequate and uniform particle size. This process usually contributes to the digestibility and acceptability of the feed by the fish. Regarding the manufacturing process, grinding eases the mixing process and the formation of pellets while the bulk density is increased. A sifter or sieve is then used for removing coarse impurities and assure the uniformity of the ingredients.

Mixing: Solid and liquid raw materials are put together. Generally, solids are mixed first and liquid materials (for instance oils and water) are added at the end and are mixed thoroughly.

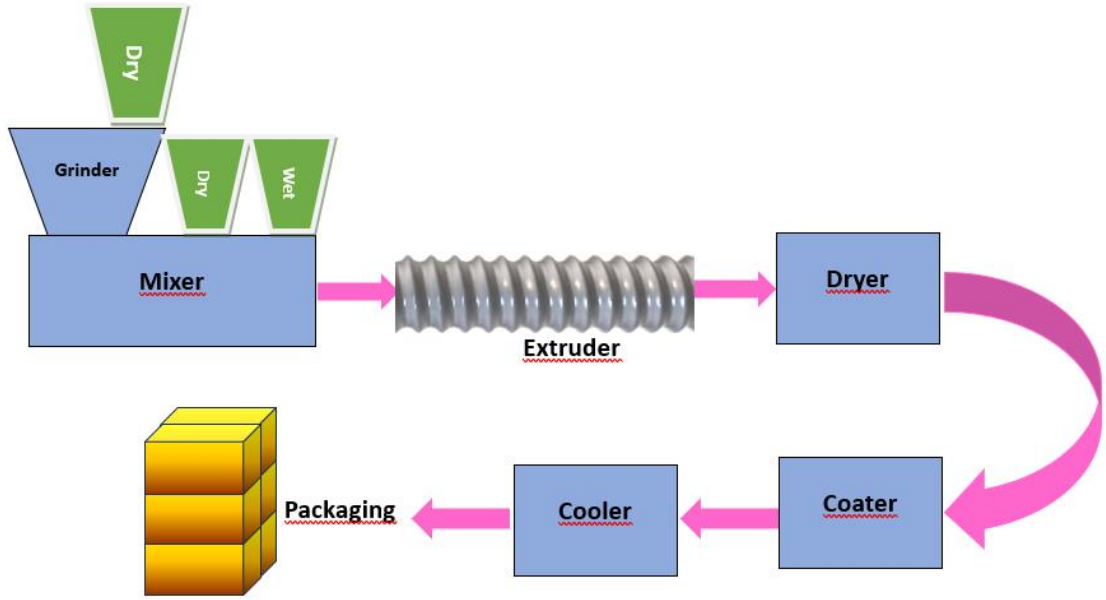


Figure 2.13 General manufacturing process for fish feed.

Pelleting: Conversion of the mixture into a compact feed, with the quality and physical characteristics needed. For this, two main processes are used:

- Extruder pelleting technology: The most common one. The extrusion process consists of cooking the mixture under high pressure, temperature, and humidity for a short period. Thus, a kind of dough is formed, which can be molded to the desired shape by being forced out of nozzles at the end of the extruder. As a final step, the material is cut to the appropriate size, producing uniform pellets full of water.
- Compressed pelleting technology: The mixture is exposed to steam for 5-20 seconds what produces 85 ºC and 16% moisture. For forming the pellets, the mix is forced in through holes in a metal die and then cut off by an adjustable knife to the desired size.

Drying: Right after the pelleting process, the feed is dried until a moisture content below 10%, what is critical for the shelf life of the product. This process must be gentle, usually at temperatures between 65-75 ºC.

Coating: This stage is done by some feed manufacturers and consists of extracting the air from the pellets of feed by using vacuum. Then, oil is added, and the vacuum is released later for the incorporation of the oil into the pores. Thus, after cooling and packaging, a feed with the desired physical and nutritious characteristics is obtained.

The oxidative stability of the feed is of paramount importance for the preservation of its nutritional value and for its effects on the fish welfare. Several studies have shown detrimental effects on fish when oxidized feed has been supplied, for instance, stimulation of the stress response, negative effects on the immune system, occurrence of skeletal deformities, among others, as well as lower levels of PUFA in the muscle (fillet), decreasing the nutritional value for the consumers.[16] Moreover, several studies have showed that the administration of fish feed including oxidized fish oil was associated with negative impact in the gastrointestinal system, apoptosis, inflammation, growth and survival of the fish.[17] When it comes to the physical characteristics of the feed, besides disagreeable changes in flavor and smell of the feed, the oxidation process entails combustion risks, as it generally occurs with the self-heating of the substrate, what can generate temperatures significantly higher than ambient, and the occurrence of combustion if this is not controlled appropriately. In conclusion, antioxidants in fish feed have two objectives: preservation of the nutritional quality and safety during storage, transportation, and shipping.[17]

The oils in the fish feed and the long chain omega-3 fatty acids (EPA and DHA), are prone to oxidation and degradation.[17] Additionally, the extrusion process has an effect in the lipid oxidation of the feed, not only during the production process but also in the storage of the final product. Pellets produced are porous materials, containing air chambers created by this structure, what promotes the contact between the material and the oxygen in the air, favoring a lipid peroxidation reaction, a direct reaction between fatty acid and molecular oxygen, which is catalyzed by free radicals. In an opposite way, fatty acids can be protected by the denaturation of enzymes involved in lipid oxidation and the addition of fats in the starch source.[16]

For protecting feed against oxidation, synthetic antioxidants have been used traditionally. Ethoxyquin, the most popular synthetic antioxidant for its high performance, was authorized in EU as feed ingredient for all animal species and categories until 2017, when the European Commission suspended its use because it was not possible to conclude on the safety of this ingredient due to the lack of data regarding the presence of p-phenetidine, an impurity that is a possible mutagen.[18] Despite of that, ethoxyquin is still usually considered as the benchmark against which other antioxidants are evaluated. Other synthetic antioxidants, as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have also been commonly used, but the transfer to these compounds to the fillets in alarming high amounts and their negative effects on health have been reported. [16]

The NAOX seem to be a promising alternative, which could contribute to eliminate or reduce the use of synthetic antioxidants and their replacement with greener and more environmentally friendly alternatives, nevertheless, research on NAOX is scarce. To the author's knowledge, the solely study available in literature regarding replacement of synthetic antioxidants by NAOX in fish feed was conducted by Hernandez et al.[16], in which the authors studied the protection achieved with the use of NAOX from rosemary and thyme extract in fish feed in comparison with BHT during the feed production: raw mix, recently extruded, and dry feed, as well as at different temperatures of storage for different times. Overall, their results suggest that rosemary extracts had similar performance than BHT, both in manufacturing and

storage stages, and superior to thyme extracts. They also found that for 24 weeks of storage, the feed with rosemary extract was the least oxidized.

Oniszczuk et al.[19] evaluated the effect of the addition of different amounts of roots from *Echinacea purpurea* on extruded fish feed. *E. purpurea* is a purple coneflower which has been found to present antioxidant activity from caffeic acid and its derivatives and which seems to have an influence in the control of diseases in some fish. The result of this study indicated that the antioxidant capacity of the feed was directly correlated with the content of roots of *E. purpurea* and the extrusion process did not deactivate the antioxidant compounds present in the roots[19], however, this study did not make any reference to synthetic antioxidants or their replacement.

There are also studies in matrices different from fish feed in which the performance of the NAOX is evaluated and compared with synthetic antioxidants. Zhang et al.[20] evaluated the antioxidant performance and the stabilization of sunflower oil with addition of different proportions of carnosic acid in comparison to synthetic antioxidants. They found carnosic acid in different concentrations showed strong protection against lipid oxidation in the oil, and the addition to the oil of the highest-concentrated carnosic acid, had notable better performance that BHT and BHA but it was less effective than TBHQ.[20] Similar results were found in a study comparing the antiradical power of extracts of rosemary, olive and grape seed, as well as a tocopherol mix, with synthetic antioxidants (BHA, BHT, TBHQ and ascorbyl palmitate) at the same concentrations[21], in which rosemary extracts exhibited a higher efficacy as food antioxidants than the evaluated synthetic compounds.

Overall, there is a lack of studies attempting to the reduction or replacement of synthetic antioxidants by NAOX even though their potential is well-known, and the evidence had suggested that some of them could even outperform the traditional synthetic antioxidants. As legislation and market trends are going towards the use of natural ingredients and greener ingredients, detailed and specific studies should be conducted to evaluate the feasibility of the industrial use of NAOX as antioxidant ingredients in fish feed from the economical and functional points of view.

2.2.2. NAOX and fish farming*:*

Organisms produce reactive oxygen species (ROS) as a response against external factors, contamination, diseases or other causes of stress. Small quantities of ROS are necessary for activating different physiological functions in the organisms but an excess causes oxidative damage in many molecules affecting protein and DNA and it causes lipid peroxidation of the membranes of the cells. Reduction in the antioxidant status not only affects the animal farming process affecting productivity, it also can affect the quantity and quality of the muscles or fillets that are the main parts of the animals that are consumed.[22]

There are many factors causing oxidative stress of fish in aquaculture. Seasonal changes, as the increase in day length and water temperature cause stress. Intensive practices intended to reach higher production rates require the farming of fish at high densities of population, which besides affecting the animal by excess of specimens per cubic meter, also affects quality of water and ease the spread of infectious diseases and viruses.[23] Moreover, as fish are cold-blooded animals, the regulation of their body temperature depends on external sources, thus, the variation in the water temperature has important effects and influences their growth and development, food consumption, defense response and subsistence. For instance, when some species are farmed in different climates to the one from which they are native and are exposed to changes in the water temperature, especially in winter seasons, oxidative

stress could be generated.[24] Even the climate change that is affecting environment in general, could impact fish farming. There are studies which report that warmer aquatic temperatures could be directly linked to higher mortality of aquatic species.[25]

Another important factor affecting oxidative conditions of the fish nowadays is the different nature of fish feed. Traditionally, the fish diet was mainly composed of fish meal and fish oil obtained from wild stocks and/or fast-growing non-commercial fish. As the demand for these products increased significantly, the risk of important impacts on wild stocks and marine ecosystems started to be a matter of concern. In consequence, in the last years, fish feeds have been constantly reformulated, looking for alternative sources of proteins and amino acids vital for the optimal development of the farming species. Mainly algae, vegetable crops and vegetable oils are now used for partial replacement of the fish raw materials, and research is focused on finding other alternatives as well, as microbial ingredients, insects, animal by-products and by-products from other production processes, for instance, trimmings and body fluids.[10,26,27] These changes contribute to the sustainability of the aquaculture sector but also originate another factor that the fish have to deal with and adapt to, thus, it is another factor causing stress.

In summary, the four factors reducing the antioxidant status in fish are: 1) Chemotoxicity-induced factors found in water, such as petroleum derivatives, pesticides, herbicides and heavy metals , 2) environmental factors, such as unusual water conditions of temperature, salinity, oxygen or ozone, 3) aquaculture factors, such as overcrowding, handling, transportation, nitrogen wastes, ammonia, 4) dietary factors, such as lack of vital nutrients in the diets, inadequate condition or composition of the feed and inclusion of challenging and new ingredients.[28]

For overcoming the issues generated by oxidative stress, synthetic antioxidants and antibiotics have been used. Besides the drawbacks already discussed for synthetic antioxidants, antibiotics also must be eliminated or at least reduced to their minimum amount, as they can induce devastating environmental problems by the development of strains resistant to these antimicrobial compounds, affecting also animal and human health.[25] Again, the PPHs are considered a good alternative to avoid the used of those synthetic ingredients as they have shown also antimicrobial activity besides their antioxidant properties. With this purpose, there are myriad of studies, in different kind of animals, attempting to evaluate impact of dietary supplementation of PPHs in their development and welfare. In aquaculture, despite being in an early development, the research about PPHs and fish has increased significantly in the last years. For instance, the available publications in ScienceDirect regarding these topics, for the period 2010 -2015, are 50 on average, reaching a higher value (63) only in 2015, while for the last years, it reaches almost 200 for 2020 and almost 300 for 2021 and 2022.[29] Different development and biological parameters have been evaluated in fish, in numerous species and with diverse sources of phenolic compounds. Research in this field seems to be led by Asian countries, although countries as Egypt, Italy and Spain also have reported studies. In Table 2.1., a small selection of scientific articles from 2023 and 2022 evaluating PPHs application in fish is shown. The type of studies varies widely, PPHs had been tested for counteracting detrimental oxidative stress effects artificially induced by compounds present in fish environment, by inadequate conditions of the fish feed, as well as by environmental conditions as water temperature, presence of heavy metal contaminants and by bacteria and other pathogenic microorganisms. Other type of studies does not use an induced stress condition but evaluate the effect of the PPHs on the fish development and welfare under different approaches, being the main parameters, and the ones found in common in all the studies, growth, and weight.

Table 2.1. Selection of articles from years 2023 and 2022 regarding application of NAOX in aquaculture

Country	Year /Ref.	Species	Source of PPHs	Study	PPHs analysis	Main results
Italy-Iran	2023 [30]	Zebrafish (Danio rerio)	Chestnut (Castanea sativa) shell, agri-food waste rich in tannins and mullein Verbascum macrurum), a plant	PPHs' effects on fish model of intestine inflammation induced by k-carrageenan.	TPC - Folin-Ciocalteu HPLC-UV	Before inflammation: PPHs ameliorate the effects of intestinal inflammation, during and post-inflammation: partial effect to counteract severity of inflammation
China	2023 [31]	Catfish (Ictalurus punctatus)	No information	Fish fed with fresh fish oil (FFO) and oxidized fish oil (OFO), with no addition and addition at 3 levels of Chlorogenic acid CGA	Not reported	OFO showed detrimental effects on fish, CGA ameliorated OFO's effects partially (regarding growth performance, intestinal inflammation, skin color, among others)
Turkey-Egypt	2023 [32]	Nile tilapia, Oreochromis niloticus juveniles	Reishi mushroom powder (Ganoderma lucidum) RMP	Feed with RMP in 3 different proportions and control (0, 5, 10, 20 g/kg). Effect on growth and at molecular level, genes correlated with development and lipid metabolism.	HPLC	Best results were obtained at supplementation with 10 g RMP/kg diet. **Optimum dosage calculated was 12.5 g RMP/kg diet .**
Spain	2023 [33]	Juvenile tilapia (*Oreochromis niloticus*)	Banana by-products, such as banana pseudo-stem and banana flower	Banana by-products in fish feed and extract of the banana flowers	Not reported	Addition of byproducts of banana was adequate until 5% for banana pseudo steam and 3%. Flower and its extract seemed to regulate levels of plasma cortisol and glucose and contribute to the antioxidant capacity in fish liver and muscle.
Egypt	2023 [34]	Striped catfish, (*Pangasianodon hypophthalmus)*	Milk thistle extract (herb) (MTE)	Feed with MET in 3 different proportions	TPC - Folin-Ciocalteu TFC - aluminum chloride method HPLC	Positive impact of MTE on growth, digestive enzymes, intestinal morphometry and improvement of the immunity and antioxidant capacity of the liver.
Egypt	2023 [24]	Nile tilapia, Oreochromis niloticus	Chia seed powder (CSP)	Feed with CSP in 4 different proportions and control (0, 1.5, 3, 4.5 y 6.0 g/kg) at temperature 16 - 19 ºC. Effect on tolerance to cold thermal stress	Not reported	Supplementation of CSP in the highest concentrations ameliorated stress effects by means of increasing survival rate, growth performance, antioxidants, immune response and lowering cortisol.

Country	Year /Ref.	Species	Source of PPHs	Study	PPHs analysis	Main results
Iran-Russia	2023 [35]	Common carp, Cyprinus carpio,	Pomegranate peel (PP)	Feed with PP in 4 different proportions and control (0, 5, 10, 15 y 20 g/kg). Effects on chronic crowding stress	TPC - Folin-Ciocalteu	**Supplementation of 5 g PP/kg decreased stress and improved antioxidant and immune parameters**. Even though the highest antioxidant and immune parameters were found at 10g PP/kg, an adverse effect on growth was found at this and the higher concentrations.
Egypt - Iran	2022 [36]	Rainbow trout (*Oncorhynchus mykiss*)	Persian shallot (Allium stipitatum) powder PSP (plant)	Feed with PSP in 3 different proportions	TPC - Folin-Ciocalteu	PSP enhanced specific growth rate SGR, weight and weight gain. **Optimum dose accordding SGR and FCR was 1.27 - 1.35%**. Overall, improved performance and inmunity.
Iran - Italy	2022 [37]	*Asian sea bass Lates Calcarifer* juveniles	Mixture of commercially available PPH (PMIX), from chestnut wood extract and olive extract (Silvafeed®TSP, and PhenoFeed®, respectively	Feed with PMIX in 4 different proportions	TPC - Folin-Cocalteau	PMIX could be beneficial for growth, immune response, and hepatic oxidative status. 2.5 g PMIX/Kg feed was optimum for improvement of growth and health
China	2022 [38]	Chinese sea bass (Lateolabrax maculatus)	Condensed tannins (CT) from grape seed	Effect of aflatoxin B1 (AFB1) with and without addition of CT in the fish diet on various biological parameters	Not specified	AFB1 in the diet caused negative effects on antioxidant status of the liver and and on the immune system. CT showed protection against these effects and reduced AFB1 content in liver and muscles.
Iran - Rusia - Italy- Thailand	2022 [39]	Rainbow trout Oncorhynchus mykiss fingerlings	Persian shallot (Allium hirtifolium) PSE	Feed with PSE in 4 different proportions	TPC - Folin-Ciocalteu	PSE was found beneficial to growth, antioxidant and immune system. **Optimum dose was between 1 and 2%.**
Iran-Thailand	2022 [40]	Common carp (Cyprinus carpio)	Cornelian cherry (Cornus mas L.) fruit extract (CCE)	Feed with CCE in 3 different proportions, also challenge with A. hydrophila, a bacterium considered pathogen for fish	Not specified	CCE had a positive effect in the growth performance, and overall health of fish, as well as in the resistance against infection by A. Hydrophila.

Country	Year /Ref.	Species	Source of PPHs	Study	PPHs analysis	Main results
China	2022 [41]	Chinese seabass (Lateolabtax maculatus)	Condensed tannins (CT) from grape seed	Feed with CT in 2 different proportions, also with a positive control of the highest proportion plus polyethyleneglycol (PEG). Evaluation of growth performance, antioxidant and immune response and muscle quality	Not specified	The highest concentration evaluated showed adverse effects on feed intake, growth, and antioxidant activity. Only the immune response of the fish was enhanced. **CAUTION for application of CT in aquaculture.**

Two important aspects can be highlighted from this review. First, the positive impact on fish when PPHs are included in the diets, that has been evidenced by many authors evaluating different parameters. However, it seems that for some PPHs, there is an optimum quantity or dosage for obtaining benefits from their use and negative effects have been reported when higher contents have been administrated.[25,41] Secondly, the approaches in aquaculture have been mainly from the biological point of view, and there is a lack of characterization and quantification of the sources of PPHs, as most of the studies do not determine phenolic profile of the source or determine them by the most common method for analysis of PPHs, the Folin-Ciocalteu, which is non-specific and references the total phenolic content to a single compound. As the biological products, such as plant extracts, have a high variability, and considering the importance of knowing and controlling the administration of phenolic compounds, it is imperative to integrate a more specific chemical analysis to the studies regarding use of PPHs in aquaculture.

2.2.3. Integration NAOX in fish feed and in fish welfare

From the analysis of the numerous studies of application of NAOX in aquaculture it is obvious that results vary a lot within the different species, sources and environmental conditions. As they are biological systems, their study is very complex and, for the safe and efficient application of PPHs in aquaculture, it is imperative to control the content of NAOX, and additionally, there is an urgent need of the study of the NAOX from a wider perspective.

If NAOX are used for feed protection, some quantities are going to remain in the feed and are going to be consumed by fish, what can have effects. On the other hand, if they are intended to contribute to the diet of the fish, the optimum quantity has to be available for consumption, that means, after manufacturing and storage of the fish feed. Those two perspectives are intimately related, and their study requires the adequate chemical approaches. This work aims to contribute to fill the actual gap in the application of NAOX in aquaculture.

2.3. Chemical analysis of PPHs and NAOX

The group of PPHs comprises an enormous quantity of compounds of different nature, which are present in plants and algae at low concentrations, in many cases are sensitive and unstable and are immerse in

complex matrices, making their analysis a challenging task. The most used methods for their analysis are based on chromatography coupled to UV/VIS or MS detector, and on nuclear magnetic resonance.

2.3.1. HPLC with UV/VIS detector:

Due to the aromatic nature of the PPHs, they exhibit absorption properties of electromagnetic radiation in the region of the ultraviolet and visible spectrum and thus, single UV/VIS f analysis has been widely used. However, there are many compounds that can absorb at the same wavelengths of the PPHs, making the approach unspecific and susceptible to overestimation of the actual levels of PPHs. Despite these drawbacks, the technique is still used for PPH quantification but not for total phenolic content (TPC) as the UV detection is based on molar absorptivity (ε) defined by the Beer-Lambert's law, parameter that exhibits very different values for each PPH, hindering the use of one single standard for the TPC quantification. [42]

Better results have been reported with the integration of HPLC-UV/VIS in conjunction with chemometric techniques, such as HPLC Fingerprint with Chemometric Analysis. As the polyphenolic profile of a type of source of NAOX varies significantly depending on time of harvesting, origin, geographical area, manufacturing, and storage conditions, among others, the chromatographic fingerprint analysis has been accepted as an adequate method for the identification and qualification of botanical products. The technique allows visualizing variations in the profiles of single species (also kwon as fingerprints) that can be differentiated from other closely related species. This technique also allows the identification of authentic products and adulterations.[43]

2.3.2. HPLC coupled to Mass spectrometry methods:

Mass spectrometry is the most sensitive method for structural analysis, which consists basically in ionizing the chemical substance of interest and the later separation of ions according to their mass to charge ratio (m/z). The representation of the abundance of the ionized species versus their m/z ratio is characteristic for each compound.[43]

There are two main classes of mass spectrometry depending on the mass analyzer:

- Low-resolution mass spectrometry (LR-MS): corresponding to quadrupoles and ion trap, commonly applied in tandem mass spectrometry (MS-MS). This technique is widely used for analysis of PPHs, determining their identity according to the theoretical mass and fragmentation patterns in comparison with analytical standards, what is known as targeted analysis. Nevertheless, the availability of the standards is limited and also the possibility of identifying and quantifying a wide variety of compounds. Although the most common ionization source is the electro spray ionization in negative (ESI-) mode, from which the deprotonated molecule $[M-H]^-$ is obtained, some applications have also used the positive mode (ESI+) to analyzed the protonated molecules $[M+H]^+$.[43] The analysis can be done in full scan (FS) for identifying ionization species without fragmentation, usually as a first step of the method development. Other types of operation for the mass spectrometers in the analysis of PPHs are: single ion monitoring (SIM), for selecting one single ionized species for analysis without fragmentation, product ion (PI), which selects one specific ionized species for fragmentation and later monitoring of the product ions, and selected reaction monitoring (SRM), which consists of the selection of one precursor ion, its fragmentation, and the posterior selection of one fragment. Multiple reaction monitoring (MRM) is also used, in which multiple precursor ions and product ions are monitored.

- High-resolution mass spectrometry (HR-MS): corresponding to time of flight (TOF) and orbitrap mass analyzers, allow the analysis of the exact mass of the species instead of the nominal mass obtained in low-resolution. For instance, the nominal mass of and atom of oxygen would be 16, and the exact mass would be 15.9949. Thus, HR-MS has become increasingly popular due to its capacity for providing more accurate information about the molecular mass and the chemical structure of the compounds. Besides targeted qualitative and quantitative analysis, this technology is also used in untargeted analysis, which means without using analytical standards, by studying of the fragmentation models. A strategy for the chemical profile of a sample is shown in Figure 2.14. Despite the benefits of HR-MS, the identification of unknown compounds is challenging, as it implies complex analysis of massive databases from MS and MS/MS analysis. The use of databases is applied for the chemical formular generated with a mass accuracy of < 2 ppm and a match factor of 70%. The processing of the spectrum and the comparison with database can be done manually, which is and extremely laborious task, or by using specialized software as Trace Finder ™, Mass Hunter, MetabolitePilot ™, among others, as well as specialized software for peak selection and chemometric techniques for data analysis.[43]

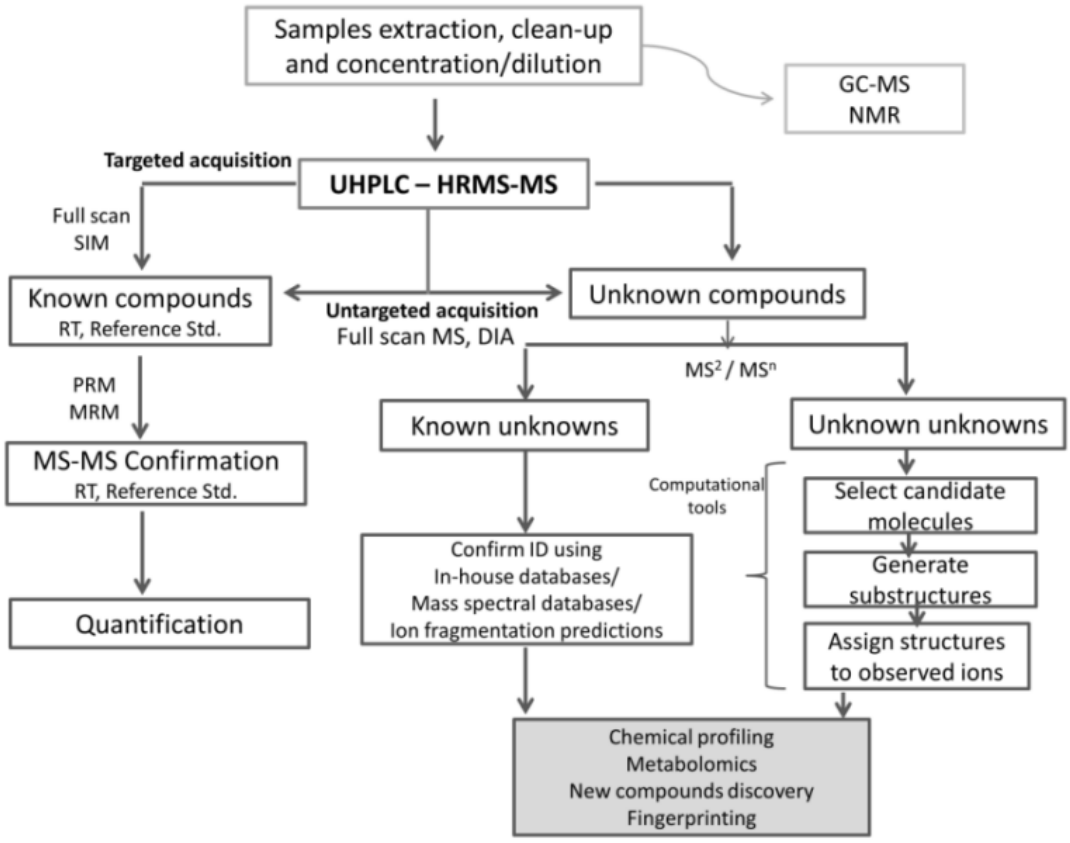


Figure 2.14. General procedure for analysis of the chemical profile using HR-MS. From Chiriac et al. [43]

2.3.3. Gas chromatography- mass spectrometry (GC-MS) using derivatization techniques:

The application of GC-MS is scarce due to the lack of volatility of the phenolic compounds, which demands complex pre-treatments and chemical derivatization to convert the hydroxyl substituents to ethers and to increase the volatility and stability, and allows the analysis by GC-MS.[43]

2.3.4. Metabolomic analysis using Nuclear Magnetic Resonance (NMR):

NMR is a technique that allows targeted and untargeted analysis, chemical profiling and molecular elucidation. It has several advantages such as simpler sample preparation, high reproducibility, and protocols of analysis that are easier to standardize than the ones for LC-HR-MS. However, NMR is less sensitive than LC-HR-MS. Despite the previous mentioned advantages and disadvantages, both techniques are considered complementary approaches.[43] The quantification analysis by NMR is not dependent on molar absorptivity, absorption of electromagnetic radiation at specific wavelengths, or calibration curves. The quantification is done based on correlation between phenolic compounds and a standard compound (internal or external), significantly different from the analyte. Nevertheless, there must be an adequate selection of the NMR peaks for quantification and some knowledge about the phenolic profile of the sample. [42]

2.4. Experimental design: factorial and fractional factorial

The experimental design is the adequate planning of experiments for studying a specific problem. By using experimental design for the variables and responses defined for a specific problem, experiments can be carried out in a systematic way, obtaining valuable information with a reasonable number of experiments. [44]

2.4.1. Factorial design**:**

In this type of design, the influence of all factors (experimental variables) and their interactions on the response(s) is studied. Usually, these experiments are done at two levels, low (-) and high (+), and the number of experiments is 2^k, where k is the number of factors to include. The – and + levels are defined based on the knowledge of the system within a reasonable interval for each variable. This definition defines the region of the study, known as the experimental domain.[44]

In Table 2.2. and Figure 2.15, the eight experiments for a design including three variables are shown, as well as the area investigated. The experiments are described as corners in a cube, which specify the level of each variable. The interaction effects have a sign, which is defined by the normal multiplication between the sign of the factors, as the example in Table 2.3. The coefficient b_0 corresponds to the mean value, that is the average of the responses of all the experiments. The complete table with the signs of the variables and the signs of the interactions is the calculation matrix, which is used to calculate based on the responses, the main effects b_n. The estimated effects define the polynomial model describing the relationship between the factors. The bigger the effect, the greater influence the variable has on the response, causing a decrease or an increase depending on the sign of the effect. In that way, it is possible to determine which are the most important variables and how their change will affect the result in the experimental domain studied.[44]

Table 2.2. Factorial design for three factors at two levels 2^3, called 2-level factorial design.

	Variables		
Experiment	**x1**	**x2**	**x3**
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

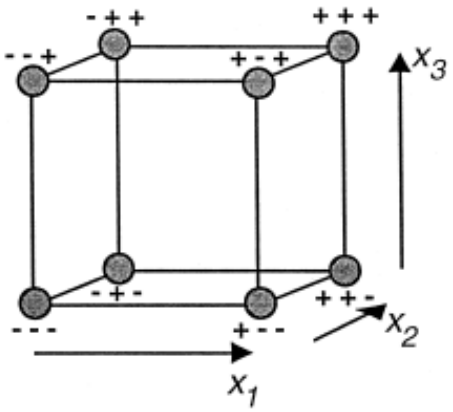


Figure 2.15. Experimental domain for three factors at two levels 2^3, called 2-level factorial design. From Lundstedt et al.[44]

Table 2.3. Sign for interaction effects for a factorial design 2^2. From Lundstedt et al.[44]

Sign of interaction effect $x_1 x_2$		
x_1	x_2	$x_1 x_2$
−	−	+
+	−	−
−	+	−
+	+	+

2.4.2. Fractional factorial designs

It is very common that the influence of some interactions in the response is negligible, especially those of third order or superior, and can be excluded of the polynomial model for the area investigated. This allows to significantly reduce the number of experiments required. Thus, the factorial design includes 2^{k-p}

experiments, where *k* is the number of factors and *p* is given by the fraction of the original experiments for a full factorial design, 1/2, 1/4, 1/8, etc., in the way:

$$\frac{1}{2^p} \rightarrow \frac{1}{2^1}, \quad \frac{1}{2^2}, \quad \frac{1}{2^3}, etc.$$

For instance, for an experiment with 3 variables, 4 experiments instead of 8 could be used to study all the variables. This corresponds to a design 2^{3-1}, also known as half-factorial design. In the fractional designs, columns from the design matrix for the factorial design are used to obtain the additional variables created by the reduction of the model. For the example of the design 2^{3-1}, the table 2.4 illustrates the levels for the 3 variables in the 4 experiments. Variable X_3 was established as the product of X_1 and X_2. [44]

Table 2.4. Design matrix for a 2^{3-1} experiment, fractional factorial design. From Lundstedt et al.[44]

Exp. no.	x_1	x_2	x_3
1	−1	−1	1
2	1	−1	−1
3	−1	1	−1
4	1	1	1

With the fractional factorial design, it is possible to investigate many variables with a low number of experiments, but the coefficients determined are confounded, what means that they do not correspond purely as the effect but include interaction effects as well. For instance, from the Table 2.2. it is possible to see that each variable changes as the product of the other two variables, and the intercept, b_0, is the product of all the three variables. If those relationships are established as equivalences and are replaced in the polynomial expression for the model, the result is that every coefficient determined contains an interaction term in this way:

$$b_0 = \beta_0 + \beta_{123}$$

$$b_1 = \beta_1 + \beta_{23}$$

$$b_2 = \beta_2 + \beta_{13}$$

$$b_3 = \beta_3 + \beta_{12}$$

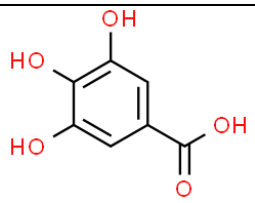
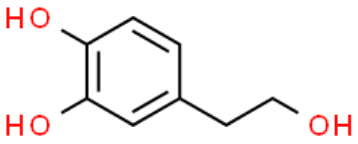
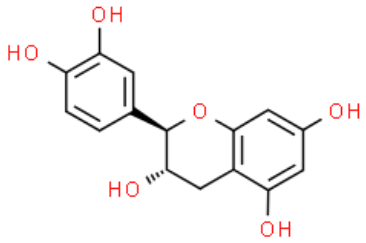
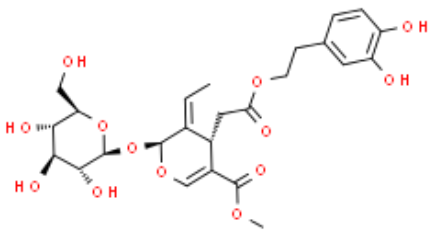
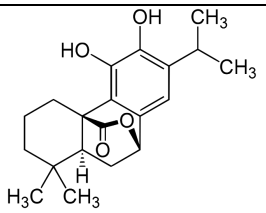
Where β corresponds to the pure coefficients. This means that every parameter b_n is an estimate of a true individual or main parameter and another interaction parameter, that is, each parameter βn is confounded with an interaction term. The resolution of a fractional factorial design is defined according to the confounding patter, Resolution III means that the main effects are confounded with two-variable interaction effects, Resolution IV means that the confounding is with three-variable interaction effects and so on. Resolution higher than V is rarely used.

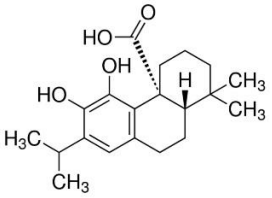
3. EXPERIMENTAL

3.1. Chemicals

Catechin hydrate ≥ 98%, 3-hydroxytyrosol ≥ 98%, oleuropein, carnosol, carnosic acid (all analytical standards), and gallic acid TraceCERT ® (certified reference material, CRM) were purchased from Merck Life Science AS, Oslo, Norway. Methanol and acetonitrile Optima ® for LCMS were purchased from Fisher Chemical. Ultrapure water for LCMS was produced from the system MilliQ Integral 5 Merck. The chemical structure, formula and mass for each compound is summarized in Table 3.1.

Table 3.1. Chemical structure, formula, average and monoisotopic masses for the evaluated PPHs

Compound	Chemical structure	Formula / Mass
Gallic acid (GA)		$C_7H_6O_5$ Average mass: 170.120 Da Monoisotopic mass: 170.021530 Da
Hydroxytyrosol (HOH)		$C_8H_{10}O_3$ Average mass: 154.163 Da Monoisotopic mass: 154.062988 Da
D-(+)-Catechin (CAT)		$C_{15}H_{14}O_6$ Average mass: 290.268 Da Monoisotopic mass: 290.079041 Da
Oleuropein (OLE)		$C_{25}H_{32}O_{13}$ Average mass: 540.514 Da Monoisotopic mass: 540.184265 Da
Carnosol (COH)		$C_{20}H_{26}O_4$ Average mass: 330.418 Da Monoisotopic mass: 330.183105 Da

Compound	Chemical structure	Formula / Mass
Carnosic acid (CA)		$C_{20}H_{28}O_4$ Average mass: 332.434 Da Monoisotopic mass: 332.198761 Da

3.2. Samples

3.2.1. Antioxidant ingredients

Samples of Ingredients A, B and C were supplied by Artic Feed Ingredients, (AFI), Brønnøysund, Norway.

3.2.2. Fish feed

Samples from different stages of the manufacturing process of the fish feed ingredient were supplied by Artic Feed Ingredients, (AFI), Brønnøysund, Norway. Three stages were analyzed: before extrusion, before drying and final feed. The experimental diets were produced at different inclusion levels of ingredient A (0.01, 0.05 and 0 % - Control) at BioMar AS pilot plant, (Tech Center, Brande, Denmark). The diets were kept at -20 °C during the experiment (3 months) and at -80 °C until the PPHs analysis.

3.2.3. Fish tissue – liver and muscle

Liver and muscle samples were supplied by AFI, Norway. The fish experiment, a tank trial, was run at Matre Aquaculture Station, IMR, from March 22 until June 29, 2021, in 9 tanks with simulated natural photoperiod and temperature at GIFAS' commercial sites (67°N). Nine tanks (1x1x0.4 m) were stocked with 35 post-smolts with an average weight of 473 ± 11g. The temperature increased from 5 to 9 °C and the photoperiod from 10:14 to 24:0 (L:D) with daily increments. The salinity was 26-29 g/L, and oxygen was kept at above 80% saturation. Fish in triplicate tanks were randomly assigned to each of the three diets (Control -without NAOX ingredient, feed with 0.01% and with 0.05% addition of Ingredient A)

At the start of the trial (March-M), 27 fish were sampled for analysis. At the end of the trial (June), 9 fish per tank, 27 fish per dietary treatment were sampled after the fish were euthanized (0.5 g L-1 Finquel vet.). Liver and muscle samples were taken and stored at -80ºC until the analysis.

The scheme of the general feed production along with the fish tank trial is presented d in Figure 3.1. The specific samples that were analyzed in the present research are indicated by stars ( )

3.3. Study of the Instrumental parameters for LC-MS/MS analysis

Initial experiments were conducted for individual solutions of the PPHs in methanol, at a concentration of 100 µg/mL for GA, HOH, OLE, COH and CA and 120 for CAT. Subsequently, A 2^k factorial design, where k represents the three studied factors (Collision energy (CE), cell accelerator voltage (CAV) and dwell time DT) and 2 represents the high and low level of those factors, was used to study the variations in the response of the main product ions identified for OLE. Finally, 2^{3-1} designs were done for GA, HOH, CAT, COH and CA, for evaluating the responses obtained when varying the same factors (CE, CAV and DT).

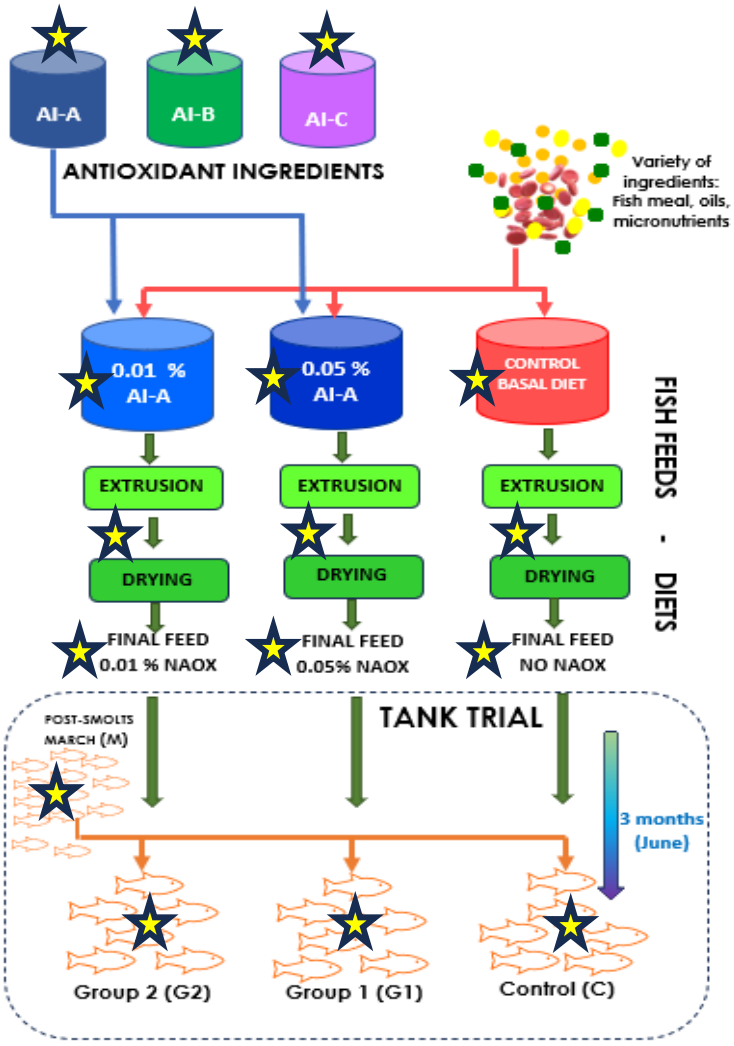


Figure 3.1. General scheme of the feed production

3.4. Stability approach

Various approaches to stability of the selected PPHs were done in the present study. First, individual solutions of CA and COH in methanol were analyzed fresh, after eight days maintained at non-controlled conditions and after fourteen days of preparation with storage at -80 °C. Secondly, three mixtures of GA, HOH, CAT, OLE, CA and COH in methanol in concentrations between 0,1 and 52 µg/mL were stored at six different conditions: transparent (white) and amber vials, and three different temperature ranges: -80 °C, -20 to -25 °C, and conventional refrigeration (around 4°C), and the responses of the PPHs were analyzed 1, 2, 6 and 9 days after the preparation of the solutions. Finally, a mixture of GA, HOH, CAT, OLE, CA and COH in methanol in concentrations between 32 and 84 µg/mL were stored in amber container at -80 °C and the signals for the six PPHs was analyzed at the day 0 (preparation day) and after 2,4 and 14 days.

3.5. Calibration curves and quantification

External standard curves were obtained for each PPH from dilutions of a stock solution of the mixture of GA, HOH, OLE, CA and COH in methanol. Ten calibration points were analyzed in the ranges 1.2 to 44 µg/mL (for GA, HOH, OLE, CA, COH) and 3.4 to 84 µg/mL (for CAT).Linearity evaluation was performed for each PPH. Subsequently, SAM calibration curves were obtained for six concentration points from 0 to 26 µg/mL for GA, HOH, OLE, CA and COH and from 0 to 50 µg/mL for CAT in equal volumes of the extraction of ingredient A at a dilution 1:15. The RME was determined in each case, according to the methodology proposed by Moreno et al. [45]. The slopes obtained by SAM were compared with the obtained by external standard calibration and ratios between 80 and 120% were considered as indication of no significant RME. As a significant RME for OLE, CA and COH was determined, all the calibration curves were obtained by SAM for the ingredients, the fish feed and the fish tissues analyzed.

3.6. Extraction of PPHs

3.6.1. Evaluation of the extraction in the antioxidant ingredient

Extraction of ingredient A was evaluated in four solvents, methanol, water, acetonitrile - 0,1% formic acid and methanol at concentrations of 1.3% w/v for 40 min and 25 min. Results were compared with 15-min extraction by ultrasound.

3.6.2. Antioxidant ingredients

The extraction of ingredients A, B and C was done by magnetic stirrer for 25 min, at a concentration of the ingredient around 1.3% m/v. The solvent used was methanol. After extraction, the solutions were centrifugated at 1620 × g for 5 min, filtered using 0.45 µm filters and put in amber vials for the analysis by SAM.

3.6.3. Fish feed

Extraction of different samples that were prepared with ingredient A: before extrusion, before drying and final feed containing different levels of antioxidant (0.01, 0.05 and 0 %) were done by magnetic stirring for 25 min at a concentration of the fees around 30% m/v. The solvent used was methanol. After extraction, the solutions were centrifugated at 1620 × g for 5 min, filtered using 0.45 µm filters and put in amber vials for the analysis by SAM.

3.6.4. Fish tissue – liver and muscle

Samples of fish liver and muscle with weights from about 537 to 2580 mg were extracted using a multi-tube vortex equipment in 2.5 mL methanol for 70 min. All the available tissue for each fish was analyzed. After extraction, the solutions were centrifugated at 1620 × g for 5 min, filtered using 0.45 µm filters and put in amber vials for the analysis by SAM.

3.7. Estimation of precision and accuracy in the quantification of the ingredients

The precision was estimated using the Goncalves et al.,[46] where the precision of the SAM method is calculated as the error in the y-axis intercept in the calibration curve when the graph is constructed in a reversed way. The error of the intercept (standard deviation) was calculated from the error of the intercept

(the concentration), obtained by using the feature "*Regression*" in MS Excel found in the command "Data Analysis".

The estimation of the accuracy was done by the percentage of recovery by cross-validation in the calibration curves constructed.

3.8. UHPLC -MS analysis

The equipment used was an Agilent 1290 LC system coupled to an Agilent 6495B Triple Quadrupole LC/MS with an ESI interface and iFunnel ionization. The software Agilent MassHunter Workstation Data Acquisition 10.0 and Agilent MassHunter Qualitative Analysis 10.0 were used for acquisition and data treatment, respectively.

A Zorbax column RRHD Eclipse Plus C18 95 Å, 2.1 × 50 mm and 1.8 µm (Agilent Technologies) was used. The mobile phase consisted of water (A) and acetonitrile: methanol ratio 1:1 (B), both acidified with 0.1% formic acid. The flow was 0.3 µL/min and the temperature of the column 30°C. The gradient program was as follows: 0 min 5% B; 3.5 min 30% B; 4.0 min 60% B; 5.5 min 95% B; 7.5 min 95% B; 9.5 min 5% B, 11 min 5% B. The injection volume was 1µL. The autosampler was maintained between 15 and 18 °C during the analysis by a thermostat. Carryover was prevented by the consecutive injections of blanks between samples.

The ESI source was used in negative mode at the conditions: temperature 120 °C, gas flow 19 L/min, nebulizer 20 psi, sheath gas temperature 300°C, sheath gas flow 10L/min, capillary voltage 4300 negative mode. The iFunnel parameters were high pressure RF 150V and low pressure RF 60V.

3.8.1. MS analysis

For the evaluation of the instrumental parameters for LC-MS/MS analysis, scan acquisition (MS2Scan) from 100 to 1000 m/z was used for analyzing individual solutions of each compound, with a scan time of 500 ms,and CAV of 5 V. This was followed by analysis in SIM mode, (Product Ion, PI), with general fragmentation parameters of CE 15 V and CAV 5 V, scan time of 500 ms, scanning from 50 to 400 m/z for all the ions, except OLE for which the scan was done until 700 m/z. Finally, the effect of CE, CAV and DWELL on the response was evaluated and the optimal parameters were established for each compound. Dwell time was set to 200ms. The software Sirius 11.5 © Pattern Recognition Systems was used in this stage.

For the analysis of the antioxidant ingredients A, B and C, and MRM acquisition method was used, for the selected PPHs GA, HOH, CAT, OLE, CA and COH and other related compounds usually found in olive and rosemary extracts. The parameters in the collision cell for each compound are shown in Table 3.2.

For the analysis of the fish feed in the different stages of the production process and for the analysis of fish tissue, the parameters of the MRM acquisition method are summarized in Table 3.3.

Table 3.2. MRM parameters corresponding to each PPH for the analysis of the Antioxidant Ingredients

Compound	Precursor Ion (m/z)	Product ion (m/z)	CE (V)	CAV (V)
Oleuropein	539	307	25	2
Oleuropein	539	275	25	2
Rosmarinic acid	359	179	15	5
Rosmarinic acid	359	161	15	5

Compound	Precursor Ion (m/z)	Product ion (m/z)	CE (V)	CAV (V)
Rosmanol	345	301	15	5
Rosmanol	345	283	15	5
12-O-methyl carnosic	345	301	12	5
12-O-methyl carnosic	345	286	12	5
Rosmadial	343	315	15	5
Rosmadial	343	299	15	5
Carnosic acid	331	287	12	5
Carnosol	329	285	8	5
Catechin	289	245	13	2
Gallic acid	169	125	13	5
Hydroxytyrosol	153	123	13	5
Tyrosol	137	119	13	5
Tyrosol	137	106	13	5

Table 3.3. MRM parameters corresponding to each PPH for the analysis of fish feed and fish tissue.

Compound	Precursor Ion (m/z)	Product ion (m/z)	CE (V)	CAV (V)
Carnosic acid	331	287	15	5
Carnosol	329	285	15	5
Gallic acid	169	125	13	5
Hydroxytyrosol	153	123	13	5
12-O-methyl carnosic	345	301	15	5

3.9. Identification and quantification of PPHs

The identification of the PPHs was done by comparison of the retention times and the fragments of the parent ions of the standard compounds and the obtained in the sample by LC-MS/MS analysis. Quantification was conducted by SAM.

For the identification of other related compounds without analytical standard, two transitions precursor ion-parent ion were monitored according to fragmentation patterns reported in literature and the semi-quantification was done by using the standards that closely resemble their chemical structure. ROS was quantified using the COH standard and RA and 12-O-MECA the CA standard.

4. RESULTS AND DISCUSSION

4.1. Instrumental parameters for LC-MS/MS analysis

In the individual SCAN experiments, ionization and detection of the six compounds, GA, HOH, CAT, OLE, COH and CA was achieved, as well as retention times that indicated a good resolution between them. For all the compounds, negatively charged molecular ions were detected. Then, in individual experiments using Product Ion acquisition, the molecular ion was isolated and fragmented (MS2) at general conditions for the collision cell and ionization source and the main ion products were identified. For almost all the cases, only a singular ion was considered as product ion of interest, with the exception being OLE for which three important fragments were found. The mass spectra obtained for GA and OLE are depicted in Figures 4.1. and 4.2, respectively. The results for GA, HOH, CAT, OLE, COH and CA are summarized in Table 4.1.

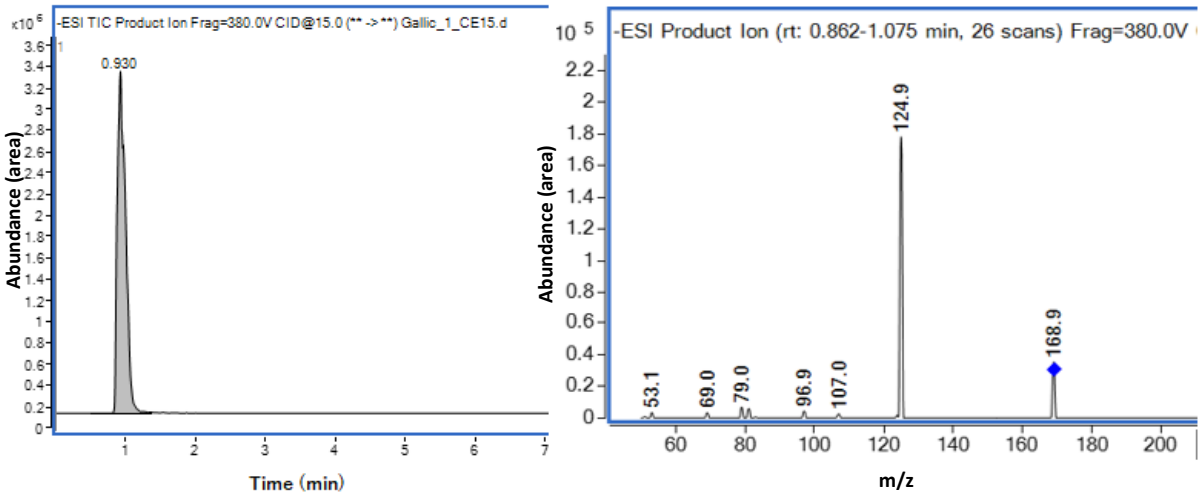


Figure 4.1. Total ion chromatogram for gallic acid (GA) and corresponding mass fragmentation pattern at CE 15 CAV5.

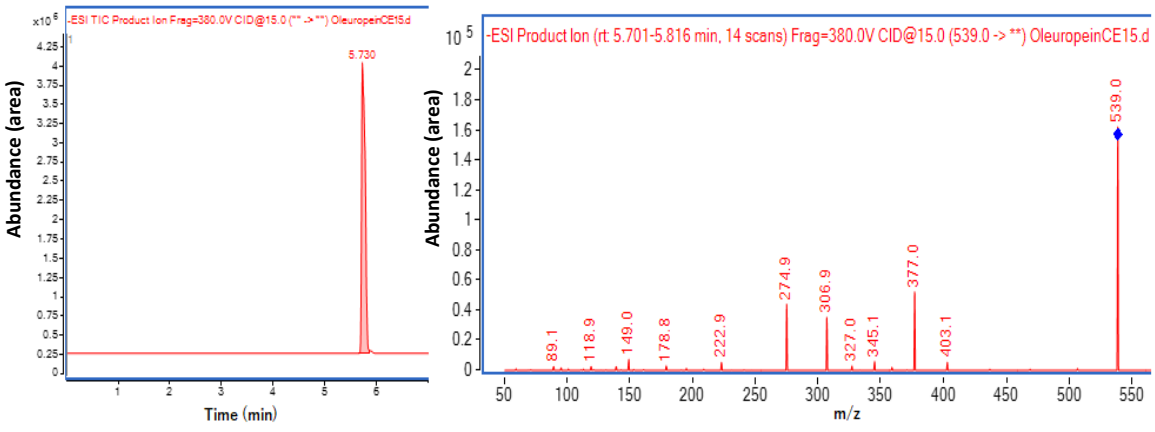


Figure 4.2. Left-TIC for OLE Counts vs. Acquisition Time (min) in Product Ion analysis at CE 15 CAV5. Right- Mass spectrum for the peak obtained (Counts vs. Mass-to-charge (m/z))

Table 4.1. Results for SCAN and SIM (product ion identification) experiments for the six PPHs.

Compound	Monoisotopic mass	Time (min)	SCAN IONS (m/z)	Main product ions (m/z)
Gallic	170.01	0.97	169	125
Hydroxytyrosol	154.16	1.97	153	123
Catechin	290.27	3.57	289	245
Oleuropein	540.51	5.73	539	377, 307, 275
Carnosol	330.42	7.10	329	285
Carnosic acid	332.43	7.57	331	287

The fragmentation in the case of GA, COH and CA corresponds to the loss of the carboxylic fraction, CO_2 (44). The results for HOH suggested that the molecular ion is produced by the liberation of the proton from the hydroxyl group in the aliphatic chain and the fragmentation is the loss of -CH_2O- from the same part of the molecule. In the case of CAT, the ionization seems to occur by one of the protons in the -OH bonded to the aromatic rings, while the fragmentation corresponded to the loss of a fragment – CH_2CHOH (m/z 44) from the central part of the molecule, the non-aromatic structure. The possible fragmentation patterns for these molecules are displayed in Figure 4.3.

OLE is a bigger molecule and its fragmentation pattern is more complex. Abbattista et al.[47], have proposed fragmentation pathways which can explain the three main fragments identified, 377, 307 and 245 m/z based on the loss of dehydrated glucose, the isoforms of the fragments of the molecule and the intramolecular proton transfer. The mechanism proposed (Figure 4.4.) also explains other ions found in the fragmentation pattern in a lesser proportion, 403.1 and 345.1 m/z.

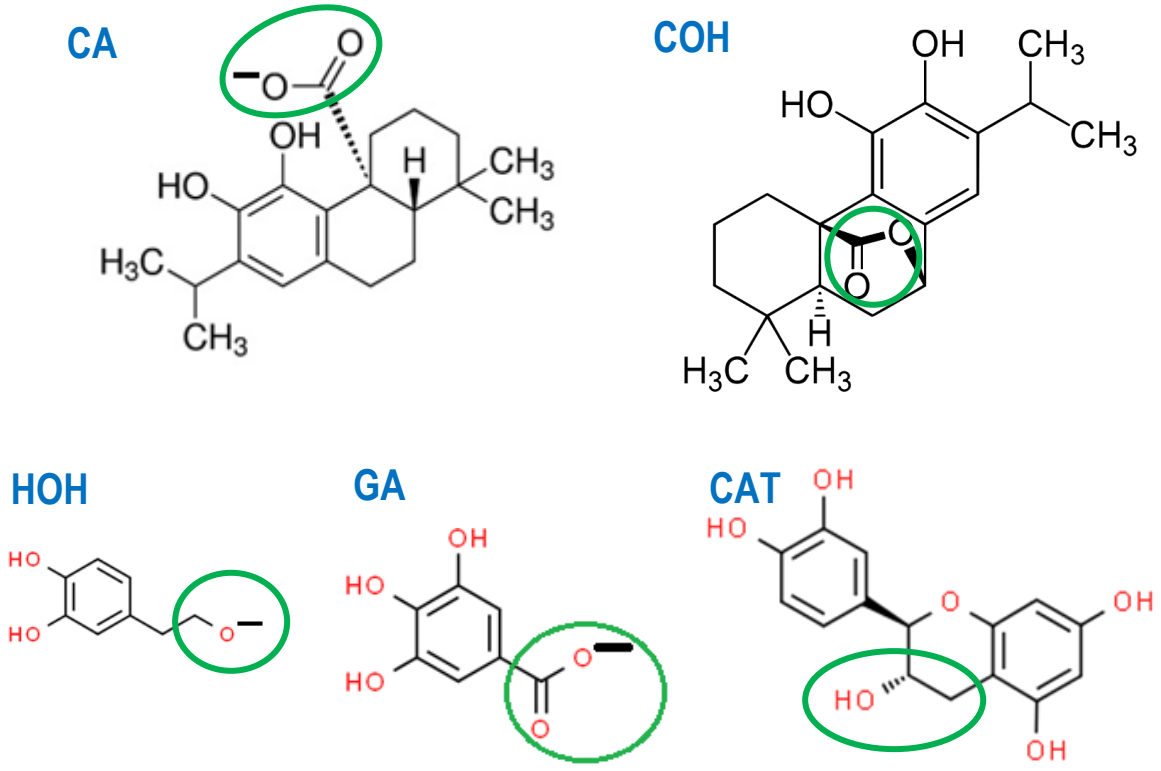


Figure 4.3. Possible fragmentation pattern for Ca, COH, HOH, GA and CAT.

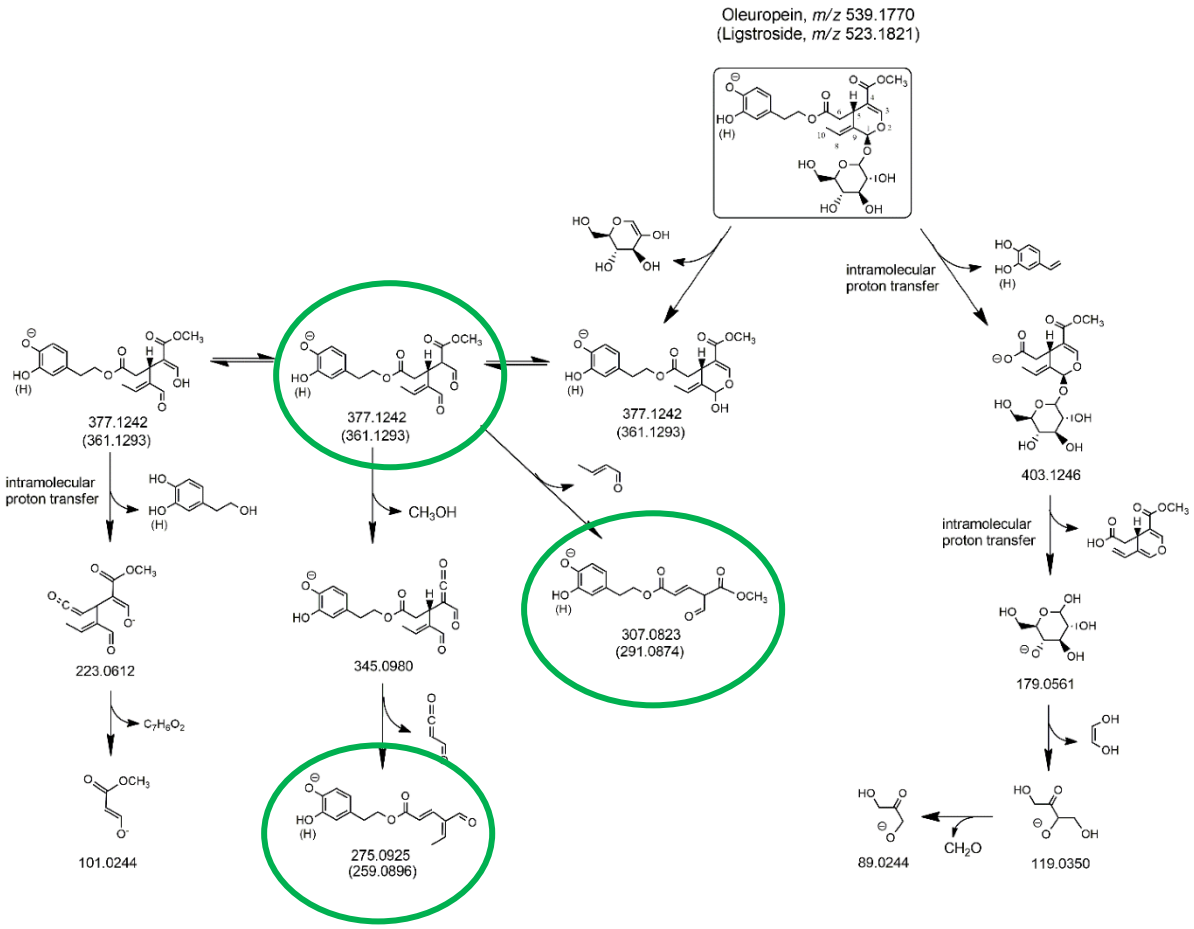


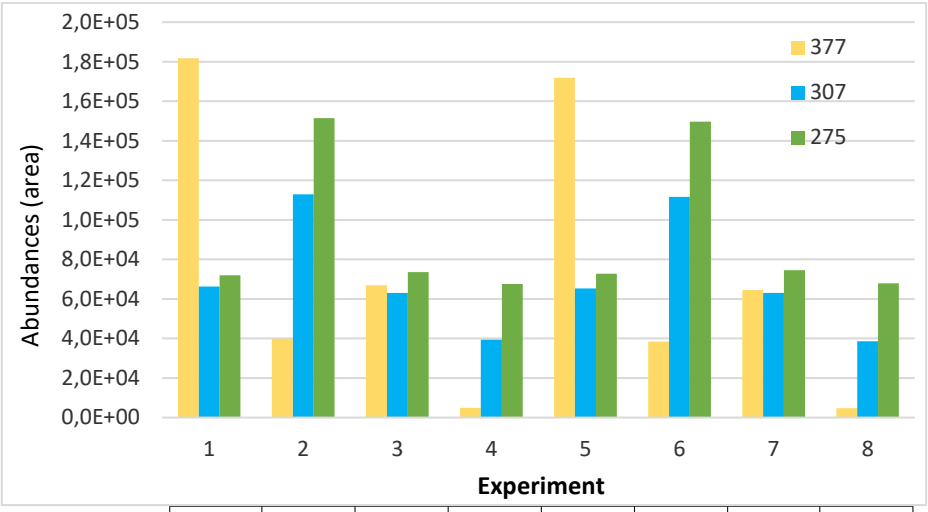
Figure 4.4. Possible fragmentation pattern for oleuropein and ligstroside. Adapted from[47]

After identifying the main product ions for GA, HOH, CAT, OLE, COH AND CA, experiments for optimizing their signal were conducted, and in the case of OLE, also for the selection of the best product ion.

Some of the most important parameters influencing the signal in the mass spectrometer are the fragmentor voltage and the collision energy, CE. The higher the CE, the higher the fragmentation of the molecule. In the equipment used, the fragmentor voltage is fixed to 380 V and thus the CE variation was studied between 10 and 25V. Collision cell accelerator voltage (CAV) is another MRM-dependent parameter that should be optimized in addition to CE. The CAV setting affects the speed at which a given product ion moves out of the collision cell. It can be set to values ranging from 1 to 8 V; the lower the value, the slower the speed.[48] The manufacturer of the LC-MS equipment suggests optimizing this parameter after CE optimization and states that the variation of this parameter may or not result in a notable improvement in the signal.

Previous results demonstrated the influence not only of the CE but also of the CAV on the abundances of the product ion for the evaluated compounds. However, the step-by-step optimization might not be the best approach as it could exist important interaction between those two variables influencing the results. Moreover, in the MRM acquisition, there is another parameter that has to be fixed for a specific method, the dwell time (DT), which is the amount of time allotted for analyzing each ion during a scan.[49]

CE, CAV AND DT at low and high levels (+ / -) of 15 and 25, 2 and 8 and 50 and 500 respectively, were evaluated in a 2-level full factorial design for OLE. The 8 experiments conducted, and the results illustrated in Figure 4.5, showed a similar behavior for ions 275 m/z and 307 m/z, different, nearly opposite for ion 377 m/z. For the former ions, best results were obtained in experiments 2 and 6, with higher CE and lower CAV, while for the latter, the maximum abundances were found for experiments 1 and 5, lower CE and lower CAV.



1	2	3	4	5	6	7	8	
-	+	-	+	-	+	-	+	CE
-	-	+	+	-	-	+	+	CAV
-	-	-	-	+	+	+	+	DT

Figure 4.5. Product ion (m/z) abundances for OLE for the 8 experiments evaluating CE, CAV and DT. MRM acquisition mode.

For the analysis of the results, the software Sirius was used to study the influence of the three factors evaluated in the signal. Analyzing the regression coefficients (Figure 4.6), as expected, CE positive effect was higher for the smallest ions whereas a higher negative effect of this parameter was found for ion 377 m/z. Regarding the other parameters, CAV has a negative impact in the abundance of the 3 ions while DT showed not significance on it. When it comes to the interaction terms, the only significant was the interaction between CE and CAV, positive for 377 m/z and negative for 307 m/z and 245 m/z. These interactions for the ions 307 m/z and 275 m/z showed an important effect, as they were nearly equal to the effect of CAV.

Even though the highest signals were obtained for ion 377 m/z in experiments 1 and 5, ion 275 m/z was chosen as the product ion for quantification because for the conditions maximizing its abundance, signal for ion307 m/z is also maximized, what is useful for the use of these par of ions as quantifier (275 m/z) and qualifier (307m/z) for OLE. Additionally, abundance of ion 275 m/z at experiments 2 and 6 approximated to the maximal signal obtained for 377 m/z. Thus, high CE and low CAV in the collision cell were defined for OLE.

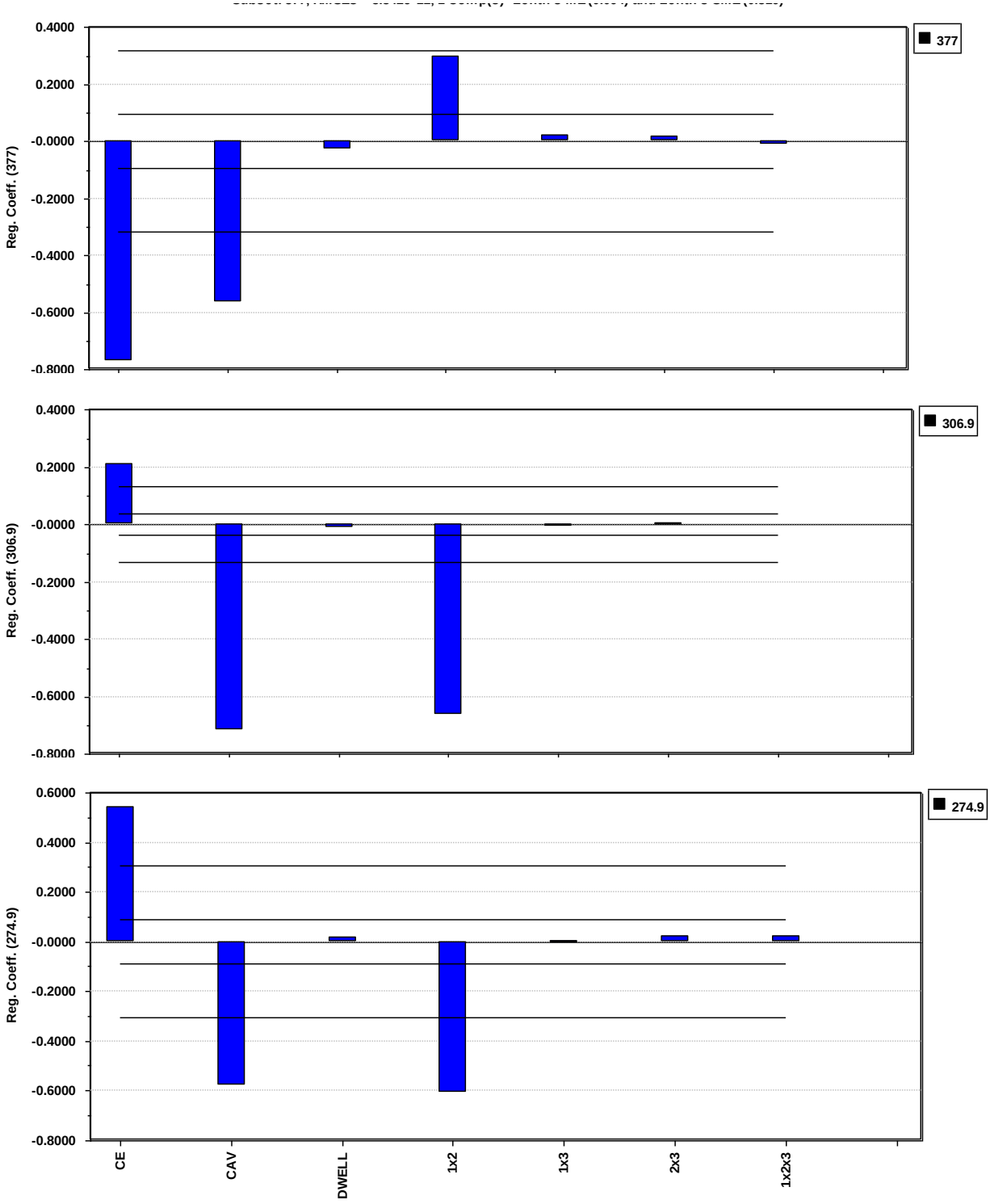


Figure 4.6. Regression coefficients obtained in Sirius for the variables and interaction terms for OLE product ions (m/z) 377,307 and 275. MRM acquisition mode.

Later experiments were conducted for analyzing the interaction between CE and CAV for the other compounds GA, HOH, CAT, COH and CA. Considering that in the experiments for OLE it was found that dwell time does not have an important influence in the signal, a reduced 2-level factorial design (resolution III) was done, that is a 2^{3-1} design. The results seemed to confirm this hypothesis and in consequence, the dwell time was fixed at an intermediate value of 200 ms. The conditions for obtaining the highest signal found in the 2^{3-1} experiments are summarized in Table 4.2.

Table 4.2. Optimum collision cell parameters for the six compounds for maximizing signal of the product ion.

Compound	Product ion (Quantifier)	Collision Cell	
	m/z	CE (V)	CAV (V)
Gallic	125	13	5
Hydroxytyrosol	123	13	5
Catechin	245	13	2
Oleuropein	275	25	2
Carnosol	285	17	2
Carnosic acid	287	17	2

4.2. Quantification of PPHs in the antioxidant ingredients

4.2.1. Evaluation of extraction in the antioxidant ingredient

According to the information provided by the manufacturer, the antioxidant ingredients contained extracts from olive and rosemary. Thus, from revision of literature and previous experiments, four solvents were chosen for evaluating the extraction of the phenolic content of the ingredients: methanol, water, acetonitrile - 0,1% formic acid and methanol. An MRM method was used for analyzing the main compounds expected, GA, HOH, CAT, OLE, COH, CA and other related compounds which are found in rosemary and olive extracts.

The four solvents were tested in extractions 1.3 % w/v of Ingredient A for 40 min using magnetic stirrer. GA, OLE and CAT were not detected in any of extraction with the selected solvents, HOH, COH, CA, 12-O-MECA, ROS and RA were detected. For HOH, the higher signals were achieved with the polar solvents, methanol and water, being methanol the solvent producing the higher responses. Acetone and acetonitrile produced signals about 60% and 95% lower than the ones produced with methanol and water. For COH, similar results were obtained with methanol, acetonitrile and acetone, but no signal was observed with water. In the case of CA, methanol and acetonitrile yielded similar results, and water did not produce any signal. With acetone, degradation of CA was suspected, as signals decreased quickly. Overall, for the three compounds, methanol yielded the best results and also exhibited acceptable extraction for the related compounds RA, ROS and 12-O-MECA, consequently, it was selected as the solvent for extraction. The chromatograms for the extraction of each compound with the tested solvents can be seen in Figures 4.7. to 4.11.

For defining the extraction method, ultrasound extraction (UE) was conducted by 15 min and results were compared with magnetic stirrer extraction. After the extraction by UE, the solutions were warm and the results showed a high variability, suggesting more instability and/or degradation of the solutions, thus magnetic stirrer was chosen. Finally, two extraction times were evaluated, 25 and 40 min and similar results were obtained, thus the extraction time was established as 25 min.

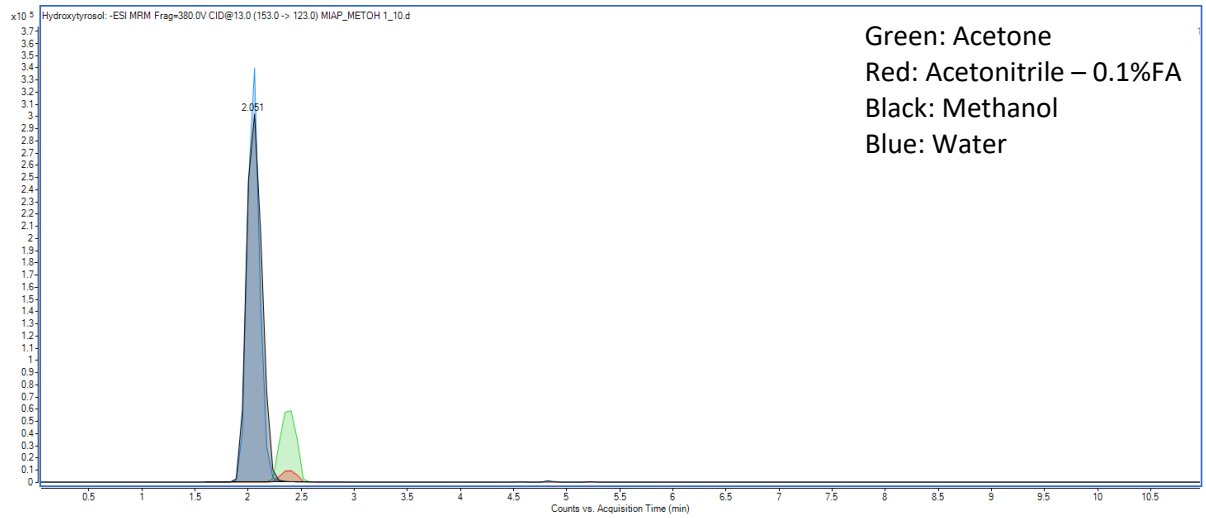


Figure 4.7. Chromatograms of the extraction of HOH from antioxidant ingredient A with different solvents: methanol, water, acetonitrile 0,1% formic acid and acetone.

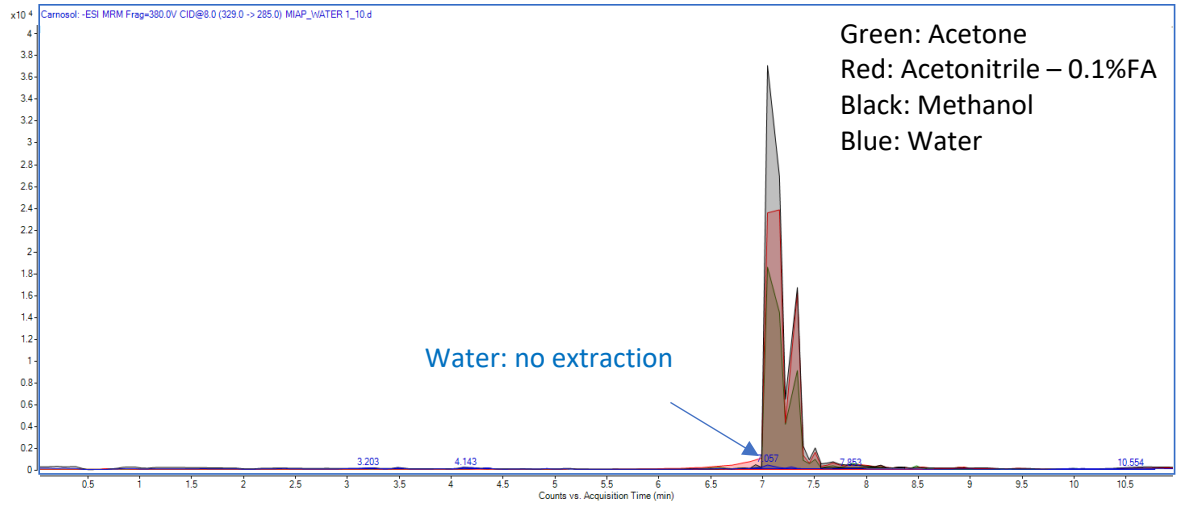


Figure 4.8. Chromatograms of the extraction of COH from antioxidant ingredient A with different solvents: methanol, water, acetonitrile 0,1% formic acid and acetone.

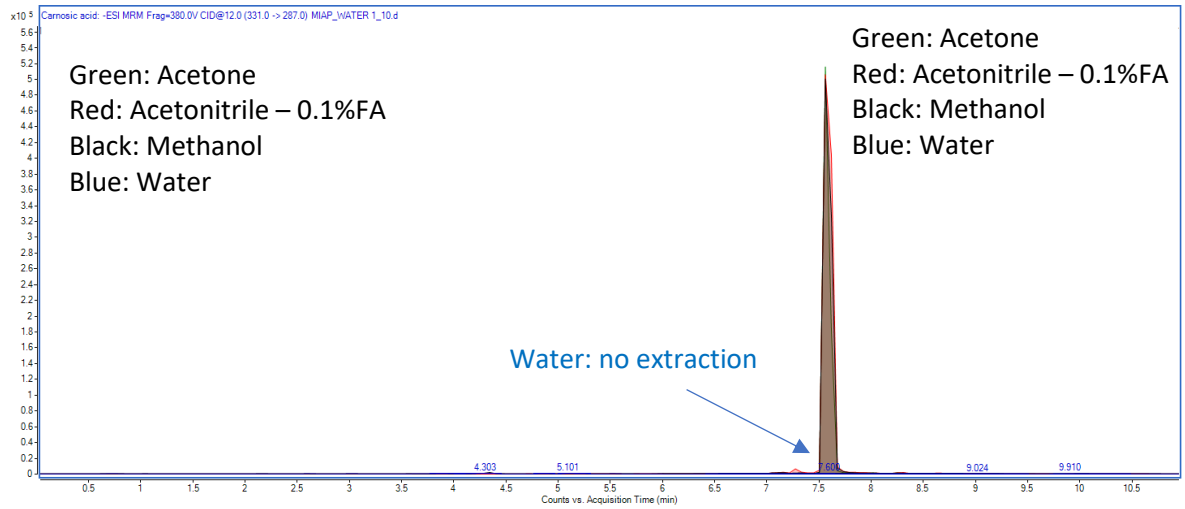


Figure 4.9. Chromatograms of the extraction of CA from antioxidant ingredient A with different solvents: methanol, water, acetonitrile 0,1% formic acid and acetone.

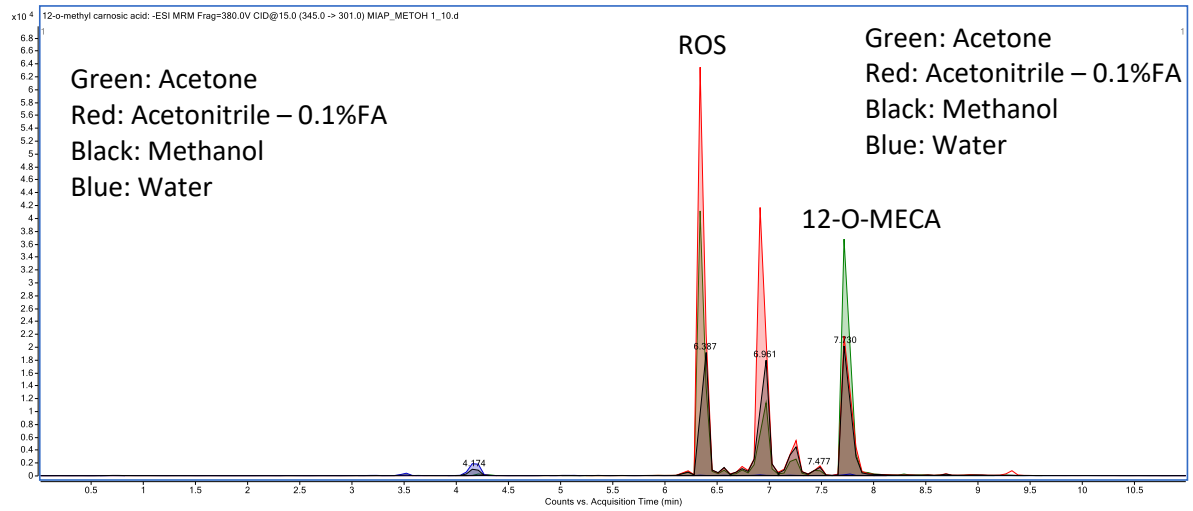


Figure 4.10. Chromatograms of the extraction of ROS and 12-O-MECA from antioxidant ingredient A with different solvents: methanol, water, acetonitrile 0,1% formic acid and acetone.

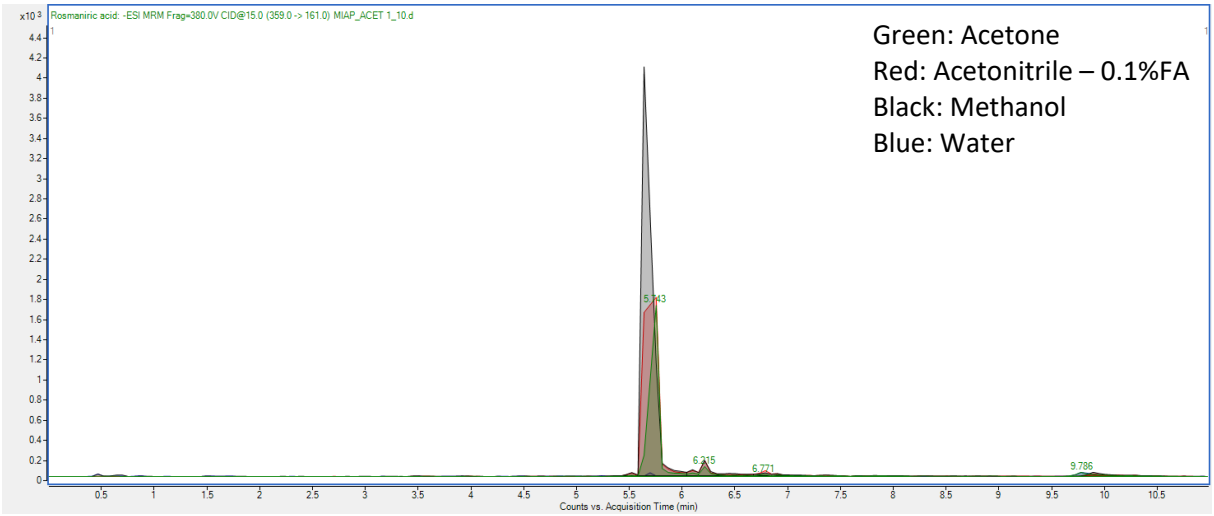


Figure 4.11. Chromatograms of the extraction of RA from antioxidant ingredient A with different solvents: methanol, water, acetonitrile 0,1% formic acid and acetone.

4.2.2. Stability approach

Instability and degradation of CA are processes already widely mentioned in the literature. These processes were also found in the first experiments for method optimization for CA and COH. Some approaches were done within this work for a better understanding of the behavior of the compounds.

4.2.2.1. CA and COH stability in methanol

Two individual solutions of 100 µg/mL of CA and COH were analyzed in transparent vials at three conditions using PI methods:

- T0: Time zero, preparation of the solution
- T8-NC: Eight days after the preparation of the solution, same vial analyzed in T0 maintained in the autosampler at non-controlled conditions.
- T14-C: Fourteen days after the preparation of the solution, stored in Eppendorf tube at -80 °C.

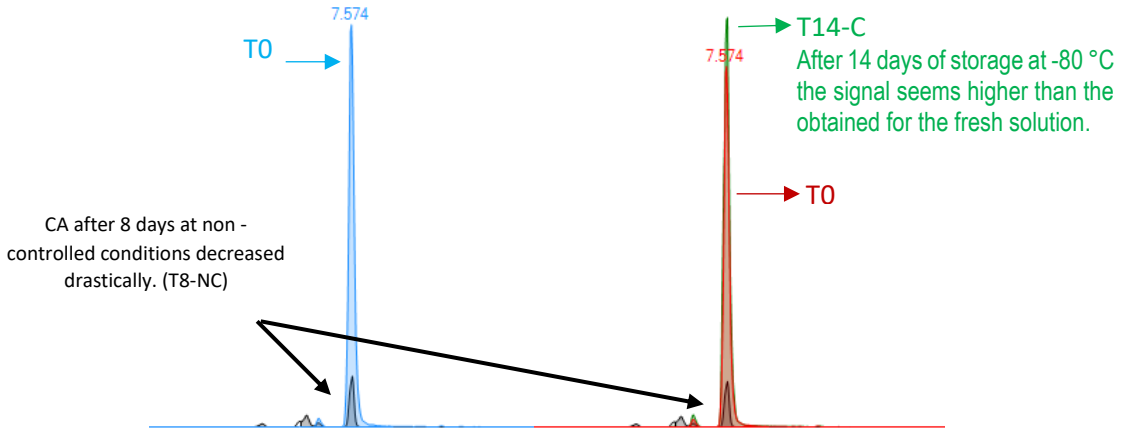


Figure 4.12. PI Chromatograms for CA at three different conditions: preparation of the solution (T0),8-day-old solution at non-controlled conditions (T8-NC) and 14-old-day solution stored at -80°C

A decrease in the concentration was detected for both compounds when comparing T0 and T8-NC, although it was significantly higher for CA, whose abundance after eight days at room temperature was less than 15% of the original abundance. Moreover, CA appeared in smaller peaks around a principal peak. The solutions kept at -80°C for 14 days seemed to be less degraded and in contrast, the abundance of the ions was higher than T0 for both compounds. Figures 4.12 and 4.13.

The later use of and MRM method evidenced that at non-controlled conditions CA was turning into COH and also COH seemed to be turning into ROS, whose precursor and product ions were 345 m/z and 283 m/z, (RT 6.4 min), Figure 4.14. Those results are in accordance with Pizani et al., who reported CA turning into COH which in turn was oxidized in its isomers epirosmanol and ROS.[8] The MRM analysis suggested that the solutions were not stable at room temperature and that the storage at -80 °C seemed to be adequate for conservation. Nevertheless, it was necessary a further evaluation as abundances for those compounds seemed to enhance with the time when stored at -80 °C.

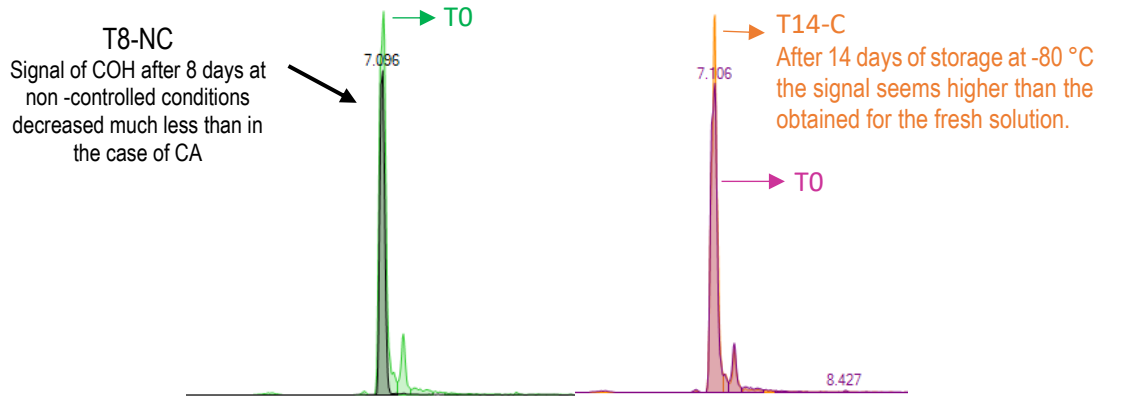


Figure 4.13. PI Chromatograms for COH at three different conditions: preparation of the solution (T0),8-day-old solution at non-controlled conditions (T8-NC) and 14-old-day solution stored at -80°C

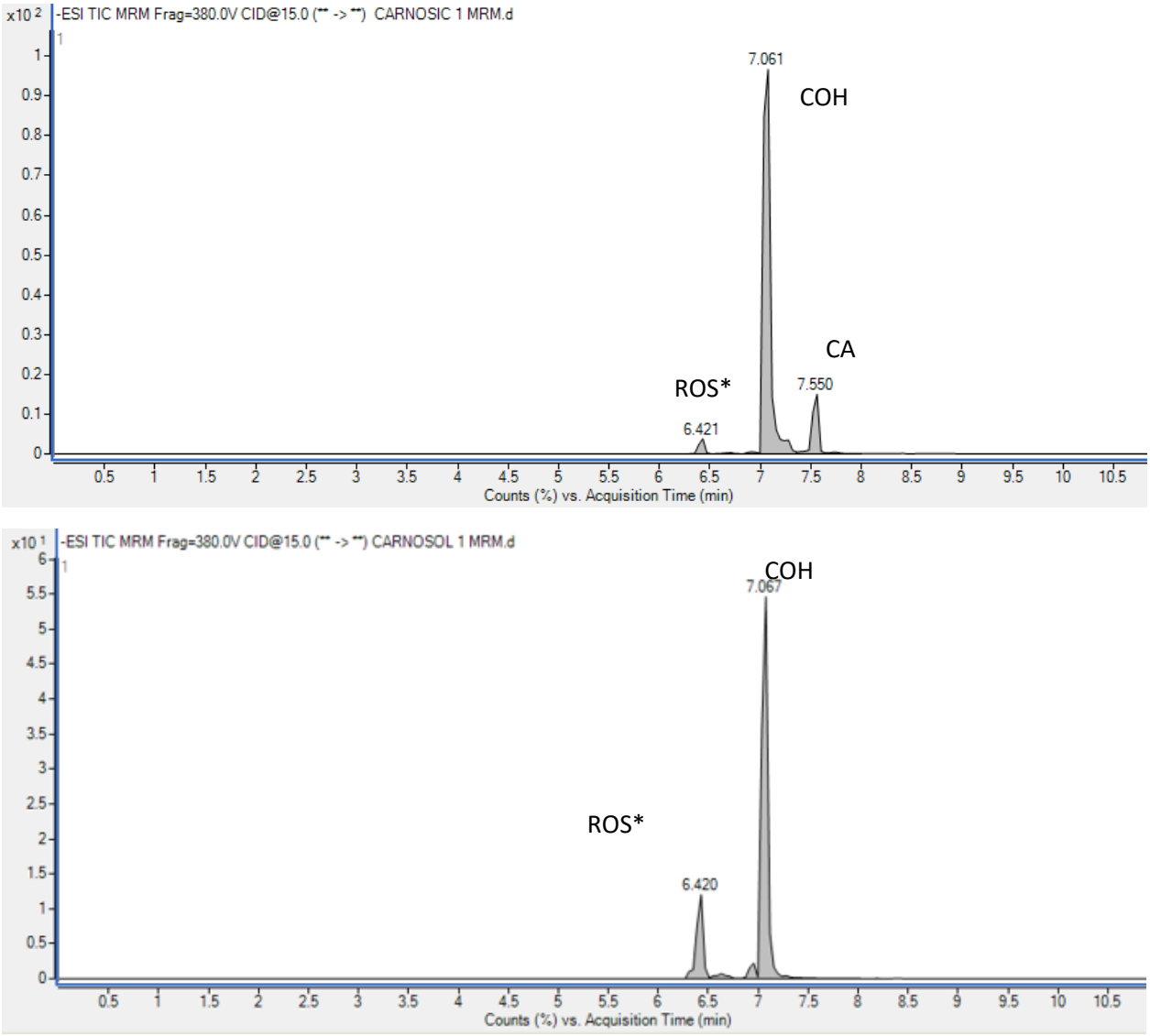


Figure 4.14. TIC Chromatograms, MRM acquisition for T8-NC

4.2.2.2. Stability for the mixture of PPHs GA, HOH, CAT, OLE, COH and CA - wide range of concentrations

Samples of solutions of *GA, HOH, CAT, OLE, COH and* CA in mixture, at three concentration levels were stored at six different conditions: transparent (white) and amber vials, and three different temperature ranges: -80°C, -20 to -25°C, and conventional refrigeration (around 8°C), and were analyzed 1,2,6 and 9 days after the preparation of the solutions. The graphs in Annex I depict the results for each PPH.

In general, for 4 PPHs, GA, HOH, CAT and OLE, in the six conditions evaluated, the signals showed a trend to decrease in all the conditions of storage, and the values obtained were within the 10 % of the initial values until day 6 of storage. The condition with the best performance was amber vial at -80°C. nevertheless, for COH and CA, the trend of the signals was different at each concentration point. At the lowest concentration evaluated, around 0.1 µg/mL, COH showed increases in the signal above 30% of the initial values, while CA exhibited higher increases, also with a higher variability between the results. At the next concentration level, around 3 µg/mL, the results for COH were within the 10 % of the initial values until day 2 of storage, while for CA there was a trend upwards since day 1, maintained for all the

conditions until day 9, except for refrigerated samples, which showed a decrease in the signal since day 2. For these samples, there seemed to be a relation between the decrease in the signal of CA and de increase in the signal for COH since day 2. Finally, for the highest concentrations evaluated (28 and 20 µg/mL for COH and CA respectively), results were more stable, the six conditions were between the 10% of variability or near until day 6 for COH and until day 2 for CA. For these two compounds, not condition was found clearly as the best, but the worst were the refrigerated samples.

These findings confirm the different behavior of CA and COH at different concentrations, indicate a lower variability for these compounds at high concentration and suggest optimal conditions of at -80 °C and amber container.

4.2.2.3. Stability solution at high concentration - mixture of PPHs GA, HOH, CAT, OLE, COH and CA

The results described in the previous section suggested a good stability of the standard mix of PPHs at high concentration. Thus, the stability of a solution stored at -80 °C in an amber recipient was monitored at the day 0 (preparation day) and after 2,4 and 14 days. Graphs with the results are included in Annex I.

The responses for GA, HOH and CAT decreased since day 2, showing similar values until Day 14, with an average variation of -20%. For OLE a higher variability was found, reaching more than 200% of the average initial value in Day 14. When it comes to COH and CA, overall, the values showed a variability within the 10% of the initial value until day 4. After it, the signals obtained indicated degradation.

Comparing these results with the previous analysis of stability, the stability for GA, HOH, CAT and OLE in general declined, as in the first evaluation, the analyses until day 9 where within the -10% of variation for all these compounds, while in the second evaluation, in Day 2 after preparation, the signals were around the -20% interval of variation. For COH and CA the results obtained were similar to those found previously for high concentration, with values within the 10% of variation until Day 4.

The use of amber vial and the storage at -80 ºC does not prevent the changes in the behavior of the compounds and does not guarantee its use for posterior days after preparation. According to these findings, the standard solution used has to be freshly prepared.

4.2.3. *Calibration curves and quantification for GA, HOH, CAT, OLE, CA and COH*

The complexity of the ionization phenomenon in LC-MS and the probability of having matrix effects caused by the presence of diverse compounds in complex matrices, especially when using an ESI source, is well known. Thus, for a reliable quantification it is necessary to evaluate the matrix effect in the analysis when it is not possible to obtain an analyte-free matrix. There are different approaches for evaluating this parameter, but the Relative Matrix Effect evaluation (RME) described by Moreno et al.,[45] was considered the most appropriate. This method consists of the comparation between the slopes obtained by the SAM with the ones from the external standard calibration, considering no significant RME for ratios of the slopes between 80 and 120%. Ratios below 80% indicate a signal suppression and above 120%, a signal enhancement or increase.

External standard curves were obtained for each PPH in the range from 1.2 to 44 µg/mL for GA, HOH, OLE, CA and COH and from 3.4 to 84 µg/mL for CAT, and linearity in each case was evaluated using the F-test according to the Analytical Method Committee approach described by Araujo, P.[50] Subsequently,

SAM calibration curves were obtained for six concentration points from 0 to 26 µg/mL for GA, HOH, OLE, CA and COH and from 0 to 50 µg/mL for CAT in equal volumes of the extraction of Ingredient A at a dilution 1:15. In the MRM method used for the LC-MS/MS analysis, the conditions in the collision cell for CA and COH were not stablished as the optimal values in Table 4.2. due to these compounds exhibited a significant higher sensitivity than the other compounds and the concentrations of the PPHs in the ingredients were expected to be high. Values for CE and CAV were established for lowering at some extent the signal obtained from CA and COH and for leveling up a bit with respect to the other compounds.

It was found that for GA, HOH and CAT there was no significant RME. However, for OLE and COH, there was signal enhancement, and for CA, there was signal suppression. The RME was even higher when the solutions used for the SAM were not fresh. According to these results and the discussion about stability presented in the previous section, all the posterior analysis were done by using SAM with fresh prepared solutions. The table 4.3 summarizes the results for the quantification of Ingredient A by SAM with a 1-day-old standard solution and with fresh standard solution, in comparison with the concentration declared by the manufacturer. Results with the freshly prepared solution are more approximate to the declared content of HOH, COH and CA. The 1-day-old standard solution caused an increase in the concentration of 38 % for HOH, 118% for COH and 123% for CA respect to the fresh solution.

No sample treatment for reducing/eliminating ME was done because the stability of CA and COH is a critical point for the analysis, and it was evident that this compound started to degrade quickly into COH, and more steps in the preparation of the sample and longer procedures could cause their degradation and alteration in the signal. Besides, as the PPHS evaluation was going to be done in three different matrices, (NAOX ingredient, fish feed and fish tissue), thus it would have been necessary to develop different sample treatments.

Table 4.3. Comparison between results obtained by SAM with 1-day-old solution and fresh solution and the values declared by the manufacturer.

Compound	Concentration declared	Quantification by SAM - 1-day-old standard solution	Quantification by SAM- fresh standard solution
GA	Not reported	Not detected	Not detected
HOH	13211	18453	13364
COH	4012	10916	4986
CA	3196	6494	2901
12-O-MECA	793	Not available standard	Not available standard

Thus, the quantification of the PPHs in three antioxidant ingredients was done by the SAM by independent measurements and preparation of fresh standard solutions and dilution 1/15 of the extracted sample. The calibration curves for the SAM are shown in Annex II for all the analytes in the three ingredients and the results are summarized in Figure 4.15. Additionally, related compounds were analyzed and semi-quantified as equivalents of the more similar/related compound, being the most important 12-O-MECA. (Figure 4.16).

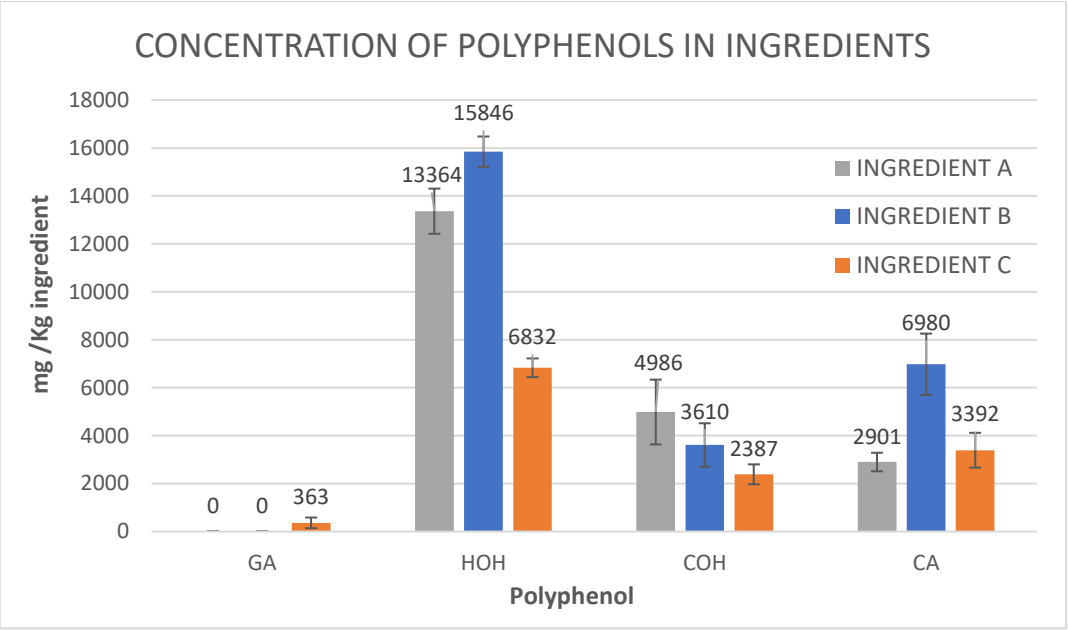


Figure 4.15. Concentration of PPHs in NAOX Ingredients A, B and C.

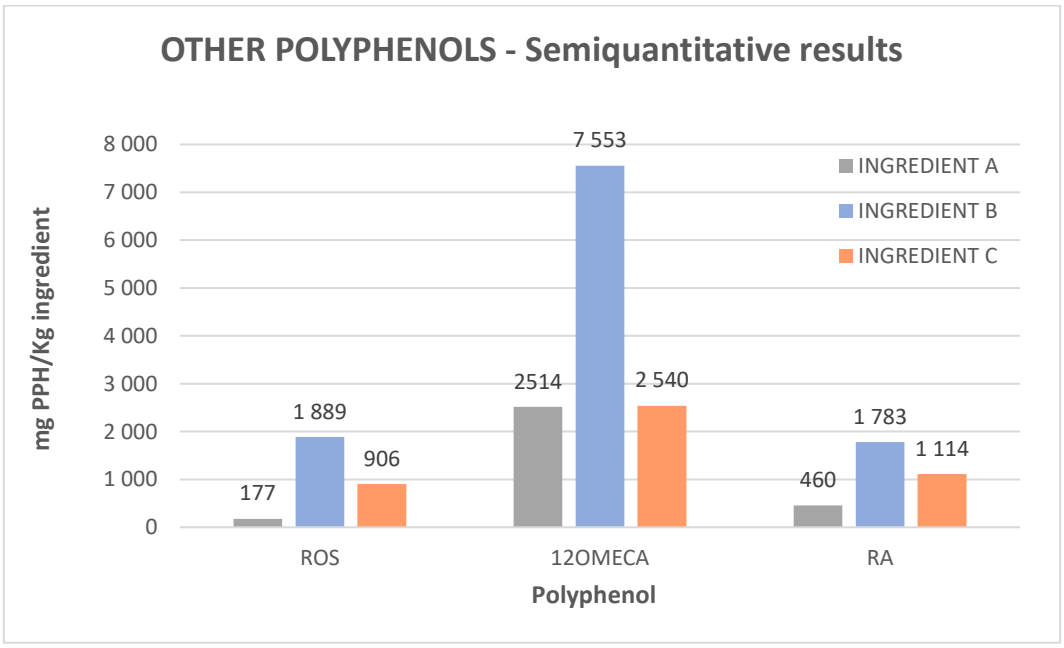
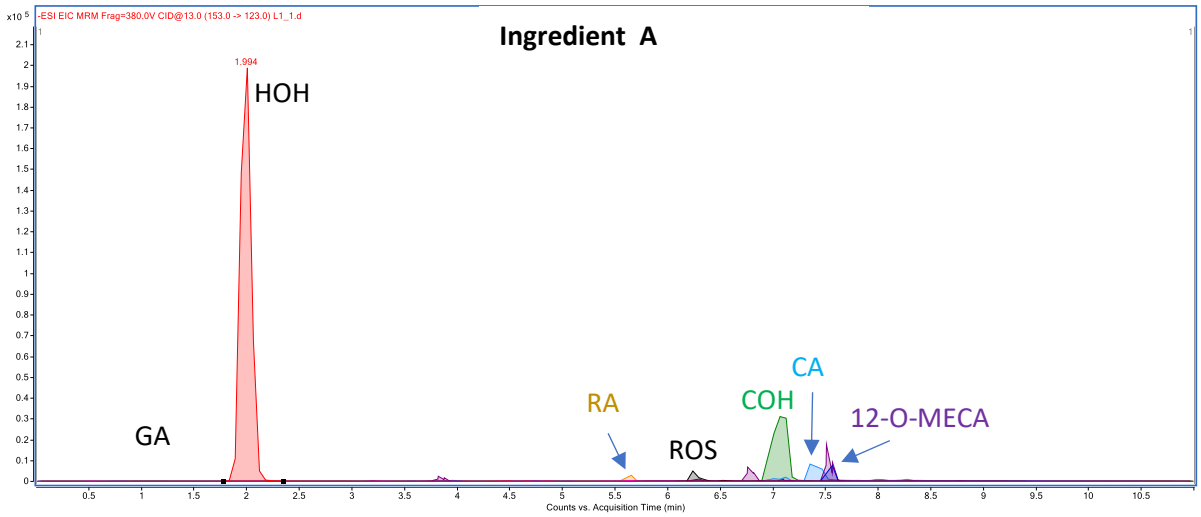
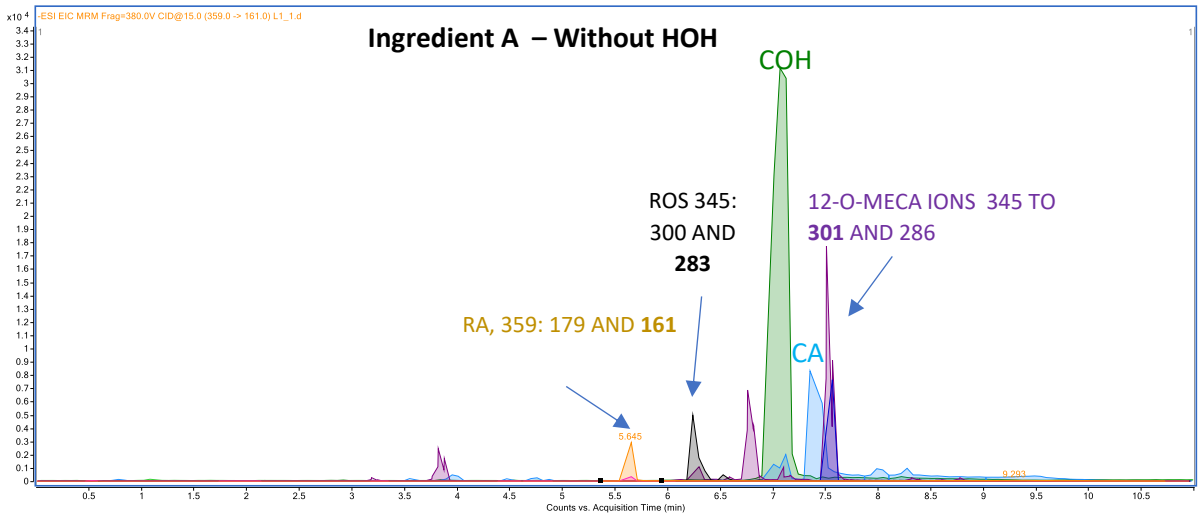
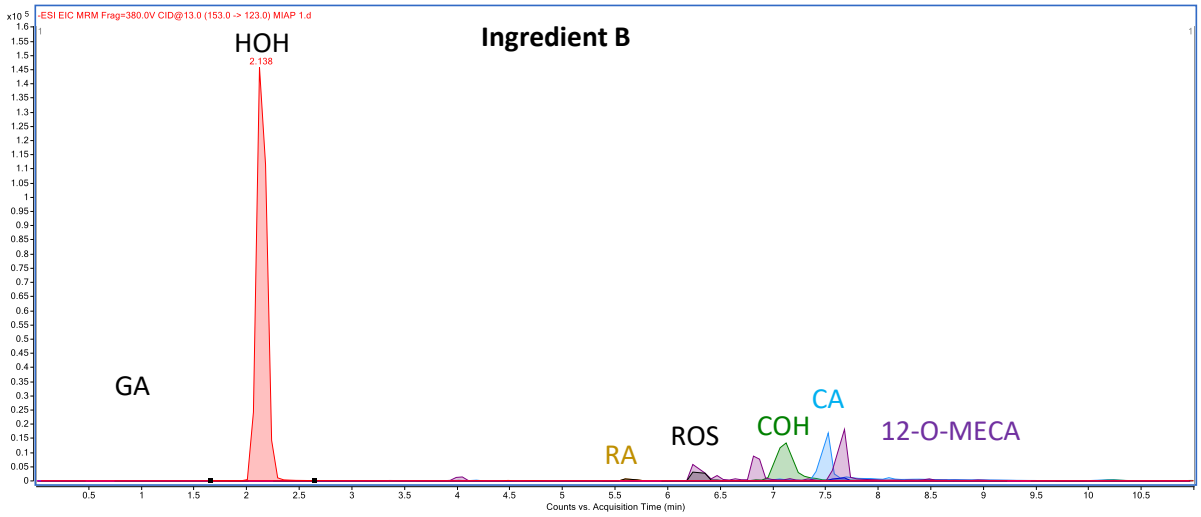


Figure 4.16. Concentration of related PPHs in Ingredients A, B and C. ROS semi-quantified as COH, 12-O-MECA and RA semi-quantified as CA.

The same four PPHs detected in the Ingredient A were detected in ingredients B and C. Overall, Ingredient B showed the highest total content of PPHs, 26436 mg PPHs / Kg ingredient, and HOH was the major compound in the three ingredients. GA on was only detected in a low quantity in Ingredient C. The chromatograms for the ingredients are presented in Figure 4.17.


-ESI EIC MRM Frag=380.0V CID@13.0 (153.0 -> 123.0) L1_1.d
Ingredient A
1.994
HOH
GA
RA
ROS
COH
CA
12-O-MECA
Counts vs. Acquisition Time (min)


-ESI EIC MRM Frag=380.0V CID@15.0 (359.0 -> 161.0) L1_1.d
Ingredient A – Without HOH
COH
ROS 345:
300 AND
283
12-O-MECA IONS 345 TO
301 AND 286
RA, 359: 179 AND 161
CA
5.645
9.293
Counts vs. Acquisition Time (min)


-ESI EIC MRM Frag=380.0V CID@13.0 (153.0 -> 123.0) MIAP 1.d
Ingredient B
HOH
2.138
GA
RA
ROS
COH
CA
12-O-MECA
Counts vs. Acquisition Time (min)

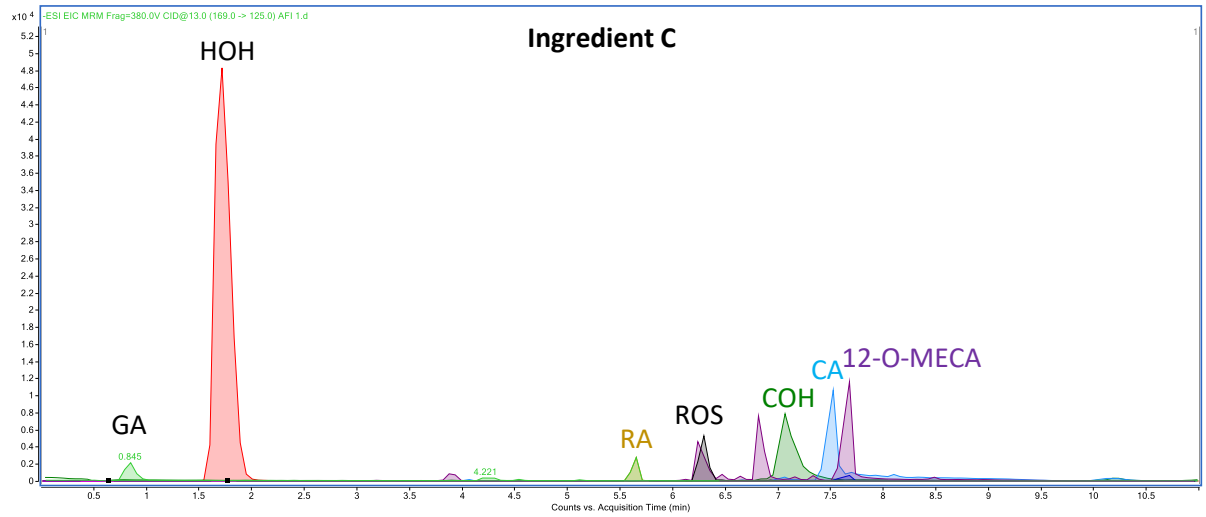


Figure 4.17. Chromatogram of the analysis of the antioxidant ingredients

4.2.4. Estimation of precision and accuracy in the quantification of the polyphenols in the antioxidant ingredients

4.2.4.1. Precision

The approach used for the estimation of the precision was the described by Goncalves et al.[46], in which the precision of the SAM is calculated as the error in the y-axis intercept in the calibration curve when the graph is constructed in a reversed way, what means, taking the x-variable as the dependent factor and the y-variable as the independent one. The feature "*Regression*" in MS Excel found in the command "Data Analysis" calculates the intercept (the concentration) and its error (standard deviation). Goncalves et al.[46], demonstrated that the precision estimated in that way is equivalent to the usual statistical methods for error propagation and extrapolation.

The described approach was used for calculating the error of the calculation of the extracted solution in the calibration curve by SAM. The percentage of deviation was calculated and applied to the values of concentration determined in the ingredients. The results are summarized in Table 4.4. and are also shown as error bars in Figure 4.15.

Table 4.4. Precision estimation in analysis of PPHs by SAM in ingredients.

PPH	Ingredient A		Ingredient B		Ingredient C		Average precision
	Precision (%)	Error (mg PPH/Kg)	Precision (%)	Error (mg PPH/Kg)	Precision (%)	Error (mg PPH/Kg)	
GA	NA	NA	NA	NA	60.9 %	222	60.9 %
HOH	7,1%	943	4.0 %	634	5.7 %	390	5.6
COH	27,1%	1352	25.1 %	907	17.4 %	416	23.2 %
CA	13,3 %	386	18.3 %	1278	21.4 %	726	17.6

From the results, it is evident a significant standard deviation for GA in Ingredient C which can be explained by the inadequate relation between the concentration in the sample versus the concentration in the addition of standard, which was too high for the value of GA in the sample, affecting precision and accuracy of the analysis. For improving this result, the analysis for GA could be done at lower dilutions, as 1/2 instead to 1/15 and with the addition of a lower concentration of this compound. Regarding the other compounds, the results for HOH are adequate, precision around 5%. However, there is a higher dispersion for COH and CA, an average around 20%, that is probably caused by the instability and erratic behavior of these compounds reported in literature and evidenced in the present work.

For improving precision of the analysis, the following measures could be considered:

- Temperature control of the tray in the autosampler at a lower temperature.
- More replicates and construction of a characteristic calibration curve for the matrix in the ranges of concentration determined and expected.
- Study other parameters of the chromatographic separation, as mobile phase, for instance, using only acetonitrile or evaluating different values of pH for trying to decrease the variation/dispersion of the signal of CA and COH.

4.2.4.2. Accuracy

For the antioxidant ingredients there is not a reference material or a quality control material for the evaluation of accuracy, thus, the estimation was done using the percentage of recovery, by cross-validation, using the calibration curves obtained for each compound for calculating for each point of the curve, the concentration obtained by the model and comparing it with the theoretical value (added value). The results are summarized in Table 4.5.

Average %R was between 101 and 104% but similarly to precision evaluation, better results were obtained for HOH in comparison with COH and CA. and the dispersion in the values of %R was acceptable ($\leq$ 10%) for GA and HOH, while for the other two compounds was higher, reaching almost 25% for CA. Those results are evident in the calibration curves for each compound and probably obey to the same reasons suggested for explaining low precision.

Table 4.5. Accuracy in PPHs analysis by SAM in antioxidant ingredients expressed as %R determined by cross-validation.

	Ingredient A				**Ingredient B**			
	GA	**HOH**	**COH**	**CA**	**GA**	**HOH**	**COH**	**CA**
Minimum %R	91 %	85 %	81 %	75 %	94 %	74 %	78 %	75 %
Maximum %R	121 %	115 %	120 %	110 %	115 %	107 %	150 %	180 %
Average %R	106 %	102 %	103 %	100 %	101 %	96 %	101 %	107 %
% CV	10 %	10 %	11 %	13 %	6 %	10 %	20 %	29 %
	Ingredient C							
	GA	**HOH**	**COH**	**CA**				
Minimum %R	92 %	76 %	74 %	61 %				
Maximum %R	113 %	123 %	120 %	158 %				
Average %R	101 %	100 %	98 %	104 %				

% CV	7 %	10 %	13 %	26 %
For the three ingredients				
Average %R	103 %	101 %	101 %	104 %
Average %CV	8 %	10 %	15 %	23 %

The composition of the ingredients was informed by the manufacturer only for Ingredient A, and accuracy was calculated based on those reference values (table 4.6.)

Table 4.6. Accuracy in PPHs analysis by SAM in antioxidant ingredients for Ingredient A respect to the concentration declared by the manufacturer.

Compound	Concentration declared	Quantification by SAM	Accuracy
HOH	13211	13364	101,2 %
COH	4012	4986	124,3 %
CA	3196	2901	90,8 %

The value obtained for HOH is very approximate to the reference value from the manufacturer. Nevertheless, for the other compounds, the differences are higher. COH showed a value almost 25 % higher than the reported value, while in the case of CA, the concentration is nearly 10 % lower. This difference could be caused by degradation of the CA in ingredient A, what according to the degradation products, would explain the increase in COH, even though the increase in COH was higher than the decrease in CA. For verifying this hypothesis it would be necessary to monitor the stability of the PPHs in the additive after production.

4.3. Fish feed production

The concentrations of PPHs in the different diets were expected to be notably lower than in the ingredient and the highest sensitivity was desired. Thus, the conditions in the collision cell for CA and COH were adjusted in order to increase the sensitivity for those analytes. It was not possible to work at the optimal values for CE and CAV, as when they were used, a high noise was observed, and the resolution of the peaks was affected. However, the analysis were done at values near the optimal determined and let the detection of the compounds.

Samples of three stages of the fish feed production using Ingredient A were analyzed: Before extrusion, before drying and final feed. The responses obtained for the 5 PPHs analyzed are illustrated in Figure 4.18. The four PPHs detected in the ingredient were found in all the stages of the feed, and additionally other PPH, GA, was detected in all the samples. The same trend of the response was observed for HOH, COH, CA and 12-O-MECA: first, an increase from the control to the sample with the higher content of ingredient (0.05%) and secondly, a decrease in the response as the process of feed production advanced, thus, the highest responses were found for samples before extrusion and the lowest, for the final feed. GA, in contrast, did not show any trend, and its content was found to vary in each stage of the feed production, being the higher presence observed in the sample before drying.

In Figure 4.19. the concentrations of the PPHs in the 3 samples from the 3 stages of the feed production are summarized. For GA, as mentioned before, a particular behavior was observed. The three different diets (control, 0.01% and 0.05% NAOX ingredient) in each stage exhibited a similar content of GA, what supports the previous finding of absence of GA in the antioxidant ingredient, and an increase instead of a decrease was observed after the extrusion process, showing the highest content of GA. In the revision of the possible sources of GA, it was found that propyl gallate was part of the basal diet and it is highly likely that for that reason, it was found in all the samples. This ester is formed by the union of propanol to the GA and is used as food ingredient to prevent oxidation of fats and oils. During the mixing process, some propyl gallate could be converted into GA, explaining the content detected in the first samples. After this, the extrusion process could have caused the rupture of the polymeric structure of this compound into GA, what explained the higher contents found in the samples before drying. Finally, it is probable that in the last stage, there had been a consumption of GA and in consequence, a new drop in the content of GA.

When it comes to the other PPHs analyzed, before extrusion, the same pattern found in ingredient A was observed in the samples with NAOX ingredient, with HOH as a major component, followed by COH. The concentrations found were higher for sample 0.05% but the ratio between 0.01 % and 0.05% was not 1 to 5 as expected but about 1 to 2. Regarding the expected values in the initial stage of the feed (before extrusion), based on the quantification of the Ingredient A, contents of PPHs in the 0.05% sample are more in accordance with the composition of the batch used on the manufacturing of this feed (2021) (Figure 4.20.).The values found for HOH, COH and CA are between the 80 and 120% of the calculated concentrations[1] and only for 12-O-MECA the found value was under the 40% of the expected value. In contrast, for 0.01% feed, the values found were from 180% and 300% of the calculated for HOH, COH and CA while only for 12-O-MECA, the determined value agreed with the expected according to the semi-quantification in Ingredient A as CA equivalents. Possible reasons for the differences in the 0.01% diet are errors in the NAOX addition during the manufacturing process, inadequate mixing of ingredients or lack of homogeneity in the NAOX ingredient and/or in the feed. Additionally, due to the lower concentration of the analytes in this matrix in comparison with the ingredient, a possible error could be caused by the method of analysis, as at lower concentrations, higher variability of the data and instability for CA and COH was found, and in consequence, it entails a higher uncertainty in the result.

After extrusion (before drying), an important loss in the PPHs content is observed. In the extrusion process the feed is exposed to high temperature and pressure and it seemed to cause the consumption of an important quantity of the NAOX compounds. While the GA content increased about 200% after extrusion, the other PPHs decreased nearly 80%. Thus, these results indicated that during extrusion there was an important consumption of PPHs.

During the drying, apart from the decrease of GA, the PPHs content did not seem to vary significantly, and even higher concentrations were found for COH and CA. Moreover, PPHs that were not detected in the samples Before drying, were found in final feed. This finding could be explained for non-homogeneous samples caused by production or sampling processes and/or by variability and errors caused by the analysis at low concentrations as discussed before. The four PPHs detected in Ingredient A were found in the final feed in both, 0.01% and 0.05% samples, although total PPHs content (without GA) was found to decrease approximately 84% and 70% respectively from the values before extrusion.

[1] Calculated concentrations refer to the expected content for each PPH in the feed based on the content of PPHs determined in the ingredient and the addition of ingredient to the feed (0.01 or 0.05%)

From these results and from the ones obtained for the analysis of the NAOX ingredient, is evident that the quantitative analysis of the NAOX products and diet is the vital importance, as many factors could cause the variation of the concentration of antioxidant compounds during the processing of the ingredient, during storage and during the manufacturing of the diet, and if those variations are not known, they cannot be controlled, and the effects on both the feed and the fish can be non-reproducible and even detrimental. The chromatograms for the two diets at the three stages of the process are shown in Annex III.

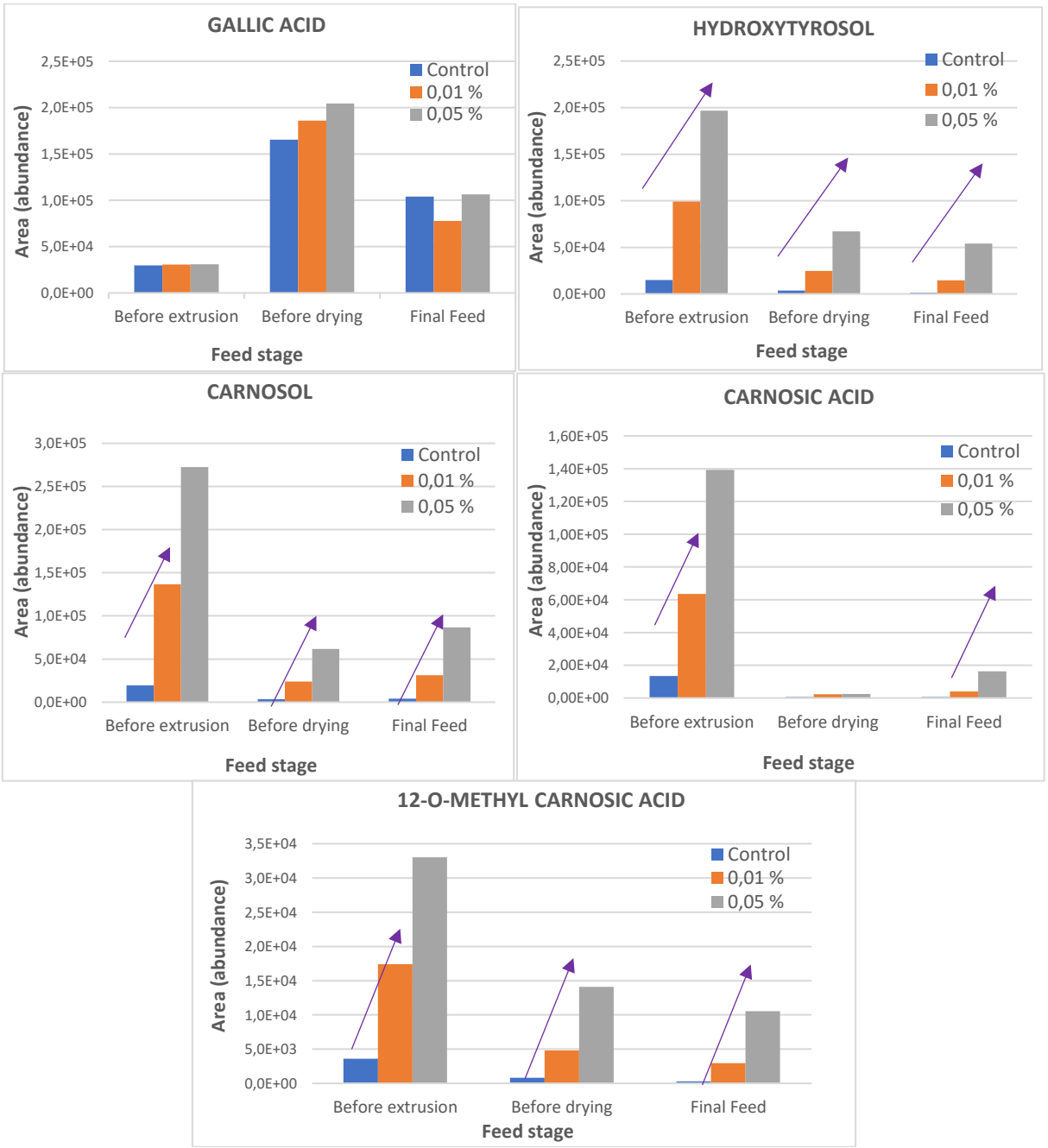


Figure 4.18. Responses obtained for 5 PPHs analyzed in three stages of the fish feed production using Ingredient A in two different proportions, 0.01% and 0.05%, compared to control samples without antioxidant ingredient.

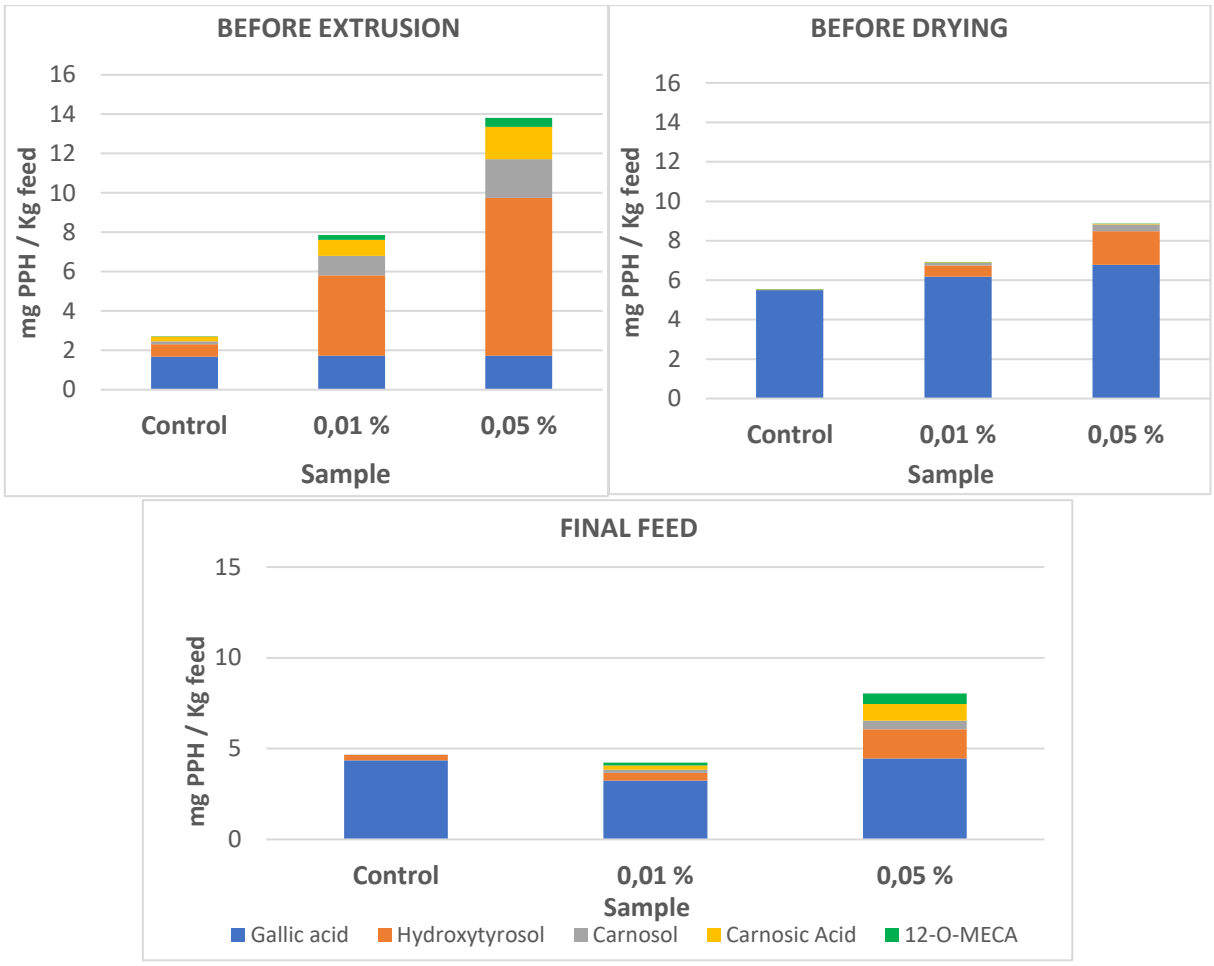


Figure 4.19. Composition of PPHs of the 3 different diets (Control, 0.01 and 0.05% of NAOX ingredient) analyzed in three stages of the fish feed production.

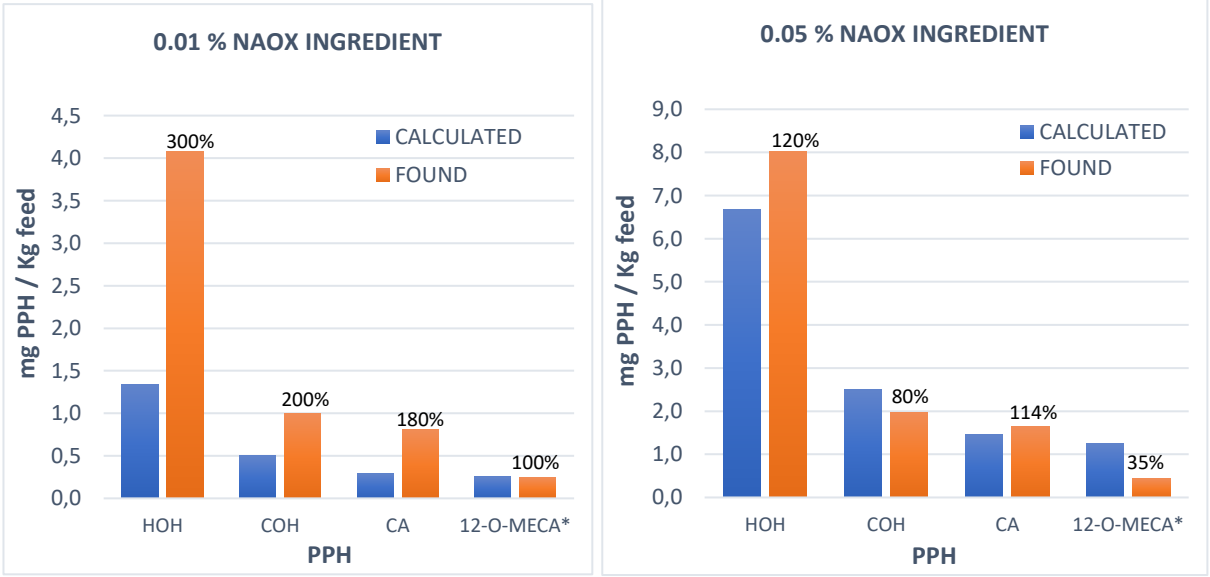


Figure 4.20. Comparison between concentration of PPHs determined in the feed before extrusion with 0.01 and 0.05% of NAOX ingredient and the expected values based on the quantification of the ingredient A.

4.4. Analysis in fish tissue

Fish from different groups from the tank trial were analyzed. The starting point was in March 2023 and samples are identified with M, after the three-month trial, samples were identified as Control (C) (feed without NAOX ingredient, group 1 (G1), feed with 0.05% NAOX, and group 2 (G2) feed with 0.01% NAOX. Preliminary analyses for method development were conducted in samples from the 4 groups (M, C, G1 and G2) and the main compounds detected in the feed, GA for all diets and HOH in 0.01 and 0.05% NAOX diets, were not detected in any of the tissues analyzed. These results were confirmed by the final analysis by standard addition of a total of 22 samples of liver and 24 of muscle in which the signals obtained for 4 compounds analyzed were overall higher for liver than for muscle. Due to the low level of PPHs expected and the limited quantity of sample, the complete available tissue for fish were analyzed and the results were normalized based in one fixed weight. The individual responses for 4 detected compounds are presented in Figure 4.21.

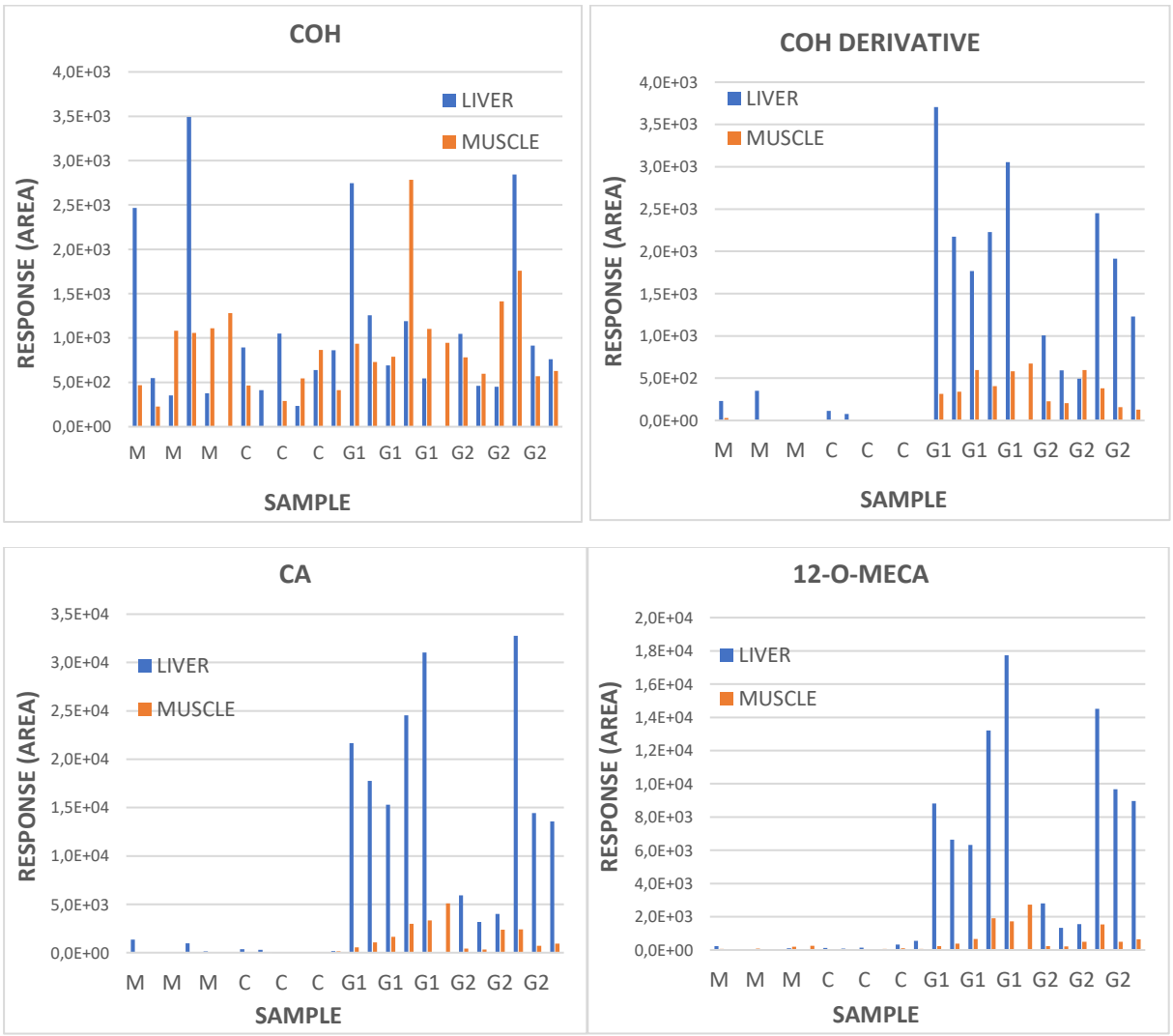


Figure 4.21. Responses obtained for 4 detected compounds in samples of liver and tissue. Data normalized to 2051.9 mg.

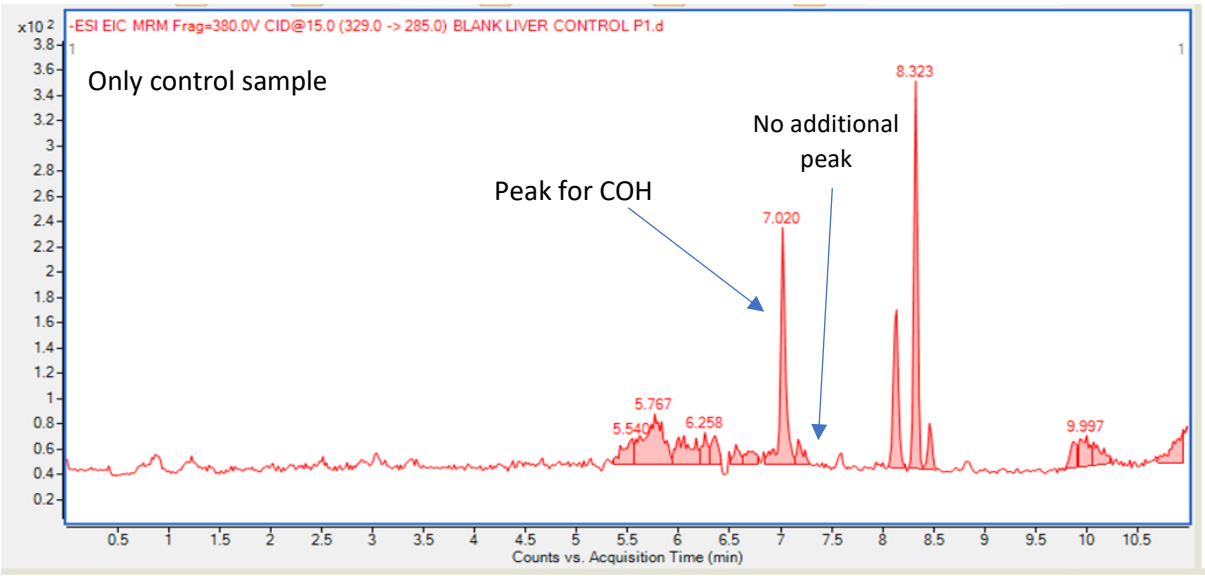
Regarding COH, low signals were obtained, and carryover was detected. In consequence, the results for this compound did not show any trend. However, it was very noticeable that in samples from G1 ang G2 there was a fragment eluting later but very close to the COH, which presented the same fragmentation pattern (329 m/z→285 m/z). This peak at 7,17 min was detected in all the samples from G1 and G2 and

not significantly in C nor M samples, what can be seen in the graph for the signals of "COH derivative" in Figure 4.21. This evidence suggested the presence of a derivative of COH in the tissue what could indicate that the COH in the fish was metabolized into a compound with the same mass and mass transition than COH. The chromatograms illustrating these findings are shown in Figure 4.22.

The first chromatogram corresponding to a control (fish not fed with NAOX), showed a clear peak for COH, identified using the analytical standard. Although this compound was not expected in this sample, its presence could be explained by the carryover evidenced in all the analyses, what made difficult to determine if the COH was present in control samples or was an effect of the carryover. No other peak was detected eluting after COH. In the second and third EICs, it was possible to observe that for G1 and G2, besides the peak of COH, there was another peak with the same transition at 7.17 min, with seemed also with a higher intensity than the peak for COH. In the last EIC, a sample from March shows that the peak called "COH derivative" is not present either.

Another aspect to highlight is the three peaks exhibiting the same transition 329 m/z→285 m/z around 8.3 min. These peaks were present in all the samples, thus they do not seem connected with the inclusion of COH in the diet and instead, they can be associated with three similar peaks observed in the fish feed that seemed part of the basal composition, as they were found in similar abundance in the different samples for the different stages of the production of the fish feed. (Annex III – Chromatogram for control samples before extrusion, before drying and final feed). These compounds cannot be identified or pointed as analytical standards would be needed.

In conclusion, regarding COH, the findings suggest a possible effect on the fish fed with the antioxidant ingredient not evident by the COH but for the COH derivative. The behavior described above was found in all the samples analyzed.



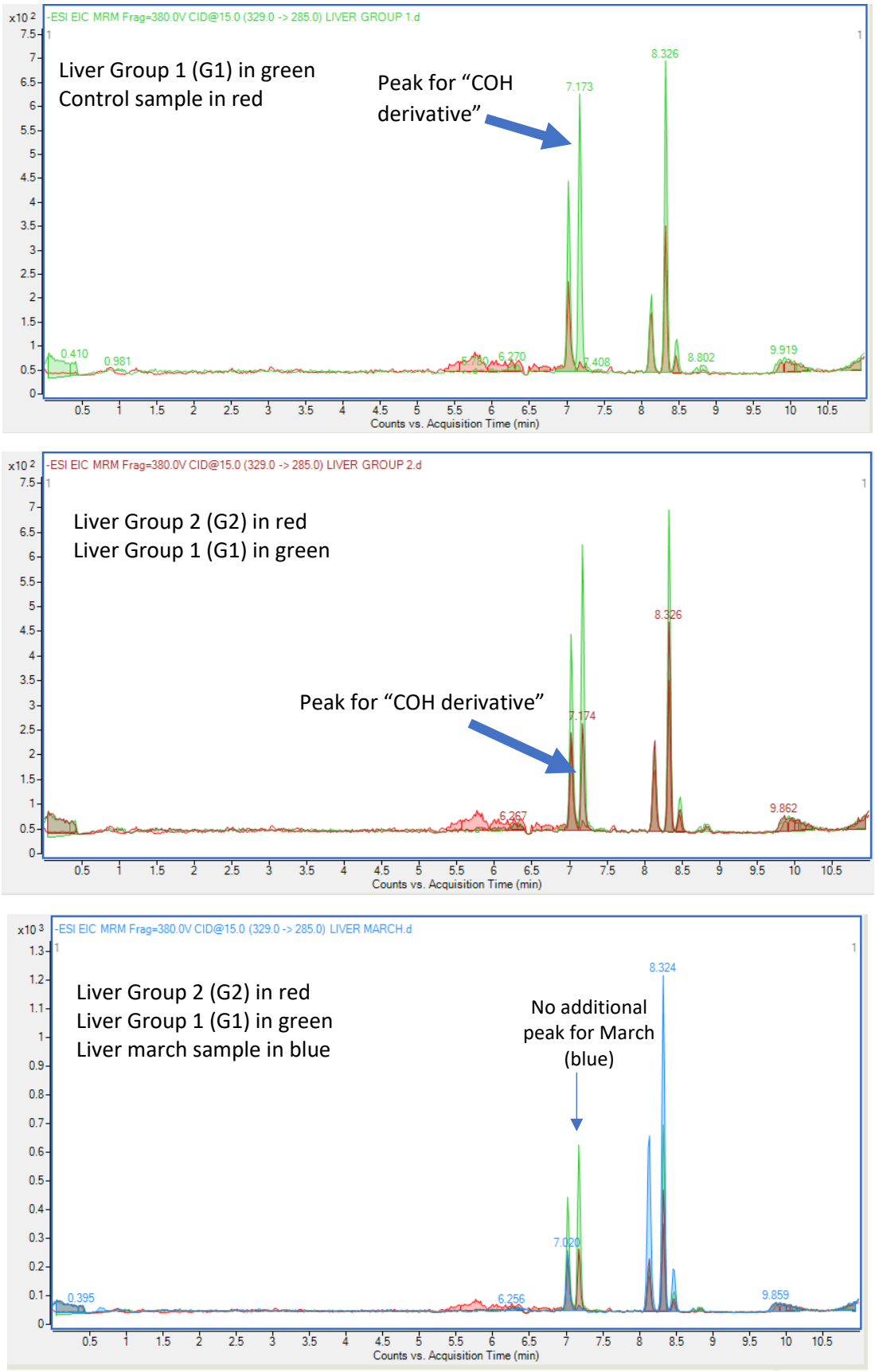


Figure 4.22. Set of EICs for the transition 329 m/z⟶ 285 m/z (COH and "COH derivative") for one sample from each group: Starting point (March), Control group (C) and 0.01% and 0.05% NAOX (Groups 2 and 1 respectively)

Regarding CA and 12-O-MECA*, the signals obtained in G1 and G2 were higher than for COH and additionally, the difference in comparison with M and C groups was evident, no CA nor 12-O-MECA were detected in those samples. These findings can be observed in the figure 4.21 showing the responses for CA and 12-O-MECA in the tissues analyzed.

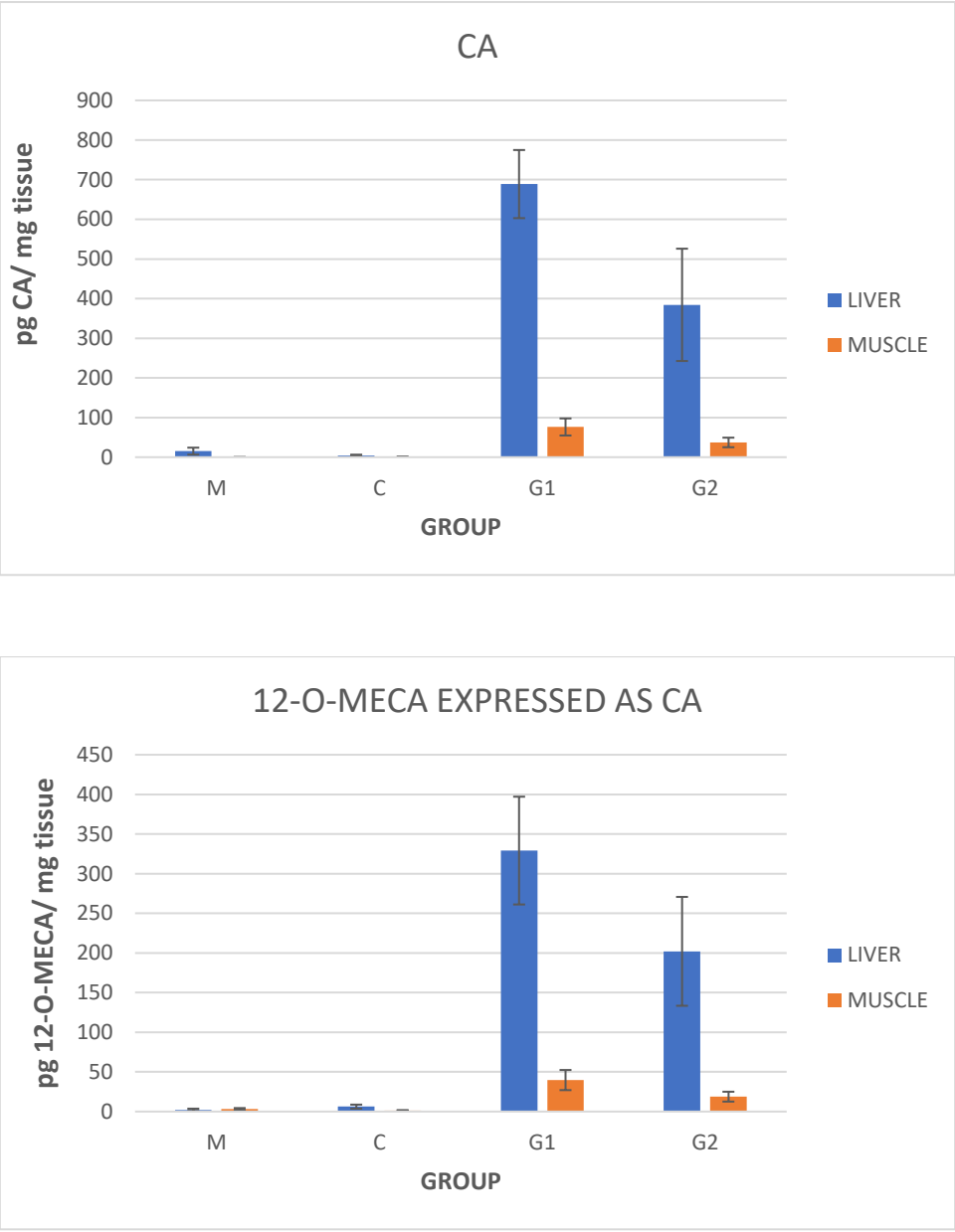


Figure 4.23. Average results for quantification of CA and 12-O-MECA expressed as CA in the tissues analyzed for the four groups.

When it comes to the quantification of PPHs in fish tissue, due to the carryover and low signals mentioned previously, COH could not be quantified in the samples. Moreover, the calibration curves constructed by standard addition showed a high variation between different days, evidencing one more time the higher variability and instability of COH at low concentrations. Better results were obtained for CA, a calibration curve could be obtained, and the samples were quantified, as well as 12-O-MECA, which was expressed as CA equivalents. This result was different than expected, as it seemed that CA, even with a longer

extraction period in this matrix, 70 min in comparison with 25 min for the other matrices, showed a notably better stability. The matrix seems to affect CA differently, avoiding its degradation and controlling in a certain way its erratic behavior.

There are many aspects to emphasize here. First, the content of CA and 12-O-CA found was higher in liver than in muscle, from 8 to 10 times bigger. For G1 and G2 the average content of CA was nearly 700 and 400 pg/mg of tissue in liver and 80 and 40 pg/mg of tissue in muscle respectively. For 12-O-MECA, the results were 329 and 202 ng/mg of tissue for liver and 40 and 19 ng/mg of tissue for muscle. This could suggest a different use/impact/effect/metabolism of the NAOX compounds in the different organs that would require further evaluation and study from the biological approach.

Secondly, the results exhibited a high variability (see error bars in the graphs), due to the fact that the samples are from living organisms and consequently they are more complex. Thus, even compounds were detected and quantified, and the analysis of the results suggested the effect of the administration of the antioxidant ingredient on fish, , it would be important to analyze a higher quantity of samples to assure a better representation of the real scenario.

Other important finding is that the ratio between the contents determined for G1 and G2 was not 5:1 but approximately 2:1, that is a similar result than from the analysis of the fish feed and thus it could also reinforce the hypothesis that the dosage in the feed with 0.01% could have been higher than the objective value as discussed previously.

Finally, and probably the most interesting point is, why the main components identified in the diets were not found in the tissues analyzed. According to the results for the diets, concentrations of GA and HOH were nearly 5 and 2 times bigger than the concentration of CA in the final diet 0.05% NAOX, and GA in 0.01% NAOX feed was 14 times bigger than CA. However, nor GA neither HOH were detected in any of the tissues. There are different hypotheses that could explain this finding:

- The consumption/use of the GA and NAOH for contributing to improve the antioxidant status of the fish is higher than for CA, COH and 12-O-MECA.
- CA, COH and 12-O-MECA have higher/better antioxidant properties and even when they are fed in a lower amount, can contribute more efficiently to the fish well-being than GA and HOH.
- The extraction method or the solvent used is not efficient enough to penetrate membranes of the cells and the compounds GA and HOH were inside the cells and could not being extracted.
- There is a different distribution of the antioxidant compounds in the organs of the fish and GA and HOH are not assimilated by liver and muscle but can be found in other organs.
- GA and HOH are much smaller and simpler molecules than CA, COH and 12-O-MECA and for that reason, it is possible that they are completely metabolized easily or transformed in different compounds, and they cannot be identified.
- GA and HOH were not assimilated by the fish and could be found in their feces.

The present results obtained in fish tissue were compared with a study where CA and COH were used to investigate the transfer of diterpenes from rosemary to liver, kidney and two classes of muscles in lamb.[51]

- The degradation of CA, COH and 12-O-MECA after the manufacturing of the feed was found between the 21 % and the 30 % for two extracts of different CA:COH ratios, much lower than the found in the present study. This could be explained for the more extreme conditions of temperature and pressure to which the feed is exposed during the extrusion process in comparison with the production of feed for lamb used in the referenced study. The final diets

contained about 700 mg of phenolic compounds (CA, COH and 12-O-MECA) per kg diet, while the diet in the present study contained about 4 mg/kg of the same compounds.

- The extraction process for CA and COH comprises initial drying, lyophilization, Soxhlet extraction for 2 hours under nitrogen atmosphere, secondary drying and re-dissolution, while the method used in this study was a simple vortex extraction for 70 min followed by centrifugation and filtration.
- Similarly to the present study, Jordan et al. suggested a metabolite of COH, the molecular formula $C_{19}H_{22}O_3$ was proposed, and the compound was detected and quantified. Higher presence of this metabolite than COH was found in both muscles but similar or less quantity was determined in liver.
- Higher content of diterpenes was found in liver in comparison with both muscles analyzed, which agrees with the findings presented in this document, CA only was detected in liver in the extract with the higher CA/COH ratio, while 12-O-MECA was not detected. Transfer of diterpenes was determined between 200 and 3100 pg/ mg of tissue for COH and 500 pg/mg of tissue for CA. In this work, the detected quantity of CA in tissue was between 40 and 700 pg/mg of tissue. The higher content of COH detected could be explained by the notably higher NAOX dosage in the study conducted by Jordan et al. The non detection of the CA in their study could be caused by the long process of extraction, that could cause CA and even 12-O-MECA degradation into COH.

To sum up, a significantly lower administration of rosemary compounds to fish led to a similar concentration of CA in tissue and higher concentration of 12-O-MECA, suggesting a higher transfer of those compounds in fish compared to lamb. Some of the results reported in this work were found similar to the reported in the referenced study, and, analyzing the results of CA in tissue of lamb, the importance of the rapid extraction and analysis of CA to prevent degradation seems to be important.

5. CONCLUSIONS AND RECOMMENDATIONS

The evaluated methods of LC-MS/MS are suitable for analyzing selected phenolic compounds in commercial feed ingredient, in fish feed at different stages of the chain production and fish tissues, using simple extraction procedures. Due to the instability of the compounds, especially of CA and COH, and the high relative matrix effect in the ingredient for OLE, COH and CA, the best alternative for the analysis was the standard addition method with solution of standards freshly prepared before use.

In the antioxidant ingredient, the average precision was between 5.6 % and 23.2 % for the main compounds HOH, COH and CA. GA was detected in one of the ingredients in a low concentration, the quantity was estimated but it is necessary the quantification at a lower dilution for increasing the accuracy and precision of the result. Within the ranges evaluated, the average %R were between 101 % and 104%, showing a higher variability for CA and COH.

Regarding the method of analysis, there are two main factors to highlight. First, the extraction and analysis of the main compounds of two very different natural extracts, olive and rosemary, was achieved in one single extraction and LC-MS/MS analysis, what for the author's knowledge it is not usual, as the compounds from each one of these natural sources exhibit very different characteristics and chemical affinities, thus are normally extracted and analyzed using different solvents and methods.

The second aspect is that it proved to work satisfactorily in three very different matrices and concentration levels, from high concentration (ingredient) to trace level (fish). However, it would be valuable to optimize the extraction methods, for instance, using the vortex multiple for the ingredient and fish feed for decreasing extraction time and/or increasing the efficiency of the extraction. This optimization, along with the enhancement of the parameters of the equipment as temperature control in the tray of the autosampler and needle wash operation, could improve the precision and accuracy of the method specially in low ranges and the number of samples processed per day. The evaluation of the method with only acetonitrile as mobile phase, as well as at different pHs could lead to an improvement in the stability of CA and COH and in consequence, in the precision of the method. It is vital to use amber vial and if possible, even amber material in the extraction process for improving stability of the compounds, specially of CA. The evaluated method was found adequate for further standardization and validation for control and studies in manufacturing and fish farming processes and/or studies.

For a better evaluation of the product and further studies, it would be convenient to evaluate the important NAOX allegedly detected in the ingredient,12-O-MECA, ROS and RA, with analytical standards as the semi-quantification done in the present work not necessarily corresponds to the real concentration. The sensitivity of each compound can be notable different even for similar molecules and can be affected by the parameters in the collision cell of the mass spectrometer. Those related compounds were neither analyzed in the feed nor in the fish and could be present in those matrices. With the appropriate standards, the optimization of the collision cell, and the improvement of extraction methods, it could be possible their quantification and a better knowledge of the ingredient composition.

It is important to mention that the conversion of CA in COH and latter in RO was evidenced, but it does not necessarily mean a lower antioxidant capacity of the product as those derivatives are said to exhibit similar properties than CA. Moreover, the other related compounds detected also have antioxidant properties. Thus, it would be beneficial to integrate the chemical analysis with an appropriate technique to evaluate the antioxidant capacity of the product, as there are multiple claims that suggest that PPHs act synergistically, and besides the group of compounds from the rosemary, the product contains a higher proportion of one of the main compounds of the olive extract.

Regarding the application of the ingredient in fish feed, it is convenient to evaluate according to the stability of the PPHs and the effects of the production process, especially the extrusion, in their composition to assure that the fish is fed with the desired amount of NAOX. The results demonstrated that only the 30% or less from the intended amount of NAOX was administered to the fish. With the real dosage, according to complementary analysis done within the project encompassing the present work, a protection of important compounds of the feed as astaxanthin and vitamin E was suggested, as well as less lipid oxidation in the fish tissue. Thus, the administration of the right dosage or even a higher one could yield better benefits for both fish and feed. Nevertheless, it is necessary to evaluate if there is a limit dosage for getting positive effects from the NAOX supplementation.

When it comes to the analysis in fish tissue, the results obtained were satisfactory as the first attempt to quantify NAOX in tissue. Despite the instability exhibited by CA in previous analysis, its behavior was stable and better in liver and muscle in comparison with the other matrices, and no carryover was detected, thus the quantification was possible. It would be interesting to explore more alternatives for the extraction process to investigate if the majoritarian compounds of the feed, GA and HOH, are definitely absent from the tissue or the extraction method was not able to extract them. For COH, it is important to improve the carryover, evaluating different solvents or times for needle wash for

The NAOX are expected in low concentrations in tissues, thus, efforts should be made to improve sensitivity as much as possible. In the present research, for the liver and muscle analysis, conditions in the collision cell for CA and COH were not stablished as the ones for optimizing the signal according to the findings in the method development, as those conditions increased the noise to a level in which the detection was affected. However, it would be convenient to evaluate this aspect and probably consider other parameters for working at the optimum conditions. For instance, the dwell time showed no effect in the signals, but the analysis was done at concentrations significantly higher than the expected in tissues and each compound was evaluated individually. The evaluation at lower concentration for various analytes could lead to conditions in which it is possible to work at the most favorable parameters in the collision cell, as the dwell time is said to influence the noise and the signal/noise ratio. Another possible improvement is the optimization of the source of the mass spectrometer, verifying that favorable conditions for all the analytes of interest are met. Finally, a high-resolution LC-MS (e.g., Orbitrap) could be considered, as their sensitivity is better than the low-resolution equipment used in the present work and can be more appropriate for tissue analysis.

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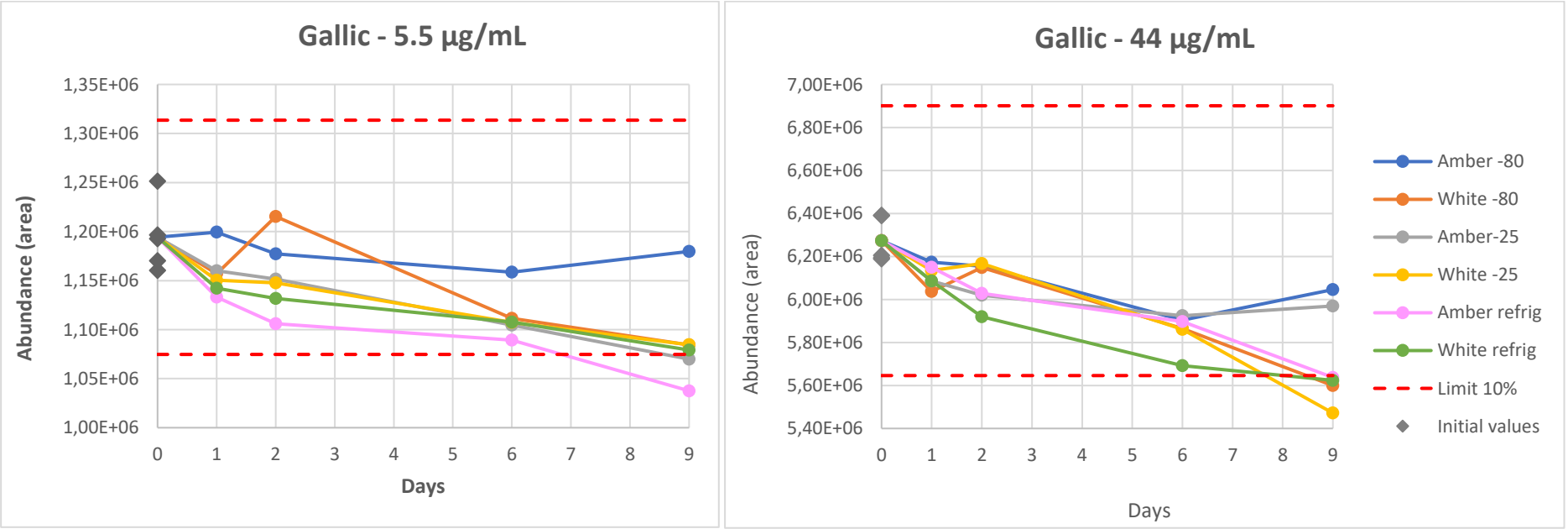
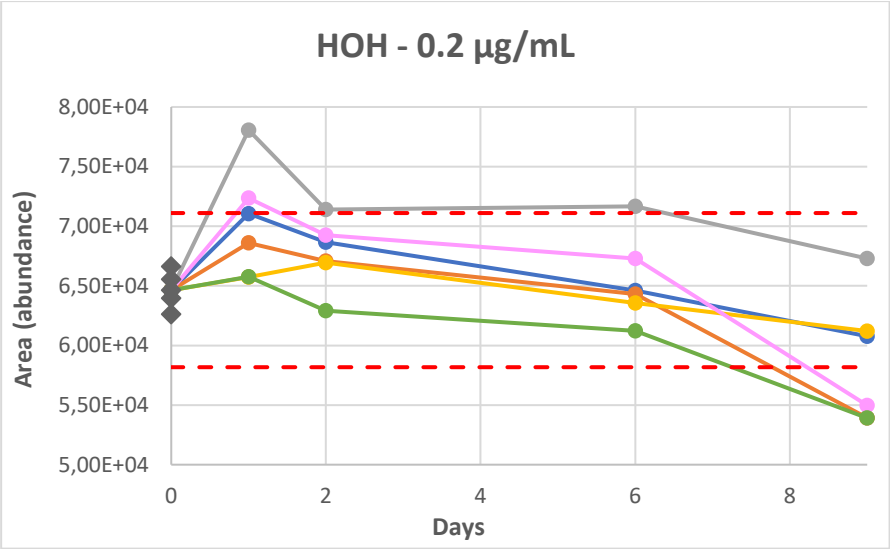
8. ANNEXES

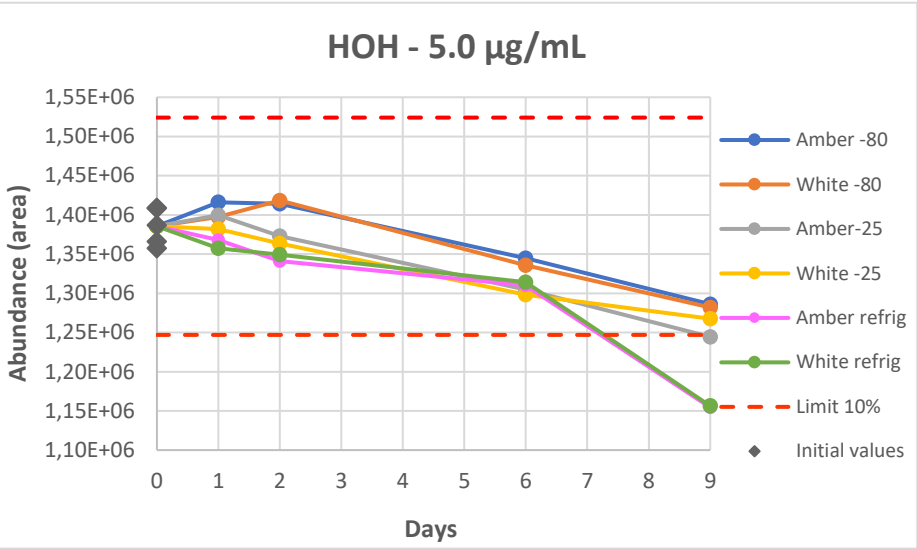
ANNEX I - GRAPHS STABILITY APPROACH

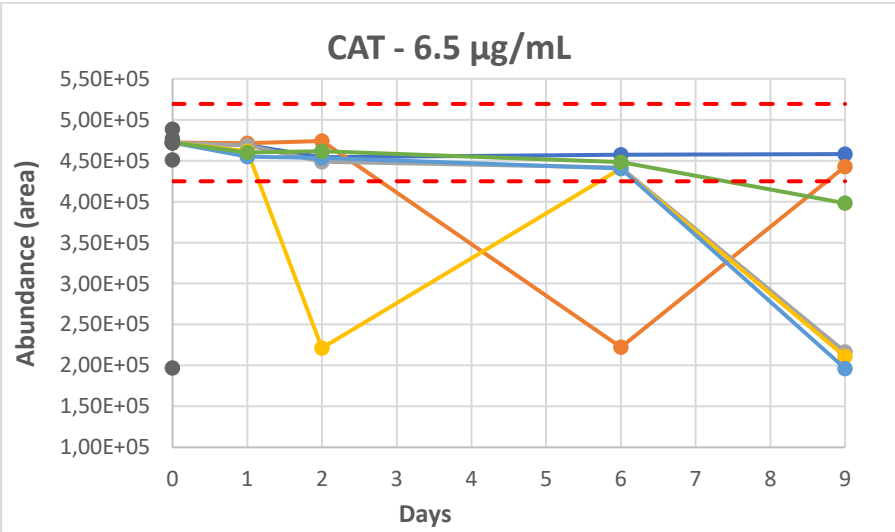
I. Stability for the mixture of PPHs GA, HOH, CAT, OLE, COH and CA - wide range of concentration

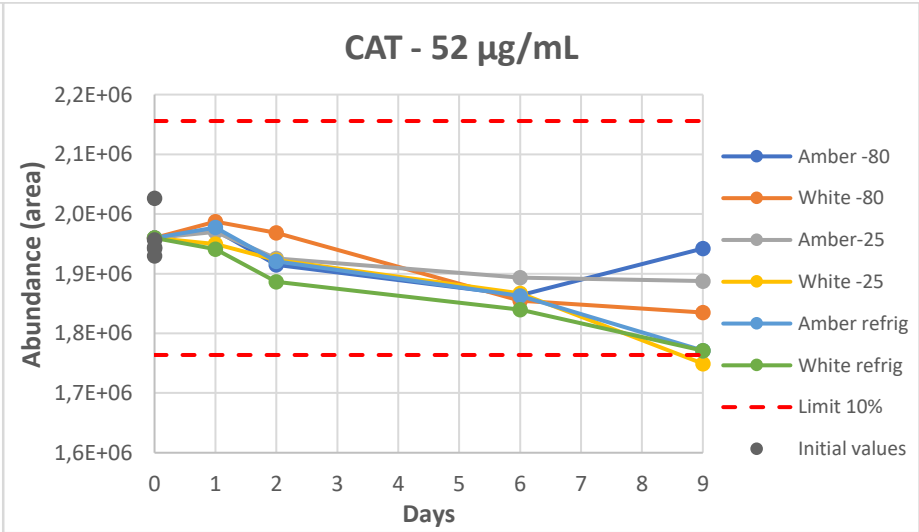
Two concentration points are shown for all the compounds except for COH and CA, for which the 3 points measured are illustrated due to their special behavior. Red dashed lines show a limit of the 10% of variation with respect to this value. For CA and COH, also green dashed lines indicate the limit for 30% of variation.

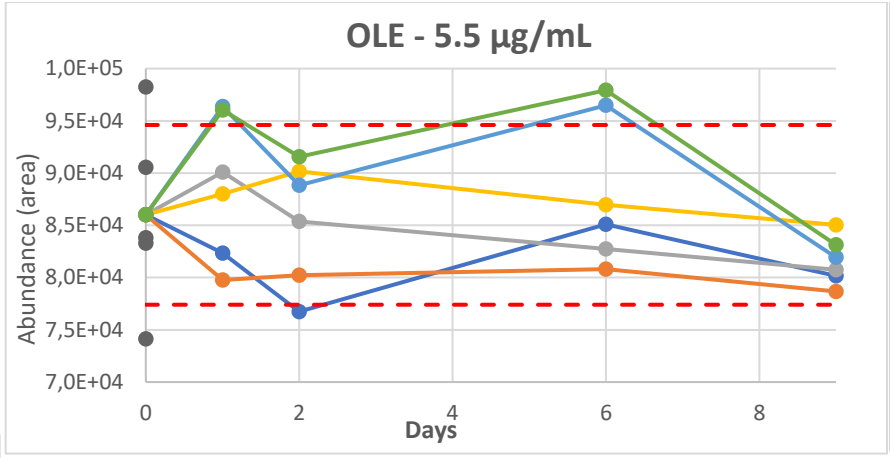


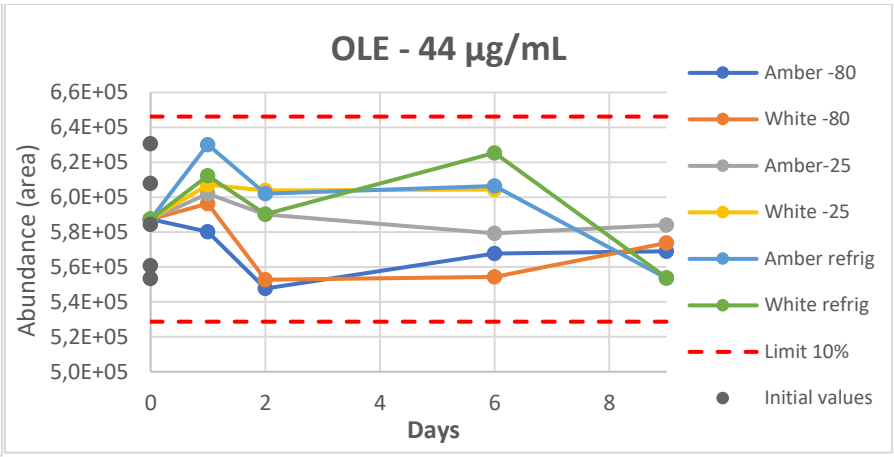

HOH - 0.2 µg/mL
Area (abundance)
8,00E+04
7,50E+04
7,00E+04
6,50E+04
6,00E+04
5,50E+04
5,00E+04
0
2
4
6
8
Days

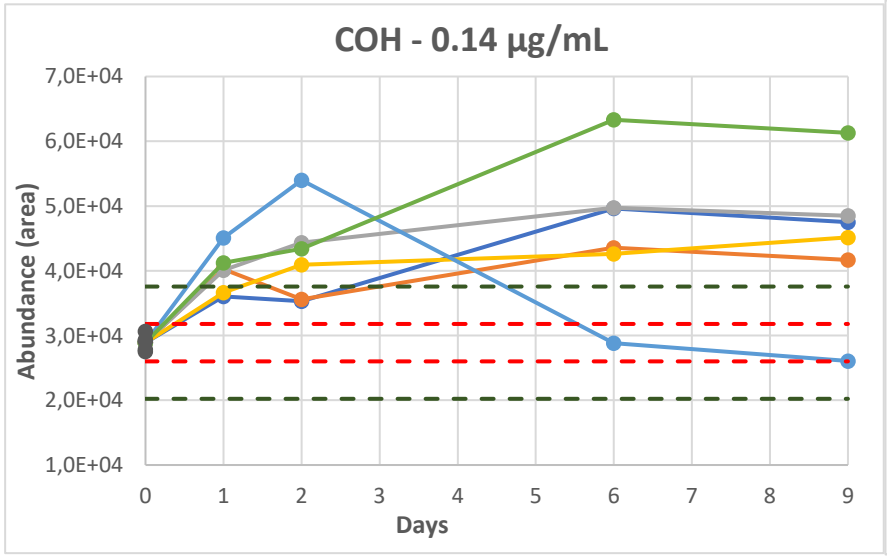

HOH - 5.0 µg/mL
Abundance (area)
1,55E+06
1,50E+06
1,45E+06
1,40E+06
1,35E+06
1,30E+06
1,25E+06
1,20E+06
1,15E+06
1,10E+06
0
1
2
3
4
5
6
7
8
9
Days
Amber -80
White -80
Amber-25
White -25
Amber refrig
White refrig
Limit 10%
Initial values

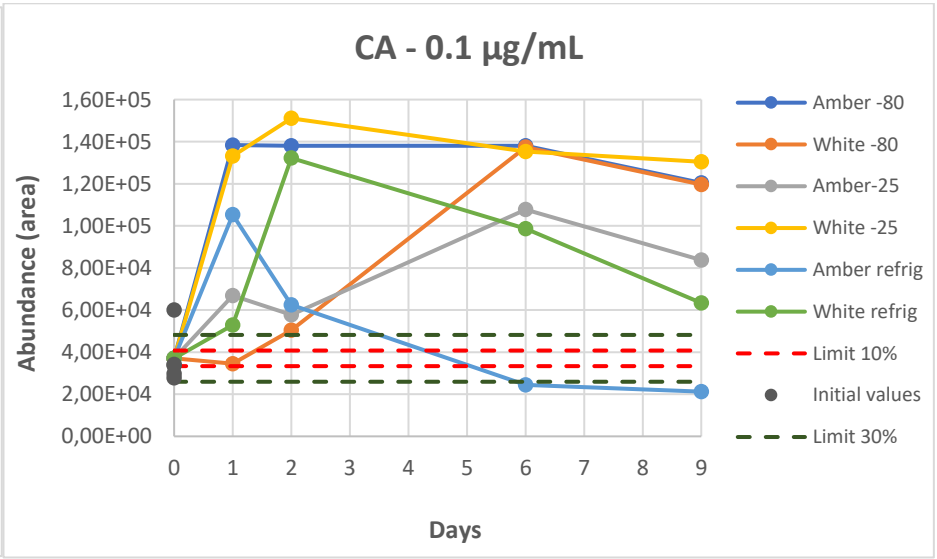

CAT - 6.5 µg/mL
Abundance (area)
5,50E+05
5,00E+05
4,50E+05
4,00E+05
3,50E+05
3,00E+05
2,50E+05
2,00E+05
1,50E+05
1,00E+05
0
1
2
3
4
5
6
7
8
9
Days

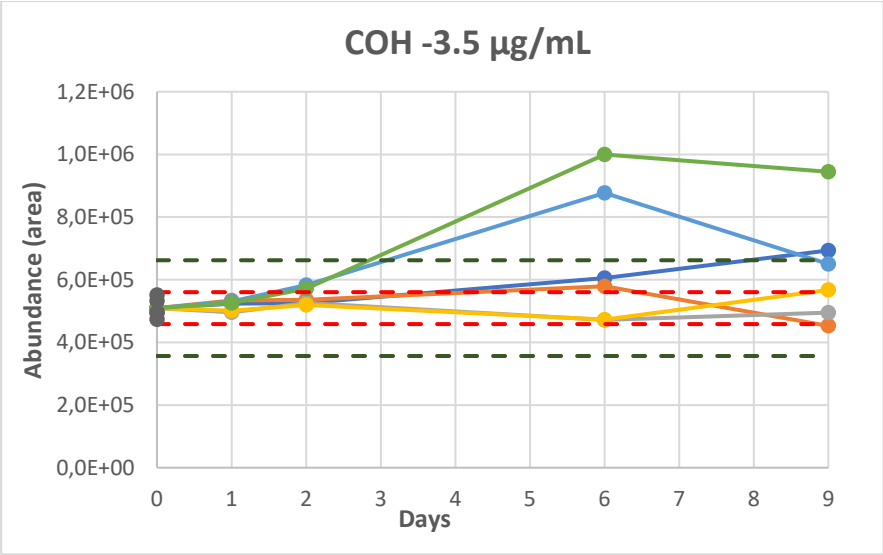

CAT - 52 µg/mL
Abundance (area)
2,2E+06
2,1E+06
2,0E+06
1,9E+06
1,8E+06
1,7E+06
1,6E+06
0
1
2
3
4
5
6
7
8
9
Days
Amber -80
White -80
Amber-25
White -25
Amber refrig
White refrig
Limit 10%
Initial values

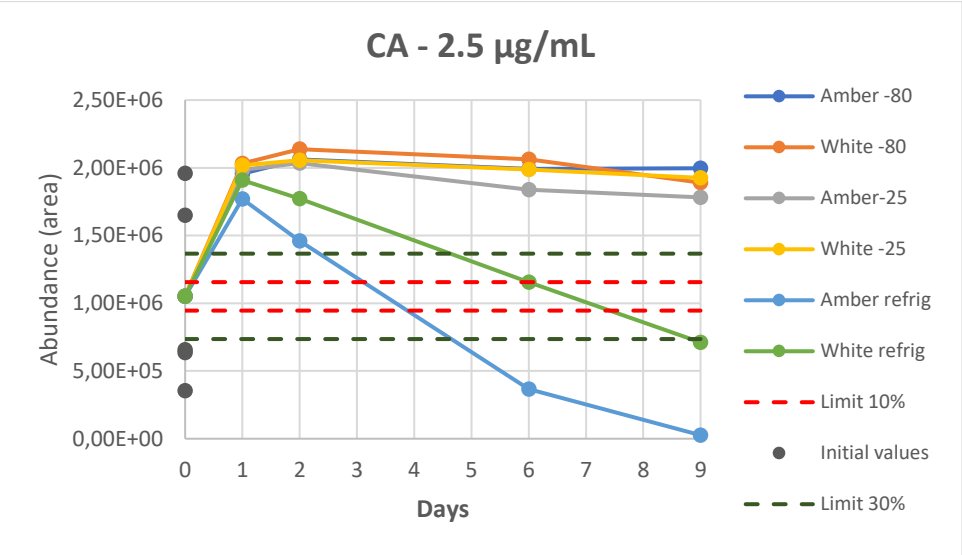

OLE - 5.5 µg/mL
Abundance (area)
1,0E+05
9,5E+04
9,0E+04
8,5E+04
8,0E+04
7,5E+04
7,0E+04
0
2
4
6
8
Days

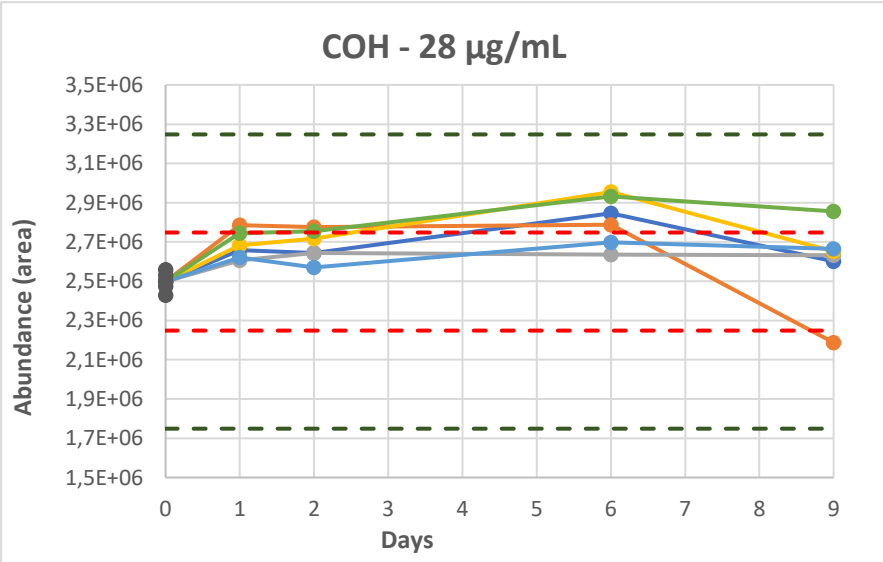

OLE - 44 µg/mL
Abundance (area)
6,6E+05
6,4E+05
6,2E+05
6,0E+05
5,8E+05
5,6E+05
5,4E+05
5,2E+05
5,0E+05
0
2
4
6
8
Days
Amber -80
White -80
Amber-25
White -25
Amber refrig
White refrig
Limit 10%
Initial values

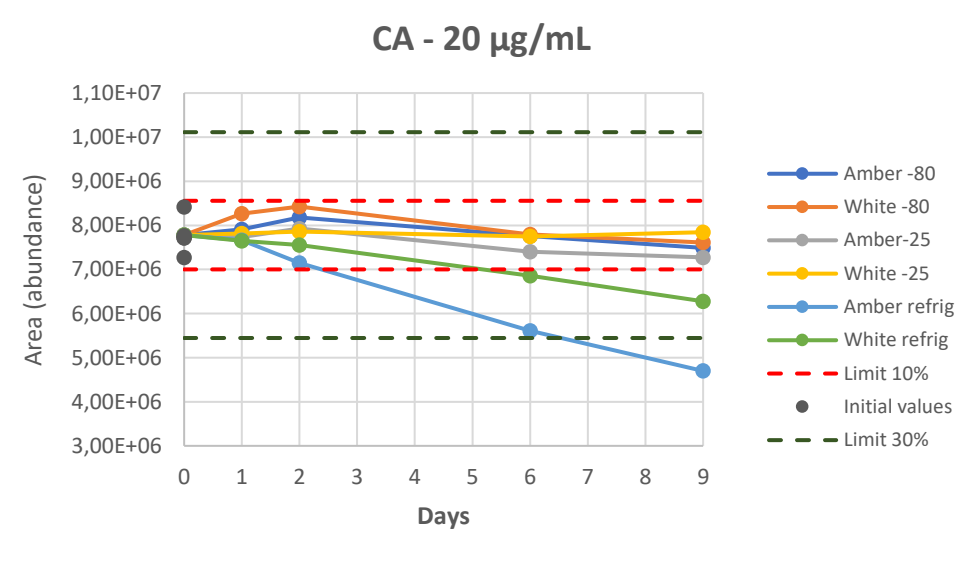

COH - 0.14 µg/mL
Abundance (area)
7,0E+04
6,0E+04
5,0E+04
4,0E+04
3,0E+04
2,0E+04
1,0E+04
0
1
2
3
4
5
6
7
8
9
Days


CA - 0.1 µg/mL
Abundance (area)
1,60E+05
1,40E+05
1,20E+05
1,00E+05
8,00E+04
6,00E+04
4,00E+04
2,00E+04
0,00E+00
0
1
2
3
4
5
6
7
8
9
Days
Amber -80
White -80
Amber-25
White -25
Amber refrig
White refrig
Limit 10%
Initial values
Limit 30%


COH -3.5 µg/mL
Abundance (area)
1,2E+06
1,0E+06
8,0E+05
6,0E+05
4,0E+05
2,0E+05
0,0E+00
0 1 2 3 4 5 6 7 8 9
Days


CA - 2.5 µg/mL
Abundance (area)
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
0 1 2 3 4 5 6 7 8 9
Days
Amber -80
White -80
Amber-25
White -25
Amber refrig
White refrig
Limit 10%
Initial values
Limit 30%


COH - 28 µg/mL
Abundance (area)
3,5E+06
3,3E+06
3,1E+06
2,9E+06
2,7E+06
2,5E+06
2,3E+06
2,1E+06
1,9E+06
1,7E+06
1,5E+06
0 1 2 3 4 5 6 7 8 9
Days


CA - 20 µg/mL
Area (abundance)
1,10E+07
1,00E+07
9,00E+06
8,00E+06
7,00E+06
6,00E+06
5,00E+06
4,00E+06
3,00E+06
0 1 2 3 4 5 6 7 8 9
Days
Amber -80
White -80
Amber-25
White -25
Amber refrig
White refrig
Limit 10%
Initial values
Limit 30%

II. Stability solution at high concentration- mixture of PPHs GA, HOH, CAT, OLE, COH and CA

Amber container and storage at -80 °C. Results for GA and CAT were similar than for HOH, thus a single graph for HOH is shown.

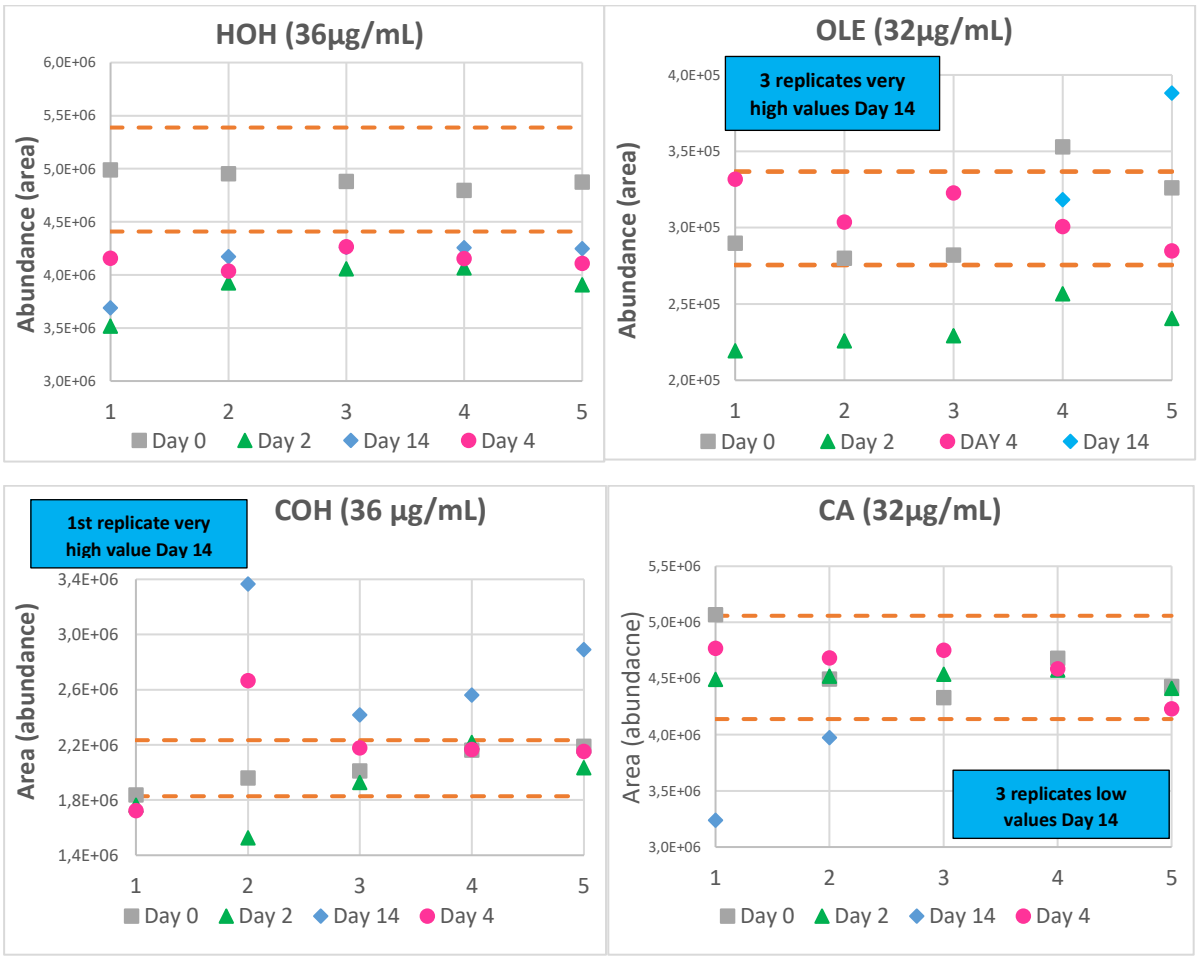
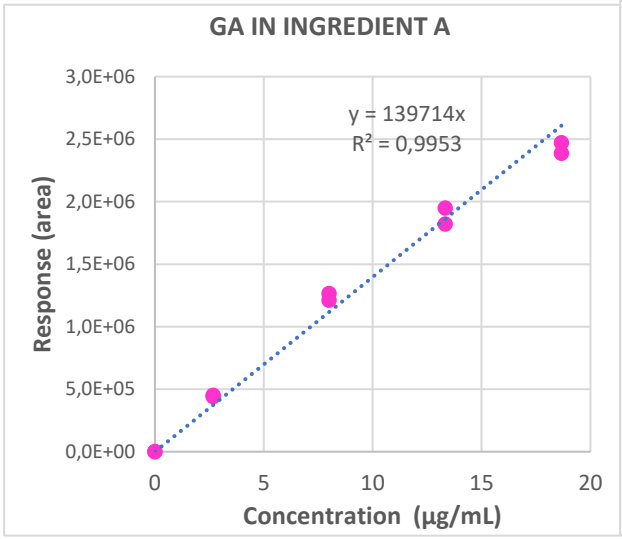
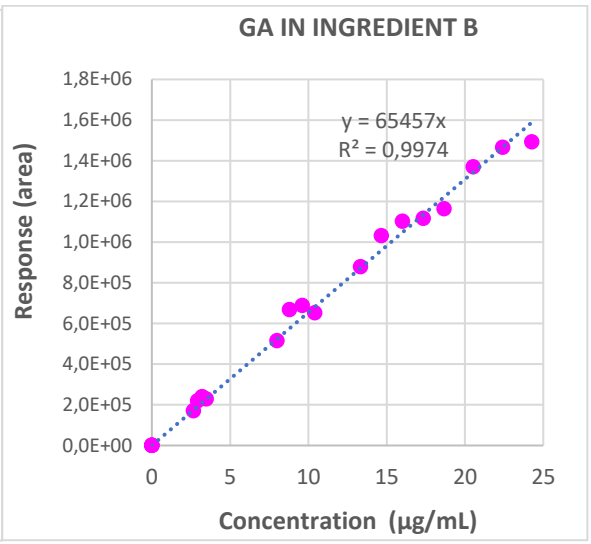


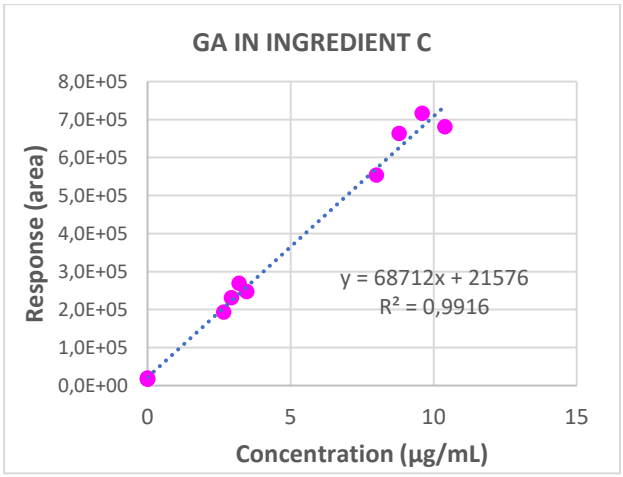
Figure 7.12. Stability analysis for PPHs in solution at high concentration store in amber container at -80 ºC. Replicate in horizontal axis. Extreme values are not shown but indicated in the blue squares.

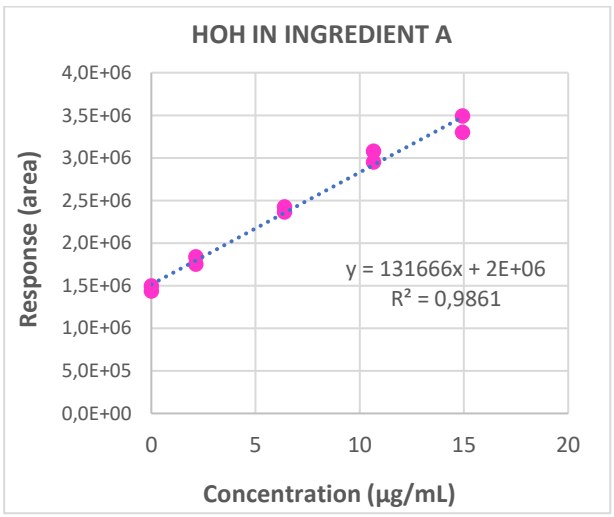
ANNEX II CALIBRATION CURVES FOR STANDARD ADDITION METHOD FOR THE ANALYSIS OF PPHS IN INGREDIENTS A, B AND C

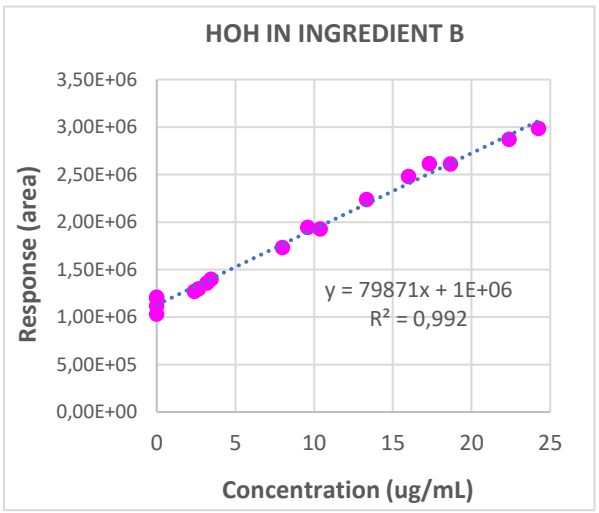
Antioxidant ingredient for fish feed (Commercial products)

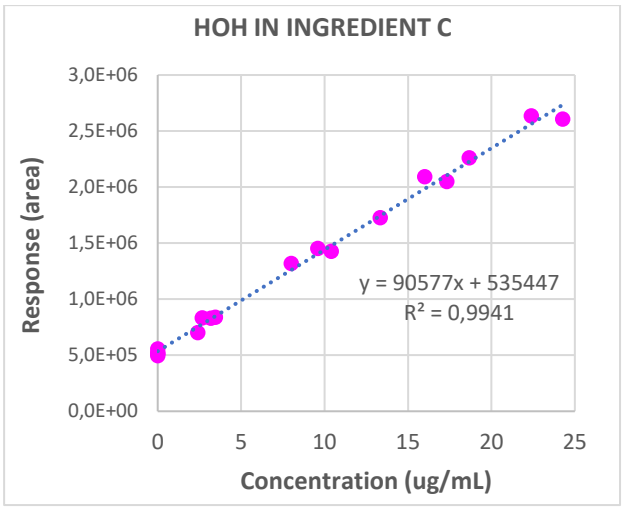


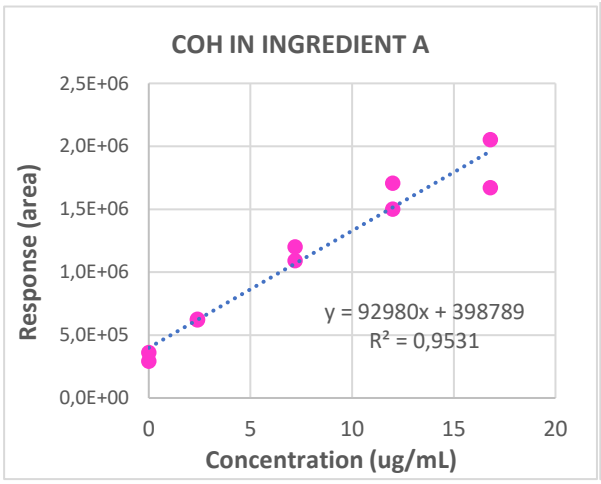


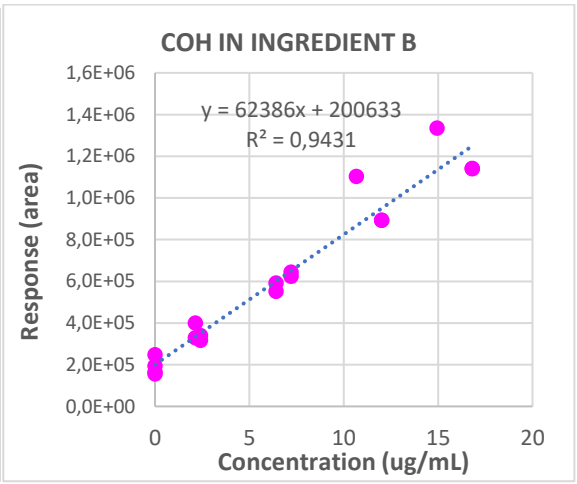


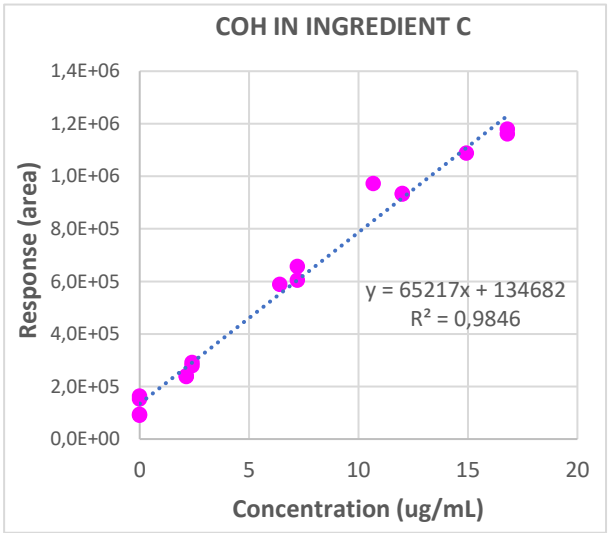


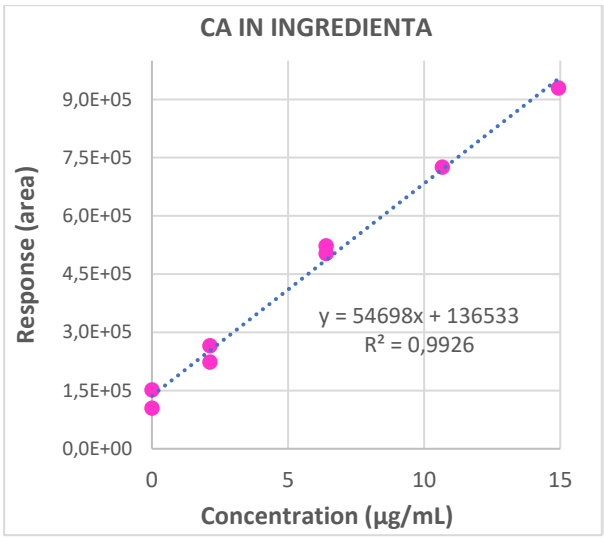


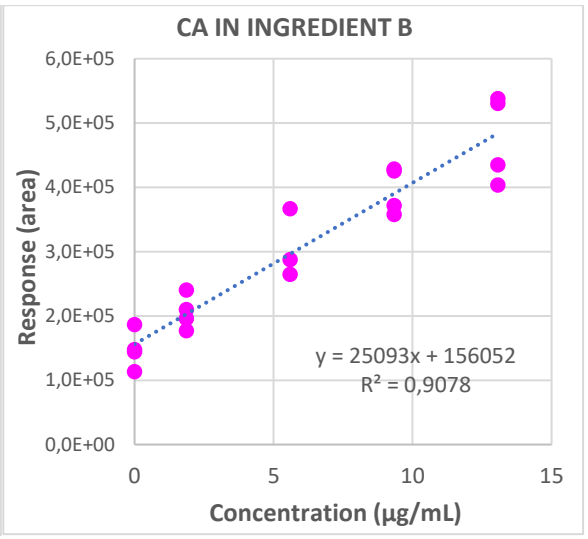

HOH IN INGREDIENT C
Response (area)
3,0E+06
2,5E+06
2,0E+06
1,5E+06
1,0E+06
5,0E+05
0,0E+00
0
5
10
15
20
25
Concentration (ug/mL)
y = 90577x + 535447
R² = 0,9941

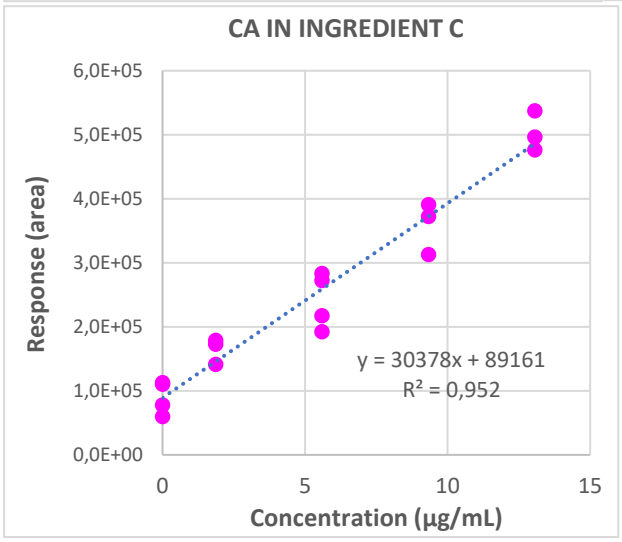

COH IN INGREDIENT A
Response (area)
2,5E+06
2,0E+06
1,5E+06
1,0E+06
5,0E+05
0,0E+00
0
5
10
15
20
Concentration (ug/mL)
y = 92980x + 398789
R² = 0,9531


COH IN INGREDIENT B
Response (area)
1,6E+06
1,4E+06
1,2E+06
1,0E+06
8,0E+05
6,0E+05
4,0E+05
2,0E+05
0,0E+00
0
5
10
15
20
Concentration (ug/mL)
y = 62386x + 200633
R² = 0,9431


COH IN INGREDIENT C
Response (area)
1,4E+06
1,2E+06
1,0E+06
8,0E+05
6,0E+05
4,0E+05
2,0E+05
0,0E+00
0
5
10
15
20
Concentration (ug/mL)
y = 65217x + 134682
R² = 0,9846

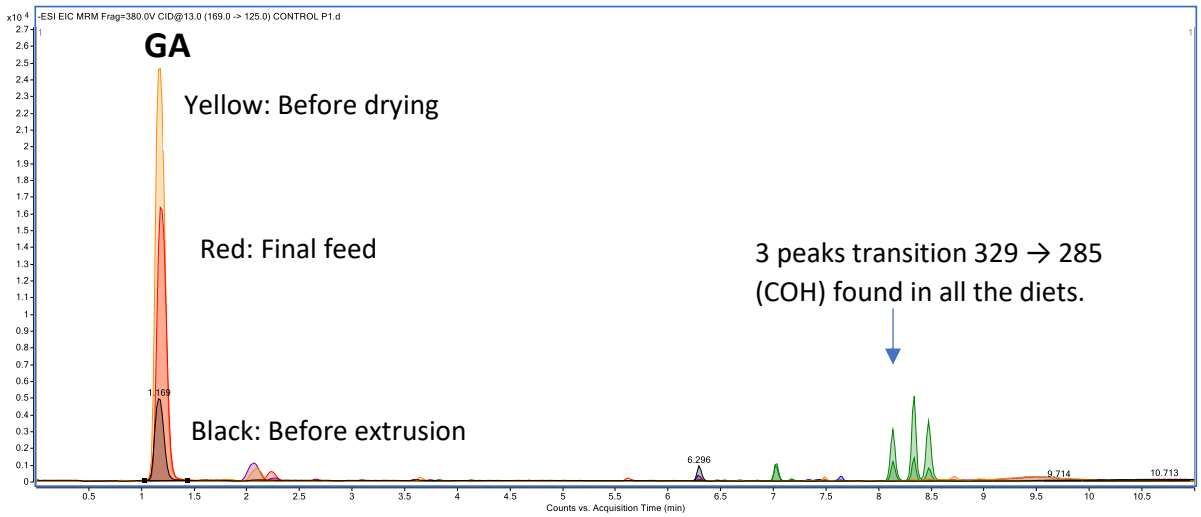

CA IN INGREDIENTA
Response (area)
9,0E+05
7,5E+05
6,0E+05
4,5E+05
3,0E+05
1,5E+05
0,0E+00
0
5
10
15
Concentration (µg/mL)
y = 54698x + 136533
R² = 0,9926


CA IN INGREDIENT B
Response (area)
6,0E+05
5,0E+05
4,0E+05
3,0E+05
2,0E+05
1,0E+05
0,0E+00
0
5
10
15
Concentration (µg/mL)
y = 25093x + 156052
R² = 0,9078

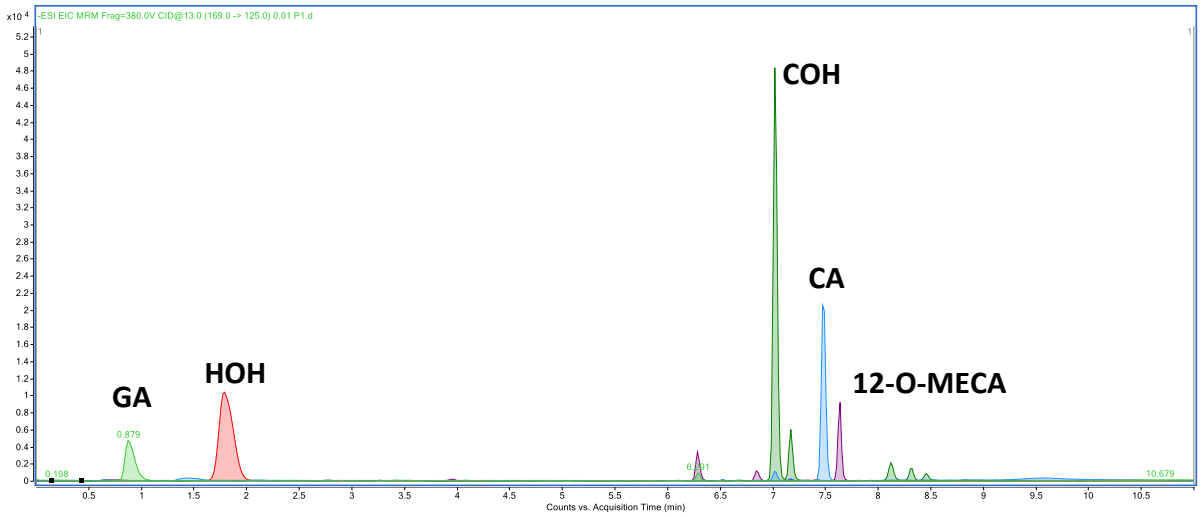

CA IN INGREDIENT C
Response (area)
6,0E+05
5,0E+05
4,0E+05
3,0E+05
2,0E+05
1,0E+05
0,0E+00
0
5
10
15
Concentration (µg/mL)
y = 30378x + 89161
R² = 0,952

ANNEX III CHROMATOGRAMS FOR ANALYSIS OF THE FISH FEED IN DIFFERENTS STAGES OF PRODUCTION PROCESS

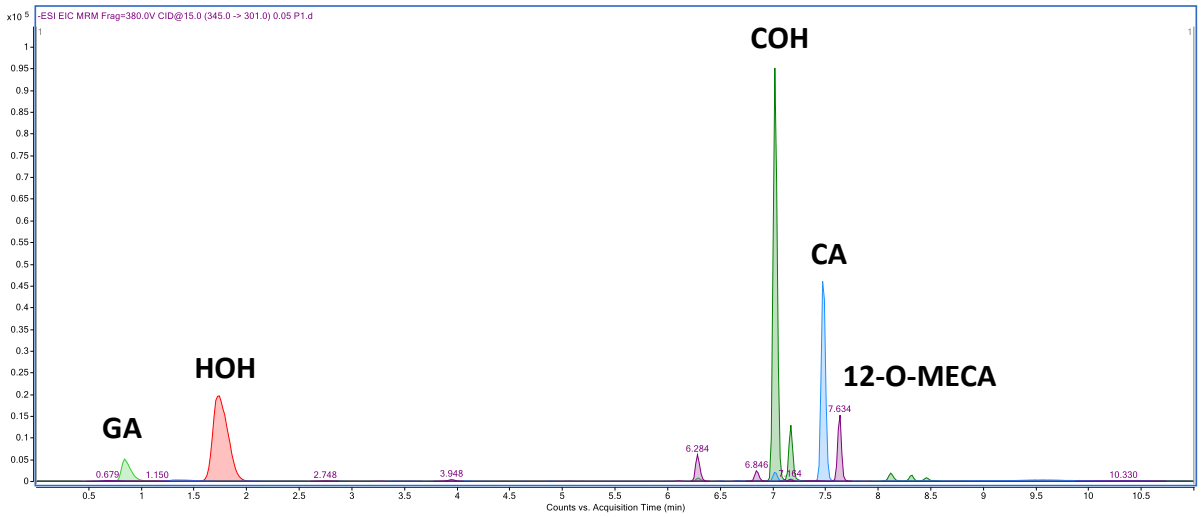
CONTROL DIETS: BEFORE EXTRUSION, BEFORE DRYING AND FINAL FEED



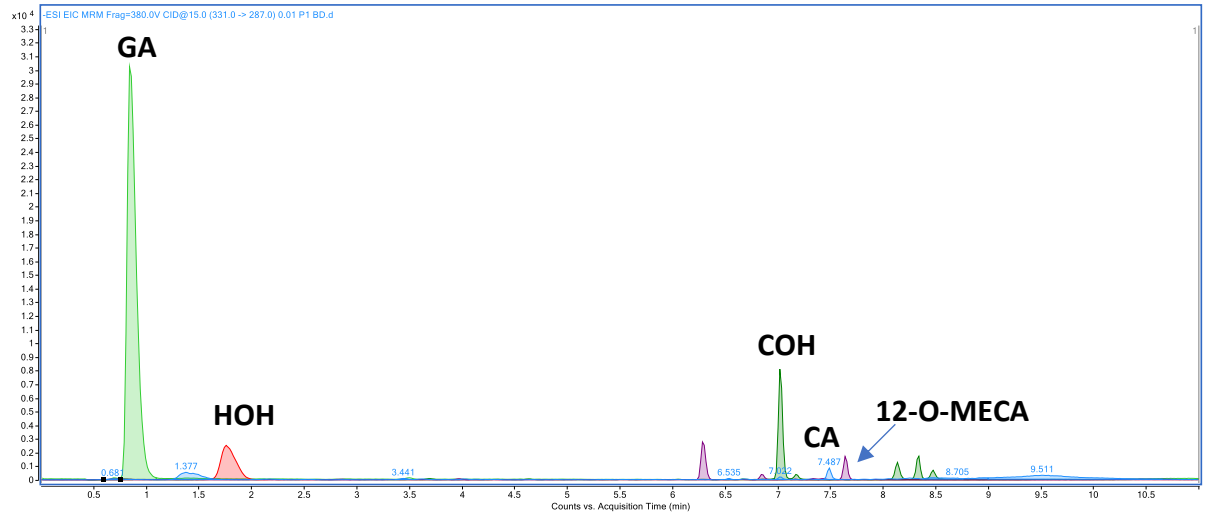
BEFORE EXTRUSION 0.01 % NAOX



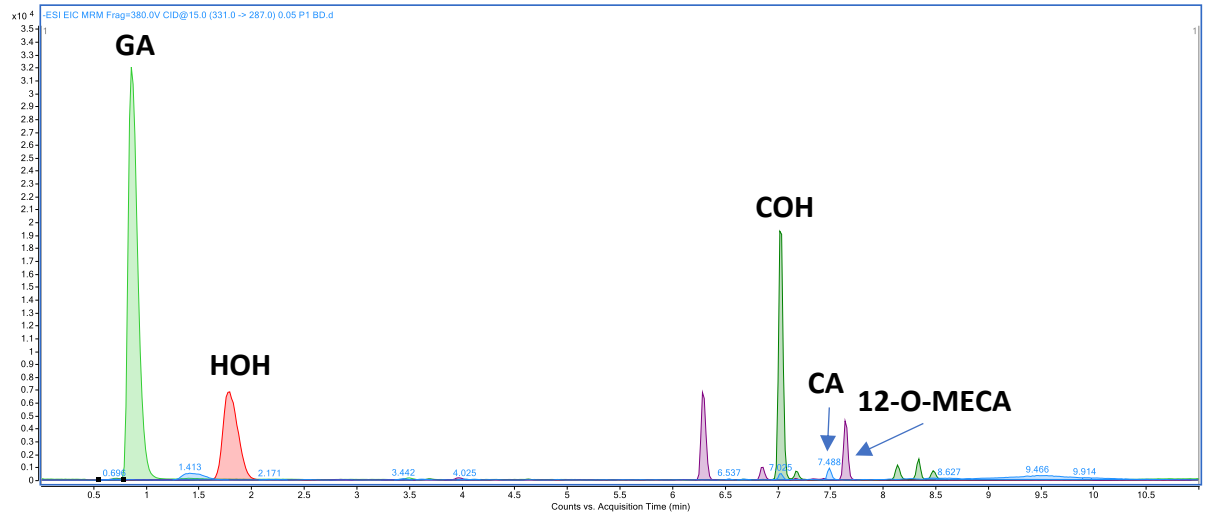
BEFORE EXTRUSION 0.05 % NAOX



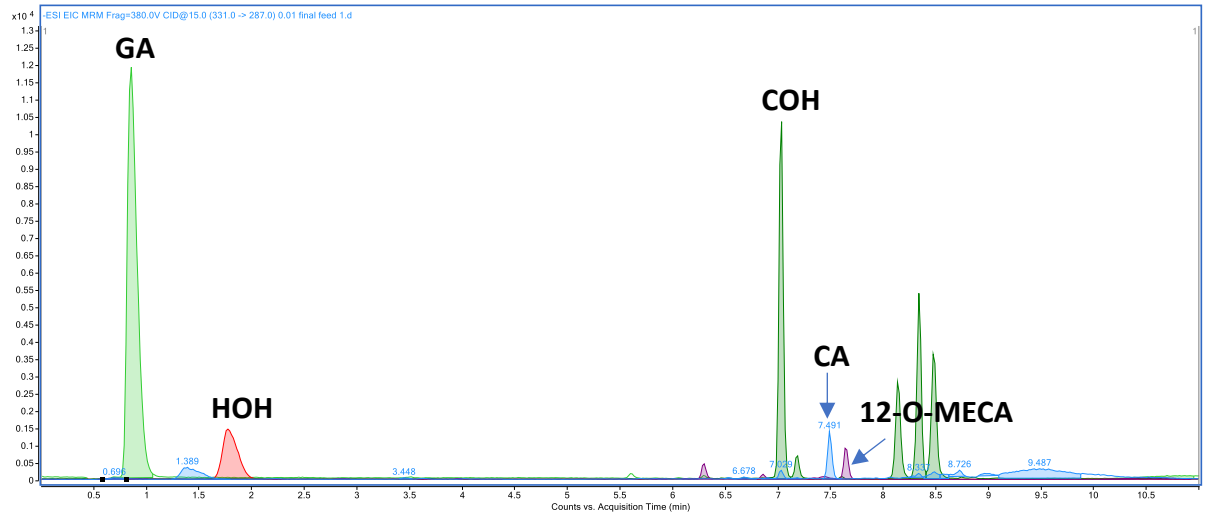
BEFORE DRYING 0.01%



BEFORE DRYING 0.05%



FINAL FEED 0.01 %



FINAL FEED 0.05 %