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PROFILES OF PARABENS AND TRICLOCARBAN IN RECIRCULATING AQUACULTURE SYSTEMS

An analytic method of development

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ABSTRACT

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Organic substances can be released from numerous sources such as feed, waste, bacteria and fish in a recirculating aquaculture system (RAS). Compounds can start accumulating in the system and affect the quality of water negatively due to the fact that the water is re-used continuously. In RAS systems, the input is water from natural sources, and it will be re-cycled through different treatment stations and instruments to control hygiene and diseases as well as biological pollution. This project analyzed feed and waste samples taken from a recirculating aquaculture system (RAS) in order to determine the presence of the target chemicals (six parabens, five parabens derivatives and antimicrobial).

The samples were collected at the Nofima Center for Recirculation in Aquaculture (NCRA) in Sunndalsøra, Norway. After that, samples were processed with numerous methods and techniques for extracting the target chemicals such as solid phase extraction (SPE) and liquid-liquid extraction (LLE). Then, the samples were analyzed by using liquid chromatography – tandem mass spectrometry (LC-MS/MS).

Key words

Parabens, Triclocarban, LC/MS-MS, TA, IS, matrix match, reagent blank, feed.

CONCEPT DEFINITIONS

EU	European Union
MM	Matrix match
RB	Reagent blank
SP	Spike sample
RAS	Recirculating Aquaculture Systems
LLE	Liquid – liquid extraction
SPE	Solid phase extraction
LC/MS-MS	Liquid Chromatography with tandem mass spectrometry
HPLC	High Performance Liquid Chromatography
Rpm	round per minute
ml	milliliter
mg	milligram
μL	microliter
MeP	Methyl paraben
EtP	Ethyl paraben
PrP	Propyl paraben
BuP	Butyl paraben
BezP	Benzyl paraben
HeP	Heptyl paraben
4-HB	4-hydrixybenzoic acid
3,4-DHB	3,4-dihydroxybenzoic acid
Vanillic acid	4-hydroxy-3-methoxybenzoic acid
OH-ETP	Ethyl protocatechuic acid
TCC	Triclocarban
MeOH	Methanol

ABSTRACT

CONCEPT DEFINITIONS

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1 INTRODUCTION

This study focuses on Recirculating Aquaculture systems (RAS) – a new alternative to traditional aquaculture systems with water quality management which allows to conceive optimal conditions for growing fish. In RAS systems, the input is a water from natural source, and it is recycled through different treatment systems in order to control hygiene and diseases as well as biological pollution. The application of recirculating aquaculture systems has not been universally approved due to various limitations such as accumulation of organic matter. This project concentrates on parabens and triclocarban, the group of chemical ordinarily used as additives in beverages, foodstuffs, cosmetics, personal care and several pharmaceuticals products. Parabens and triclocarban have been considered as contaminants because of some of their properties.

This bachelor's project analyzed feed and waste samples from a recirculation aqua system for presence of five parabens derivatives, six parabens and antimicrobial. The samples were collected at the Nofima Center for Recirculation in Aquaculture (NCRA) in Sunndalsøra, Norway. It houses seven experimental sections with water recycling for rearing the post-smolt of Atlantic salmo (Salmo salar).

A number of techniques and methods were used on the samples in order to extract target chemicals, namely solid phase extraction (SPE) and liquid-liquid extraction (LLE). Following this treatment, samples were analyzed with liquid chromatography – tandem mass spectrometry (LC-MS/MS).

2 THEORY

In the beginning of theory part, an overview of aquaculture and recirculating aquaculture system (RAS) is introduced, together with the properties of organic chemicals compounds, which are the target analytes (paraben, triclocarban, their microbials and derivatives) in this study project and the conceivable utilizes as well as effects of those compounds. Then, a concise description of analytical techniques and a brief sample preparation which were occupied in this study is presented. After that, qualification and quality assurance are provided in details description, and information about statistics processes and correlations as well as theory of the data transformation is introduced in the end.

2.1 Aquaculture

FAO (2015) currently defines aquaculture as "the organisms for farming of aquatic containing aquatic plants, crustaceans, fish and molluscs". In this definition, "farming" means some degree of interference into the rearing process in order to increase and improve the results. The interference can include feeding, protection from predators and stocking. Aquaculture was growing very rapidly during 1980s and 1990s, nowadays it is slowing down slightly, but it still is showing growth rates that are higher than other food production areas. During the period between 2000 and 2016 the average annual growth declined to 5.8 percent however in a small number of individual countries the growth was a double-digit number, particularly between 2006 and 2010 in Africa. In 2016 other major producers were Indonesia, India, Viet Nam, Bangladesh, Norway and Egypt. The most often farmed aquatic plants are seaweeds, and microalgae are farmed as well but in much smaller volumes. In 2016 Indonesia and China were the major aquatic plants producers. Seaweeds and marine bivalves can remove waste, including waste from the fed species, and lower the nutrient load in the water, thus benefiting the environment. This is why they are sometimes called extractive species. In aquaculture development is encouraged to keep extractive species together with fed species in the same marine site. In 2016 extractive species production was 48.5 percent of a total aquaculture production in the world. (FAO 2018.)

Antibiotics are used in aquaculture primarily as prophylactic agents, for therapeutic purposes. There are a number of antibiotics, authorized for using in aquaculture such as florfenicol, sara-floxacin, premix, oxytetracycline, erythromycin sulphonamides potentiated with ormethoprim or trimethoprim (Serrano 2005). In 2008 antimicrobial drug usage in fish farming in aquaculture of Norway during the 2000 – 2005 period was researched (Grave, Hansen, Kruse, Bangen & Kristoffersen 2008). It demonstrated an increase in antibiotics usage from 2003 to 2006. The increase was attributed to using new fish species for farming in Norway (before Atlantic salmon and rainbow trout were used almost exclusively), especially Atlantic cod. Unfortunately, most of other countries do not have this kind of specific data. In the USA the aquaculture is not expected to contribute a significant percentage of the non-human use of antibiotics. The status of antibiotics usage in aquaculture in other countries is not clear (Grave el at. 2008.)

Recirculating Aquaculture Systems (RAS) has an important role on the future of aquacul-ture. This system incorporates usage of indoor tanks with a controlled environment to rear fish at high densities, contrary to the traditional methods of growing fish in raceways and open ponds outdoors. Recirculating systems are used to clean and filter the water, taken from the fish tanks, and then recycle it back to the tanks. Fish producers can get many important ben-efits out of using RAS instead of open pond culture. Including methods for maximizing produc-tion using only a limited amount of land and water, flexibility in production facilities placement near large markets, effective and fast disease control. Besides, almost complete control over the environmental conditions for maximizing production all year-round and convenient harvest-ing process, allowing to collect 99% of the produced culture also included. (FAO 2015.)

Sizes of RAS can vary from small systems with 50000 pounds per year production through me-dium, 500000 pounds per year systems and up to large scale systems that can yield more than 100000 pounds per year. It is possible to use these systems for a variety of purposes, such as eggs and fingerling sport fish production, growing ornamental fish for home aquariums or pro-duction of food fish. (FAO 2015.)

2.2 Recirculating Aquaculture Systems (RAS)

A recirculation system requires a constant water treatment for adding oxygen to keep the fish alive and removing the waste products excreted by the fish. A recirculation system is fairly straightforward. The water goes from the fish tank outlet into a mechanical filter, then into a biological filter, then excess carbon dioxide is removed, it is aerated and then the water flows back into the tank (FIGURE 1). Additional stages can be added, like ozone or UV light disinfection, heat exchanging, oxygenating with pure oxygen, pH regulation, denitrification, based on given requirements.

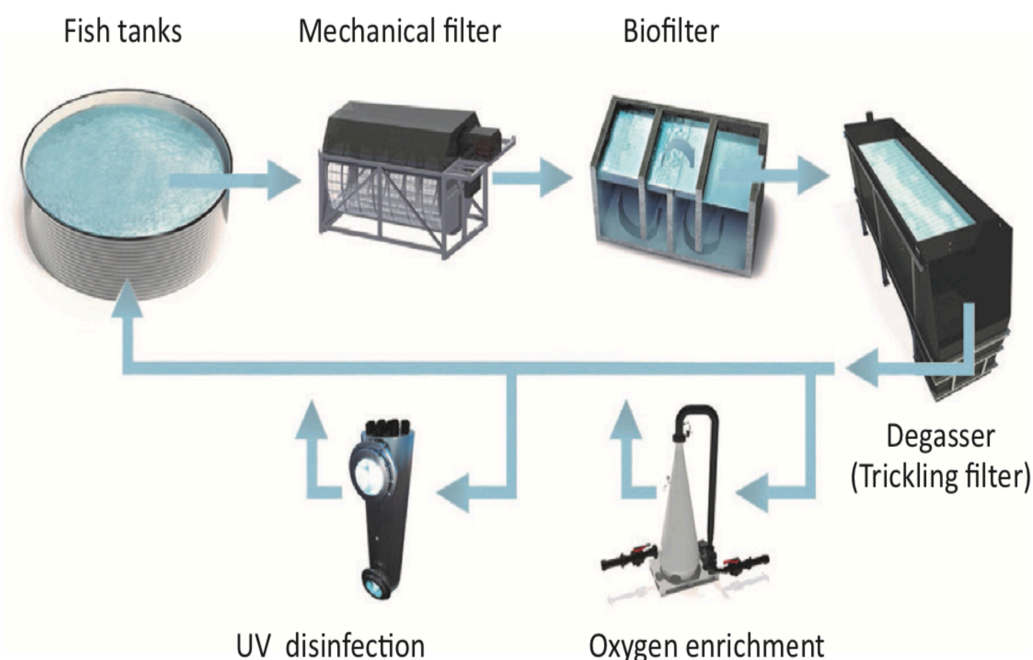


FIGURE 1. Principle of a recirculation system (FAO 2015).

On a fish farm fish needs to be fed multiple times a day. The fish eats and digests the feed, metabolises it and uses it for nourishment and energy supply for growth and other physiological needs. Oxygen (O2) is extracted by the gills and used for energy production and for breaking down the protein, resulting in carbon dioxide (CO2) and ammonia (NH3) being generated as waste products and excreted from the gills back into the water. Undigested feed is released

back into the water in form of faeces, organic matter and termed suspended solids (SS). So, the fish consume feed and oxygen and pollutes the water with ammonia, faeces and carbon dioxide. (FAO 2015.)

The only recommended feed type for a recirculation system is dry feed. Trash fish must not be used in any form because it will heavily pollute the system and is very likely to infect it with diseases. Dry feed is safe and can be designed specifically to meet biological needs of the given fish. Dry feed can have ingredients combined for fry, brood stock, grow-out, and it is distributed in different pellets sizes so it can be used for all stages of fish. (FAO 2015.)

High utilization rate of the feed is very advantageous in a recirculation system because it minimized the volume of excretion products and decrease the impact on the water treatment system. A professionally managed system has minimal amount of uneaten feed, which means that all the feed that was added will be consumed by the fish. Amount of feed per kilo of produced fish is called feed conversion rate (FCR). Higher feed conversion rate means the farmer secures higher production yield and the impact on the filtering system is smaller. There are special compositions of feed with maximized protein intake, so they minimize the ammonia excretion into the water and are especially good for recirculation systems. (FAO 2015.)

2.3 Organic chemicals

Parabens are para hydroxybenzoic acid esters, often used as preservatives in cosmetic, pharmaceutical and food products. The most common are methyl- and propyl-parabens. They have a wide range of activity against bacteria, yeasts and molds even at low concentrations. They usually have low degree of systemic toxicity and are stable chemically under physiological conditions. In the end of 1990s several reports were published about carcinogenic potential and estrogenic activity of parabens. Following it, FDA regulated maximum content of parabens in cosmetic products to 0.4% for a single ester and to 0.8% for parabens altogether. (Anjan Nan 2015.)

The six widely marketed para-hydroxybenzoic acid esters are: heptyl paraben, propyl paraben, butylparaben, benzyl paraben, methylparaben and methylparaben (FIGURE 2). Each paraben

ester is provided with a spectrum of antimicrobial activity and different solubility by the chemical substitutions. The oil solubility increases, and water solubility decreases with the increase of alkyl chain length (Lee & Kim 1994). Epidermis penetration of parabens increase at the same time as its lipid solubility increases. Thus, ester chain length causes the increase of penetration. (Twist & Zats 1986.)

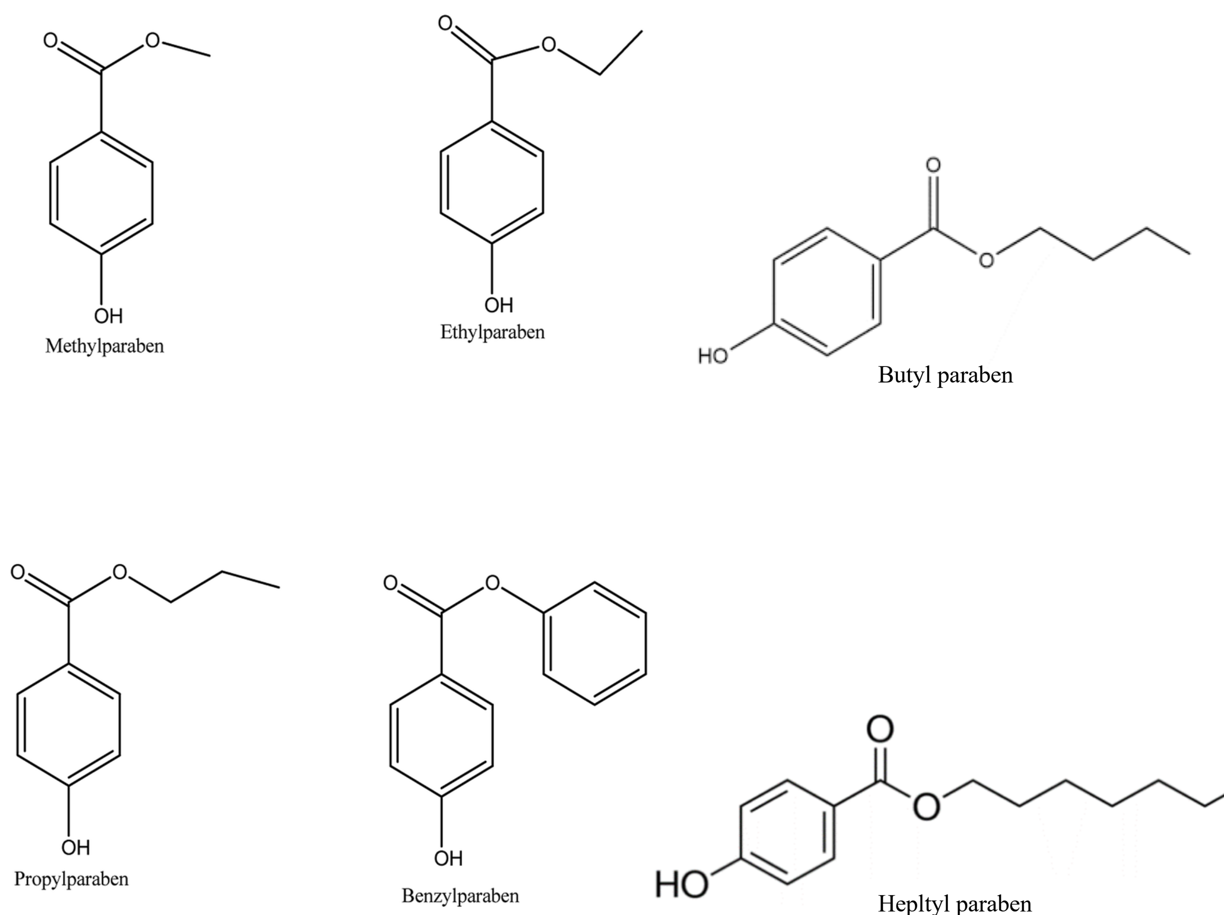


FIGURE 2. Parabens ring structure

Parabens are partially metabolized by four carboxyl esterases found in skin and subcutaneous fat when they are applied to skin. The parabens are hydrolyzed by these esterases to their respective side chains and para-hydroxybenzoic acid. Esterases residing in keratinocytes are more active against parabens with longer chains while carboxyl esterases residing in the sub-cutaneous fat are more active against parabens with shorter chains (Lobemeier, Tschoetschel, Westie & Heymann 1986). It was thought that parabens absorbed by the body are fully metab-olized esterases in the kidneys and liver and then excreted by the urine; however, recent stud-ies suggest that they are accumulated in the body. It is estimated that in the Unites States the average parabens exposure is about 76 mg per day for an individual (1.3 mg/kg/d for a person weighing 70 kg). The most significant part of the exposure (50 mg per day) comes from per-sonal products and cosmetics, the next significant part (25 mg per day) comes from drugs, and food is accountable for about 1 mg per day. Usually, food does not have parabens concentra-tions above 1%. In the past the typical products in the United States had parabens concentra-tion as high as 5% (Soni, Burdock, Taylor & Greenberg 2001). Nowadays most of the cosmet-ics products have parabens concentrations less than 0.3%, although local concentrations can be as high as 1% (Mowad 2000).

All parabens have higher bioavailability in oil-in-water preparations than in petrolatum. Relative distribution of the oil/water phases of formulation affects the potential for the percutaneous absorption of parabens, and it is affected also by the presence of various surfactants (Kon-matsu 1984). Parabens with shorter chains are more soluble in water and parabens with longer chains are more soluble in oil, although all parabens partition into the oil phase. Thus, methylparaben requires a higher concentration in oil-based formulations in order to maintain effectiveness (Han & Washington 2005). It was not investigated what kind of effect does vari-able concentrations of parental formulations have on the development of paraben hypersensi-tivity reactions.

This study also focuses on some chemicals that have structures similar to parabens. Ethyl-protocatechuic acid (OH-EtP), 3,4-dihydroxy benzoic acid (3,4-DHB, protocatechuic acid), 4-hydroxy-3methoxybenzoic acid (Vanillic acid) and 4hydroxybenzoic acid (4-HB) are included. Protocatechuic acid (3,4-dihydroxy benzoic acid) is a phenolic compound and it can be often found in natural products like plants, flowers and fruits. It is often used as an additive because it has antidiabetic, antioxidant and antiulcer applications. Also, there are reports of antiviral and

antibacterial activities of protocatechuic acid (Rasheeda, Bharathy & Nishad 2018). Vanillic acid (4-hydroxy-3methoxybenzoic acid) is also used in production of food and drugs as a an intermediate of vanillin and as flavoring agent. It has antioxidant, cardioprotective and antimicrobial properties and is a plant product (Antony, Fion, Kailas & Wasewar 2018).

2.4 Antimicrobials

Antimicrobials are a class of chemicals present in more than 2000 products including clothing, carpets, toys, soaps, detergents, toothpastes, paints and plastics. Triclosan (TCS) and Triclocarban (TCC), categorized as polychloro phenoxy phenols (FIGURE 3) are used in that role. Triclosan and triclocarban are sources if cancerogenic and toxic compounds like chloroform, chlorinated anilines and dioxins, and they persist in the environment. There are evidence suggesting that triclosan and triclocarban pose other hazards to the ecosystem and humans, so it is recommended to prevent future harm from them and antimicrobial substances with similar effects and properties as triclosan and triclocarban. Antimicrobials should be used only when evidence suggest that they will provide measurable health benefits, because of their wide and unintended environmental and health impacts. Before incorporating an antimicrobial into a product, a long and thorough investigation on ecological and health impacts should be conducted, and product formulations need to be transparent about antimicrobial components. (Halden 2014.)

The risk posed by triclosan and triclocarban have been recognized by several jurisdictions and these jurisdictions had taken several steps to restrict and lower triclosan and triclocarban use. After the Biocidal Products Committee of the European Chemicals Agency (ECHA) evaluated triclosan, the European Commission (EC) in 2016 decided that triclosan should not be used in human hygiene biocidal products anymore (ECHA 2015). Starting from February of 2017 such products in the EU will not include triclosan. Also, usage of triclosan in consumer products was banned by the state of Minnesota starting from January 2017 (State of Minnesota 2016). FDA issued a rule in September 2016, becoming effective in 2017, prohibiting marketing of over-the-counter consumer antiseptic wash products that have the antibacte-

rial active ingredients triclosan and triclocarban in them, because they “are not generally recognized as safe and effective”. The FDA controls the use of antimicrobials in medical devices and personal care products in the USA, and U.S EPA controls the use of antimicrobials for pesticidal purposes in other products (Johnson, Koustas Vesterinen, Sutton, Atchley, Kim, Campbell, Donald, Sen, Bero, Zeise & Woodruff 2016).

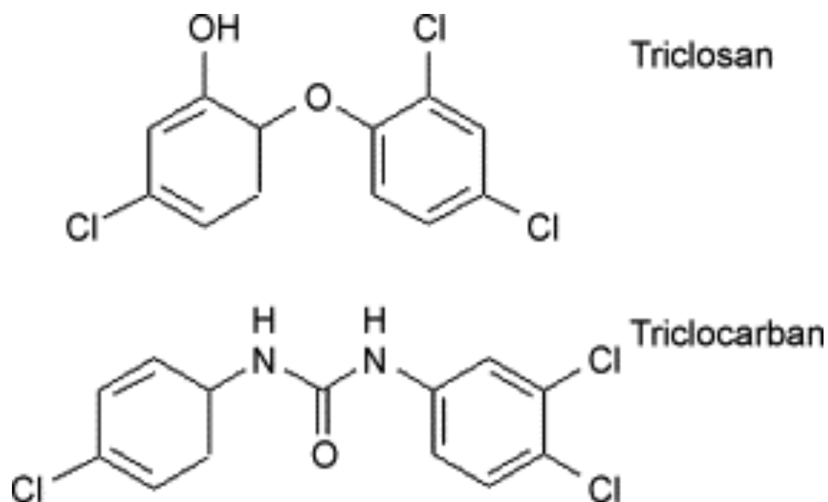


FIGURE 3. Chemical structure of triclosan and triclocarban

2.5 Liquid-Liquid extraction (LLE)

Liquid–liquid extraction (LLE) is a method of separating compounds or metal complexes. It exploits their relative solubilities in two different immiscible solvents, usually polar (water) and non-polar (organic solvent). It is also known as solvent extraction and partitioning (Henry & Yonker 2006). The LLE method is effective for removing waste solutions from the environment because the process itself can be performed without polluting air and water (given that the plant is designed properly).

LLE process consists of two steps – mixing and phase separation, so it is very important to balance them when designing the process. Too much mixing can produce emulsion and negatively affect the separation step. During the separation step the equilibrium is reached between the two phases when the chemical potential of the extractable solute gets to the same level. This can be used to define a “distribution coefficient” K as equation (1):

$$K = \frac{C\ organic}{C\ aqueous} \quad (1)$$

Where the $C_{organic}$ and $C_{aqueous}$ represent concentration for equilibrium in the two phases of the solute. (Zeki 2018)

2.6 Solid phase extraction (SPE)

Sloid phase extraction – SPE known as a well-established method utilized in many different purposes in sample preparation, such as analyte concentration, cleanup or analyte derivatization. Solid phase extraction works as the concentration and isolation of analytes or for the simplification of matrix samples with adverse properties for continuous analysis by an outstanding available approach. The extraction is accomplished by using a monolithic or a particular sorbent packed into a cartridges (a short length columns) or immobilized in the thin disc form, referred as a disk technology or disc. (Poole 2015.)

In the preparation of samples, they can come from various sources such as biological fluids, for instance saliva, plasma or urine. Another possible source is the environment, where samples can be taken from air, soil or water. Food products can be used as samples too, as well as pharmaceuticals, industry products or beverages. FIGURE 4 is an example of a solid phase extraction method.

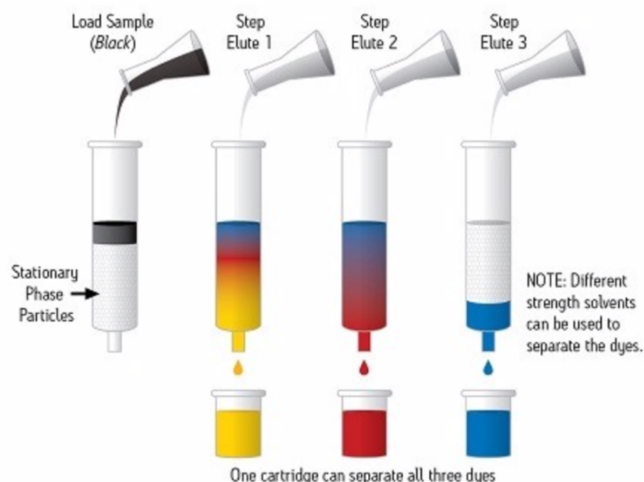


FIGURE 4. Solid phase extraction method example (waters 2020)

2.7 Analytical techniques

Liquid chromatography is a technique, widely used for substances separation. High performance liquid chromatography (HPLC) is a method, suitable for performing analysis in a wide range of application areas. In this part, a High Performance Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) method with electrospray ionization which focused on peak in dilution tandem mass spectrometry was used to determine environmental parabens in feed and waste samples from Nofima centre. (Meyer 2010.)

2.7.1 Principle of LC-MS/MS

HPLC coupled with a mass spectrometer (LC-MS) is a widely used technique due to its performance, automation and robustness (Lundanes, Reubsaet, & Greibrokk 2014). LC-MS is a combination with high sensitivity, and it provides a “fingerprint” of a particular eluent, which eliminates the necessity of relying on the retention time as in a conventional HPLC (Skoog 2003). The LC analysis system is entered around the chromatographic column, which performs

the actual separation (FIGURE 5). The essential step of analyzing compounds in a complex matrix is the ability to separate sample compounds using chromatography. Very important step in reducing or even avoiding background noise in the analysis is the efficient separation of different target analytes from matrix components and from each other. It also largely reduces the risks of false negative or false positive results (Kuster, de Alda & Barceló 2009). Separation of the compounds in the sample during HPLC is based on the difference in their polarities.

HPLC utilizes the distribution of the analyte (sample) between a stationary phase (packing material of the column) and a mobile phase (eluent) for the separation. The molecules are retarded when they are passing the stationary phase depending on the chemical structure of the analyte. The “on-column” time of the molecules of the sample is defined by the specific intermolecular interactions between them and the packing material of the column. Thus, different sample constituents are eluted at different times, and this is how sample ingredients separation is achieved. The analyte is recognized after leaving the column by a detection unit (e.g. UV detector). Data management system (computer software) converts and records the signals and shows them in a chromatogram. After the mobile phase passes the detector unit it can be subjected to additional detector units, a fraction collection unit or to the waste. Generally, HPLC system consists of the following modules: a pump, a solvent reservoir, a column, an injection valve, a data processing unit and a detector unit (FIGURE 5). The pump delivers the solvent (eluent) at constant speed and high pressure through the system. A constant and pulseless flow from the pump is crucial for keeping the drift and noise of the detector signal as low as possible. The injection valve is used to provide the analyte (sample) to the eluent. (Böcker 1997.)

Typically, an LC column is 15 – 25 cm in length with 2 – 5 mm internal diameter (Lundanes et al. 2014). The most common packing material for the LC tube is small silica particles, 3 to 10 µm in diameter. Different interactions between the components in the sample and the stationary phase in the column result in different components migrating through the system at different speeds and eluting from the column at different retention times. The retention time of a compound is the time between the moment of injection of the compound until the moment of its detection. Therefore, it represents the time the analyte is in stationary and in the mobile phases. The retention time is specific to a given substance and under the same conditions should always be the same. The acidic condition for the mobile phase is the most effective way

to improve the peak shape in chromatography with mixture of acetonitrile-water or methanol-water with gradient elution. A powerful attempt to improve the sensitivity of MS detection is adding the formic acid, acetic acid or ammonium acetate into the mobile phase to get modification. (Ramos & Fernando 2013.)

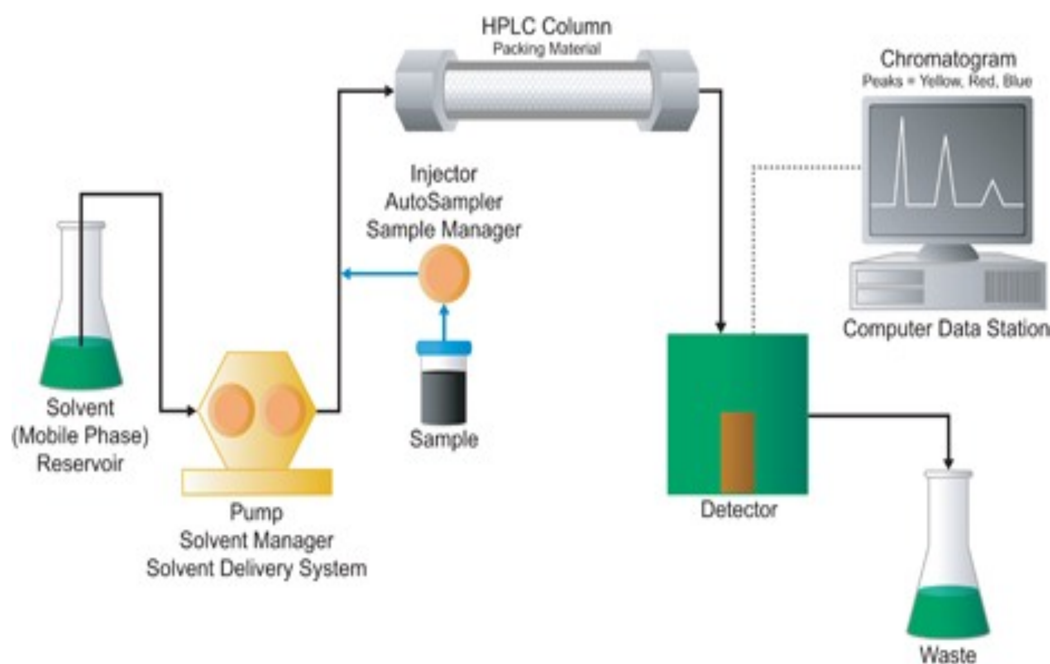


FIGURE 5. Schematic diagram of the HPLC systems (waters 2020)

A tandem mass spectrometer (triple quadrupole) was used in this study for the detection and quantification of the target analytes. Molecular weight and retention times information from the total ion chromatogram of a mass spectrometer are often insufficient for complete identification of all the compounds present in the chromatogram of the effluents. LC-MS/MS system with collision-induced fragmentation of the molecular ion to produce daughter ions can provide higher sensitivity than a mass range scan. Daughter ions fragmentation is caused by fragment-induced cleavage and rearrangements resulting in the loss of neutral molecules. Daughter ions structures can be estimated by examining isotopic patterns and mass intervals between the product ions. (McMaster 2005.)

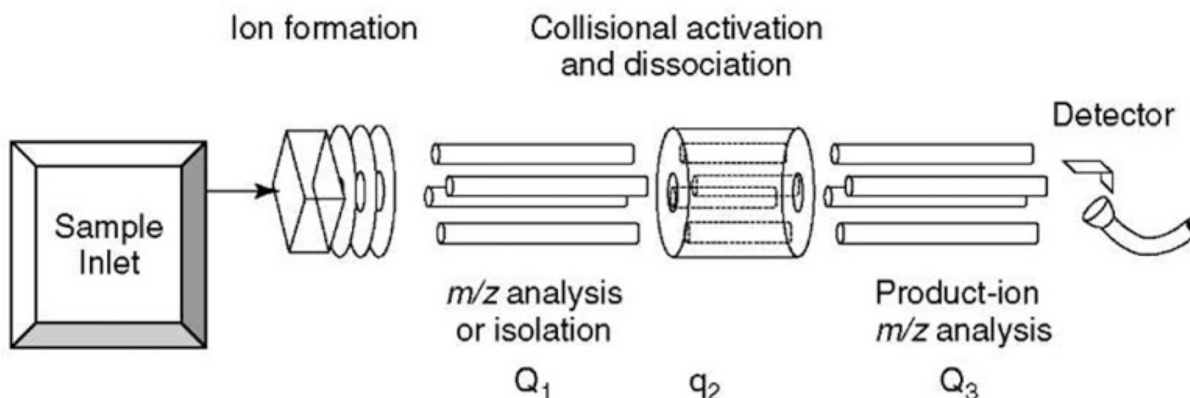


FIGURE 6. Schematic diagram of triple- quadrupole mass spectrometer (McMaster 2005)

The triple-quad LC-MS/MS system (FIGURE 6) was designed for cleaving ions into their daughter ions. The system comprises a scanning quadrupole analyser Q1 for separation of the original precursor ion(s), an un-scanned quadrupole Q2 that serves as a collision cell for fragmenting the ions sent there by collisions with heavy gas molecules and a scanning quadrupole Q3 which separates the fragments produced by the Q2 unit. The first unit (Q1) can be operated in a full-scan or SIM mode for selecting the ions that would be passed to the other analyzers. The second quadrupole (Q2) is fluted with a heavy inert gas, xenon or krypton, and the fragmentation is induced when ions from Q1 pass to Q2 and collide with the heavy inert gas molecules. The last unit (Q3) can be run in either SIM mode or full-scan mode also. (McMaster 2005.)

There are four possible combinations of Q1 and Q3 analysers operation modes: Q1 scan / Q3 SIM (daughter mode / precursor scanning), Q1 SIM / Q3 scan (parent mode / product scanning), Q1 scan / Q3 scan (neutral loss scanning mode) and Q1 SIM / Q3 SIM (multiple reaction monitoring (MRM) mode). (McMaster 2005.)

The scan / SIM mode allows to see which primary fragments are related with each other. Q1 is scanned over the mass range and all the fragments produced enter the collision unit Q2

for forming secondary fragments. The Q3 is set at a given mass / charge position so that only primary fragments broken down to a specific m/z value will be detected. The common daughter ion reveals interrelated primary fragments and simplifies understanding which fragments are formed when a large primary fragment breaks down. (McMaster 2005.)

2.7.2 Electrospray ionization

One of the most widespread interfaces in LC-MS is an electrospray ionization (ESI), and it is suitable for many applications. ESI is performed under atmospheric pressure and is usually chosen when compounds have polar groups. Neutral components can donate or accept a proton to yield negative or positive ions under given conditions. This occurs either during the ESI process or in the mobile phase, (Lundanes et al. 2014). The ionization process happens in the mobile phase for bases and acids via pH adjustments.

During the ESI process the mobile phase with the analytes goes into a capillary where a high voltage is applied (usually -5 or +5 kV). At the capillary outlet a nebulizing gas (usually N2) is mixed with the mobile phase in order to facilitate the formation of droplets. In the opposite direction of the flow a dry gas is introduced. The drops disintegrate into smaller droplets when the repulsive forces inside them exceed the surface tension. Detection of the deprotonated ions can be performed in a negative detection mode, and detection of the protonated ions can be performed in a positive detection mode (Lundanes et al. 2014).

2.8 Quantitation and Quality Assurance

The quantification methods incorporated in HPLC are mostly based on the gas chromatography methods. The quantitation process involves the measurement of a peak area or a peak height. The concentration of a compound can be determined by plotting the peak area or height versus the concentration of the substance. In this part, an overview of quantitation and quality assurance was carried out, which included the definitions of retention time, relative retention time, matrix effect and internal standard method. (Harris 1995.)

2.8.1 Retention time (RT) and relative retention time (RRT)

Retention time (RT) shows how much time does it take for a solute to go through a chromatography column, from the moment of injection until detection. Retention time of a compound is affected by many factors, such as column length, column degradation, the difference between oven and column temperatures, flow rate of the gas, and thus it can fluctuate even when using the same column during several days (Le Maux, Nongonierma & FitzGerald 2015). This problem can be solved by using not the absolute retention time of a compound but its relation to a standard retention time. It is called relative retention time (RRT), and it shows the ratio between an internal standard and the retention time of the sample analyte by using the Retention sample divided into the Retention time of internal standard. (Verbeken, Suleman, Baert, Vangheluwe, Van Dorpe, Burvenich & De Spiegeleer 2211.)

2.8.2 Matrix effect

While LC-MS is among the top analytical techniques in terms of sensitivity and selectivity it still can often suffer from matrix effects. This is the reason why it is so important to evaluate matrix effects, especially when LC-MS is used for analysis. The most common cause for matrix effects is the alternation of ionization efficiency of target analytes when co-eluting compounds are presented in the same matrix. Matrix effects can cause ion suppression (a loss in response) as well as ion enhancement (an increase in response), and both of them do affect accuracy and reproducibility of the method. (Harris 1995.)

Matrix effects can be dealt with using isotope labelled internal standard (Van De Steene, Jet, Willy & Lambert 2008). Matrix factor (MF) shows how the analytical signal of the main analyte is affected by the other compounds in the matrix . Where $\mathrm{Area}_{\mathrm{Post-ext-spike}}$ - the area of the post-extraction spiked sample divided into $\mathrm{Area}_{\mathrm{IS}}$ - the area of analyte in the standard solution (in solvent matrix) corresponding with same concentration as spiked sample (Silvestro, Luigi, Isabela & Rizea 2013). The matrix effect percentage (ME%) can be calculated by equation (12) (Asimakopoulos, Thomaidis & Kannan 2014).

$$\mathrm{ME\%} = (\mathrm{MF} - 1) \times 100\%$$

2.8.3 Internal standard method

The internal standard method (IS) consists of adding a predefined amount of the IS to calibrators and unknown samples, and then perform the calibration based on the ratio of response between the analyte and the IS instead of basing it on the absolute values of analyte response. In situations where volume errors are difficult to control and predict, using the internal standard method can improve the accuracy and precision of the results. (Asimakopoulos, Alexandros, Madhavan Elangovan & Kurunthachalam 2016.)

The internal standard is a compound, picked so that it is very similar to the interested chemical species of the sample, but does not correspond to them. By comparing the signal from the analyte with the signal from the internal standard the amount of the analyte in the sample can be estimated because the amount of internal standard added to the sample is well known (Asimakopoulos el at. 2016). This technique can be very useful when signal can vary between runs, and it allows to compensate for any spilled actions while preparing the sample (Harris 2006).

3 METHOD AND EXPERIMENT

A total of 12 feed and 8 waste samples were collected at Nofima Center for Recirculation in Aquaculture (NCRA) through 2019. Samples of waste were collected in different tubing and were stored at -20 Celsius degrees. Samples were also categorized according to the time of their collection for accurate data in the final result.

3.1 Chemicals and materials

Analytical standards of EtP (99%), MeP, BezP (≧98%), HeP, BuP (≧99%), PrP (≧99%), 3,4-DHB (≧97%), 4HB (99%), OH-EtP (97%) and Vanillic acid (97%), as well as TCC (99%) were prepared and obtained from Sigma-Aldrich. Internal standard of mix solution of Paraben contained $^{13}C_6$ – BuP, $^{13}C_6$ – MeP, $^{13}C_6$ – PrP and $^{13}C_6$ – EtP was purchased from the same place. Ethyl acetate, ammonium acetate as well as MeOH were used and purchased from Sigma – Aldrich. Besides, Milli – Q water was purified by distributed system of Millipore water from Merck Millipore in the US.

3.2 Internal standard (IS)

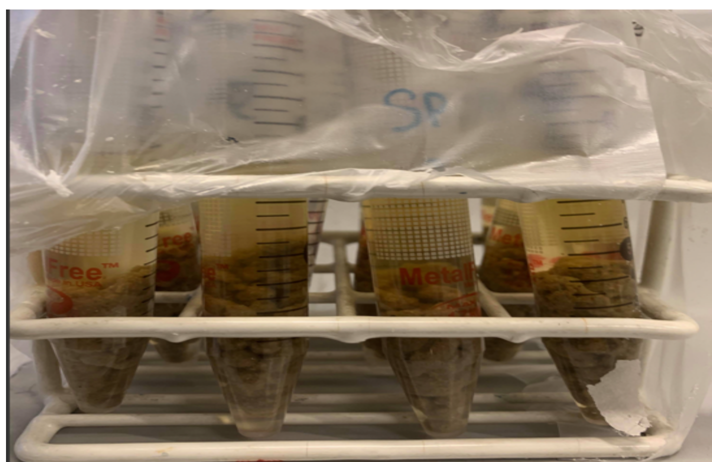
For spiking, paraben internal standard mix solution was used. The stock solution was 10 ppm, and to prepare 1 ppm solution, 100 µL of the stock solution was transferred to a glass vial (for LC) by an Eppendorf pipette mixing with 900 µL of MeOH. 100 µL of 10 ppm paraben, 20 µL of 50 ppm perfluorooctanoic acid (PFOA) and 20 µL of 50 ppm perflouroctane sulfonate (PFOS) were prepared and mixed together in 2 ml vial. Then 860 ml of distilled water was added into the vial so that the total of four substances is equal to 1000 µL.

3.3 Target Analyte

All 12 compounds of parabens, 14 compounds of bisphenol and benzophenones and PFCs with 16 compounds were mixed into the vial, 10 µL of each. The resulting volume of all the compounds was 420 µL in total. 580 µL ml of distilled water were added into the vial after that to dilute solution, so that the total volume of all the substances is equal to 1000 µL. Two similar vials were prepared.

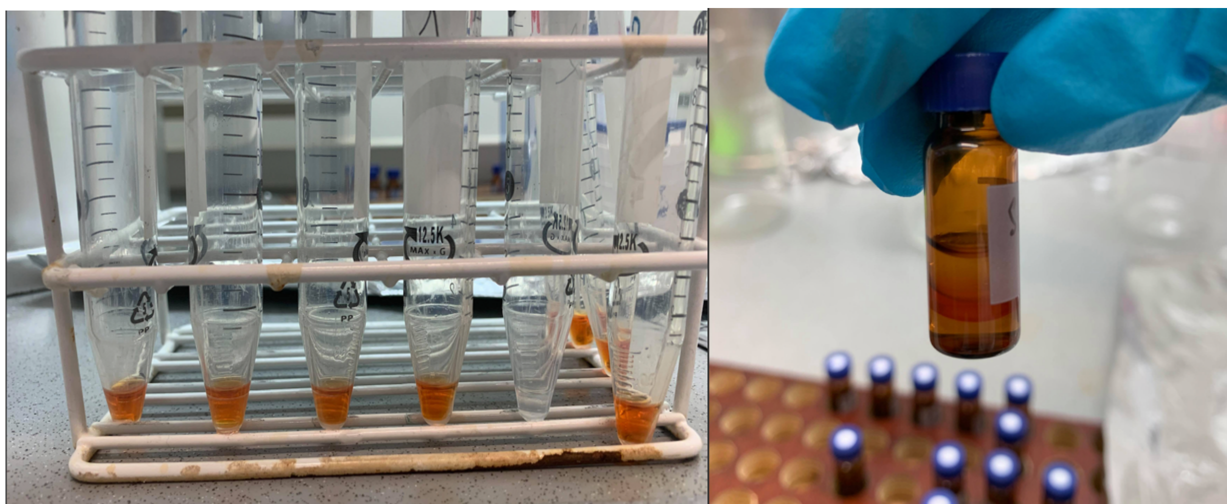
3.4 Liquid-liquid extraction method

The extraction method was developed prior to the beginning of the experiment. The parabens with their derivatives and antimicrobial were extracted from the fish feed using the liquid-liquid extraction method. In short, first approximately 1 g of the feed sample was weighted and transferred to a 15ml PP tube. After that about 3.58g of the solution of 1M ammonium acetate was added to the tube. Then a mixture of labeled IS (10 µL of 1 ppm IS solution, section 4.2.2.1) with a known concentration was spiked, then vortexed and then allowed to equilibrate. For spiking the samples ethyl acetate was used, 6mL were added and then shake using a mechanical shaker (KS501 digital, IKA). The shaking was performed for 60 minutes, then the PP tubes were put into a centrifuge set to 4000 rpm for 5 min as shown in PICTURE 1. Then the supernatant was moved into another 15mL PP tube.



PICTURE 1. The feed after shaking

The extraction process described above was performed again with 6 mL of ethyl acetate, and the second supernatant was added to the PP tube with first supernatant, resulting in about 12 mL in total. In order to remove the salts 1 mL of Milli-Q water was added to the PP tube and shaken for 1 minutes. Following this the tube was centrifuged and the water removed. The PP tubes were stored for 24 hours at -20°C in the freezer for separating the lipid layer. The lipid phase was removed after centrifugation and the remaining solution (with the analytes) put in the TurboVap® Classic LV (Biotage) so that it is concentrated near dryness, showed in PICTURE 2 (left) . Following this step matrix match solution was added for matrix matching the PP tube. Finally, it was reconstituted using 1 mL 50:50 methanol and water mixture, removed the lipid phase and the result was put into vials for HPLC analysis, as showed in PICTURE 2 (right)



PICTURE 2. Dryness lipid phase and HPLC vial with solution

3.5 Solid phase extraction

The waste samples were collected in Nofima Center for Recirculation in Aquaculture (NCRA) in different tanks, so before starting the protocol, all samples needed to be

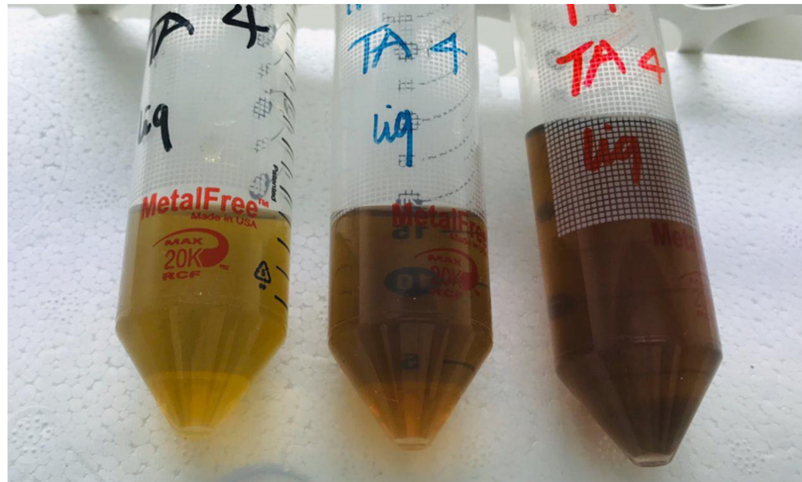
transferred into PP tubes with proper labels. The samples of disinfection with peracetic acid will be divided in three experimental periods in TABLE 1.

TABLE 1. Collected data of different experimental period

Date	***Experimental period***	***Tanks***	***Sample name***
23.09.19	Initial	Tank 1 (212)	PAA_initial_tank1
23.09.19	Initial	Tank 2 (213)	PAA_initial_tank2
23.09.19	Initial	Tank 3 (214)	PAA_initial_tank3
23.09.19	Initial	Tank 5 (215)	PAA_initial_tank4
23.10.19	Transition	Tank 1 (212)	PAA_transtion_tank1
23.10.19	Transition	Tank 2 (213)	PAA_transtion_tank2
23.10.19	Transition	Tank 3 (214)	PAA_transtion_tank3
23.10.19	Transition	Tank 5 (215)	PAA_transtion_tank4
05.11.19	Final	Tank 1 (212)	PAA_final_tank1
05.11.19	Final	Tank 2 (213)	PAA_final_tank2
05.11.19	Final	Tank 3 (214)	PAA_final_tank3
05.11.19	Final	Tank 5 (215)	PAA_final_tank4

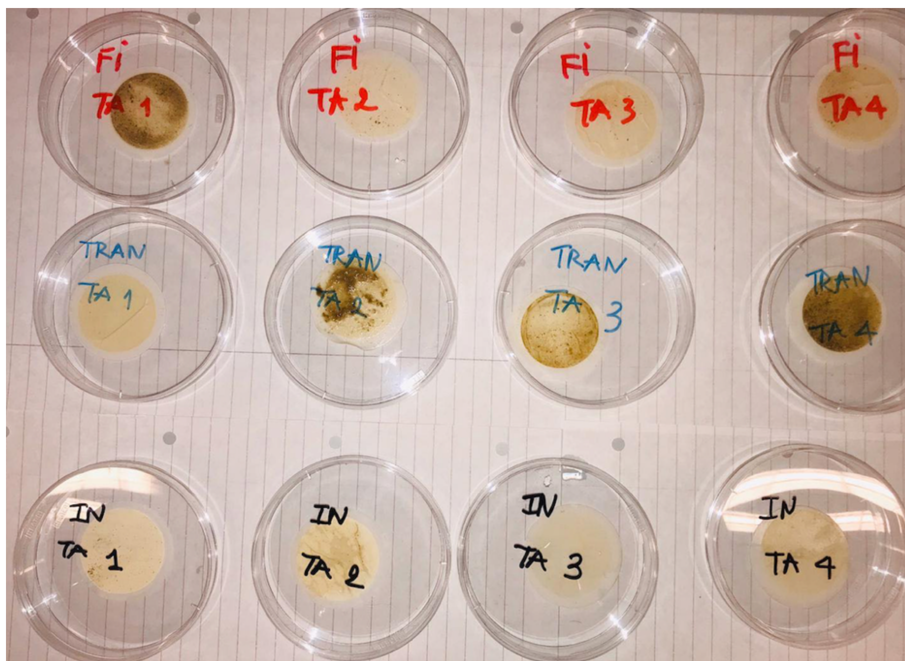
The process started with the first batch of samples, initial period. The samples were taken out of the freezer at the same time and left to defrost slowly (all the samples were treated consistently to avoid errors in the measurements) and then new PP tubes were labelled. Then the samples were weighted and transferred to the corresponding PP tubes. After that the samples were centrifuged in order to simplify the filtration process of supernatant. Next, 2 reagent blanks were included, centrifugated Milli-Q water and after that it was filtered as the samples. The next step was filtration of the sludge. An important part of it was ensuring the tweezers are always in MeOH to avoid samples contamination and cleaning them with a special tissue paper. After that Whatman membrane filters (Cellulose Acetate Circle WCA Range 0.2 µm) were weighted and placed into the filtration equipment with clean tweezers. Then the vacuum was turned on and the sludge filtration process started. The

flask was cleaned three times with Milli-Q water and dried with MeOH before filtering new samples in order to avoid the contamination. The filtered liquid was transferred to new PP tubes, and labelled properly as shown in PICTURE 3.



PICTURE 3. Filtered of sludge liquid

The used 0.2 µm filter was taken with tweezers, pre-cleaned in MeOH and transferred to a new properly labelled PP tube. Then it was weighted, and a name was added to it. The filtered liquid was then acidified with hydrochloric acid (HCl) to pH 2.5 $\pm$ 0.1 and keep in the freezer at -20 Celsius degrees for further analysis. To acidify samples a beaker was used placed there, and acid was added at the top. Then the acid was added drop by drop using a glass pipet, and the change of pH was checked with pH paper every time. The solid and filter sample were frozen at – 20 Celsius degrees and freeze dried. Then both the filter and samples were weighted. After that, each solid sample was ground into a fine powder separately using a mortar and pestle. Then the powdered solid in each sample was weighted. 200 mg of dried sludge was taken and stored at -20 Celsius until analysis as shown in PICTURE 4.



PICTURE 4. Solid dry of sludge sample

3.6 LC-MS/MS

Chromatographic separation was done with an Acquity HPLC Thermo system with a column manager, a flow thorough needle manager and binary solvent manager (Waters, Milford, USA). The LC column was Kinetex C18 column (2.1 mm × 50 mm, 1.3 mm; Phenomenex Inc., Torrance, CA, U.S.) connected to a Security Guard ULTRA C18 guard column C18 (2.1 mm × sub-2 mm, coreshell column; Phenomenex Inc.). Also, a tandem mass spectrometric system was used, ZSpray ESI (Waters, Milford, USA) with a Xevo TQ-S (triple quadrupole mass analyzer). Direct infusion and the IntelliStart software (Waters, Milford, USA) were used to determine the mass spectrometry parameters. The parent and fragment ions of each target chemicals can be found in the TABLE 2.

TABLE 2. Special Analyte of MS/MS parameters

Component	***Quantification transition(CE[a] [eV])***	***Confirmation transition (CE[a][eV])***	***Cone V (CV[b] [V])***
4 – HB	137 >93 (14)	-	30
MeP	151>92 (20)	136 (14)	36
PrP	179>92 (20)	136 (16)	28
Vanillic	167>152 (20)	108 (18)	20
BzP	227>92 (22)	136 (14)	14
EtP	165>92 (20)	137 (14)	38
HeP	235>92 (24)	136 (20)	20
OH – EtP	181>108 (22)	153 (14)	18
3,4 – DHB	153>109 (14)	-	30
BuP	193>92 (22)	137 (14)	46
TCC	313>160 (12)	126 (24)	8

Kinetex® C18 column was used for the chromatographic separation. Gradient elution program with water (acidified with 0.1 % v/v formic acid) and methanol as binary mobile phase mixture at a flow rate of 200 µL/min was used for the chromatographic analyses. The increase of the gradient elution, starting from 1 %(v/v) water is shown in TABLE 3.

TABLE 3. Gradient parameter

Time	*Flow*	*%A*	*%B*
Initial	0.2	85	15
3	0,2	1	99
3,1	0.2	1	99
3,4	0,2	1	99
3,6	0.2	85	15
4	0,2	85	15

+1.8 kV voltage was chosen for electrospray ionization, and the flow rate of the collision gas was 0.15 mL/min. The injection volume was set to 3 µL. The desolvation gas had a temperature of 350 °C and the source temperature was 150 °C. MassLynx and TargetLynx software packages (version 4.1 SCN871, Waters, Milford, USA) were used to acquire the data, and then it was processed in Microsoft Excel Microsoft Office, 2019.

4 RESULT OF PARABENS CONTENT DETERMINATION BY LC/MS-MS

This chapter shows the experiment results in the form of mean value and its standard deviation. Also, it mentions problems and phenomena observed in the experiments, as well as additionally includes presentation and explanation of the comparisons of different methods. The LC/MS-MS method can be used for detecting additional compounds or impurities that are soluble in the feed sample.

The chromatogram averaging process is straightforward, it involves transferring all the chromatographic data files to Microsoft Excel followed by the simple averaging of the data for each time point in order to produce a composite average chromatogram file. Then that composite average chromatogram file is saved in a format that allows it to be integrated and analyzed by a variety of chromatographic data processing software suits. LC/MS-MS method was used as a basis for the analysis of paraben and triclocarban, and the result is shown in TABLE 4. The Reagent Blank (RB), Areas of Spike Sample (SP), Retention time (RT) and Matrix match (MM) data was collected.

TABLE 4. Analysed results of Parabens and Triclocarban

	Areas			
	Retention time	**SP**	**MM**	**RB**
4-HB	1,20	396393,73	497562,84	656,07
MeP	1,61	302287,70	605211,50	426,53
EtP	2,0	102251,75	230602,70	51,46
PrP	2,0	340460,30	436200,10	171,72
Vanillic	1,63	286130,60	504045,30	291,77
Triclocarban	3,01	209464,76	499612,20	11,71
BezP	2,17	14169,62	43462,87	44351,08
OH-EtP	1,28	98921,50	119939,60	24,90
BuP	2,16	12408,57	65369,20	32,01
HeP	2,67	8592,36	31782,32	31622,04
3,4 - DHB	1,0	9116,69	34914,88	35771,08

TABLE 4 shows that the matrix match data always have high value because it was an important element in the evaluated LC/MS method. The data can have poor precision or become biased if these components co-elute with the subject analyte. Matrix matching is a process that helps to ensure that all the test samples, quality control (QC) samples and standards are in an identical matrix and the ion suppression is constant. At the same time the reagent blank (RB), used to analyze free matrix carried through the complete preparation and analyze procedure should usually hit the lowest point to ensure that the purity of the sample in the extraction project is not affected. This is because the reagent blank is used for evaluating the contamination introduced during the whole preparation and analysis process. In order to determine the recovery efficiency or for other quality control purposes, the sample can be spiked by adding a known mass of target analyte to identify the process. The higher is the value of spiked sample, the higher is the percentage of the compound, appearing in the samples. The retention time can be changed slightly compared to the standard retention time depending on the different extraction methods. FIGURE 7a and FIGURE 7b demonstrates data about the comparison of parabens and triclocarban in LC-MS/MS analytic.

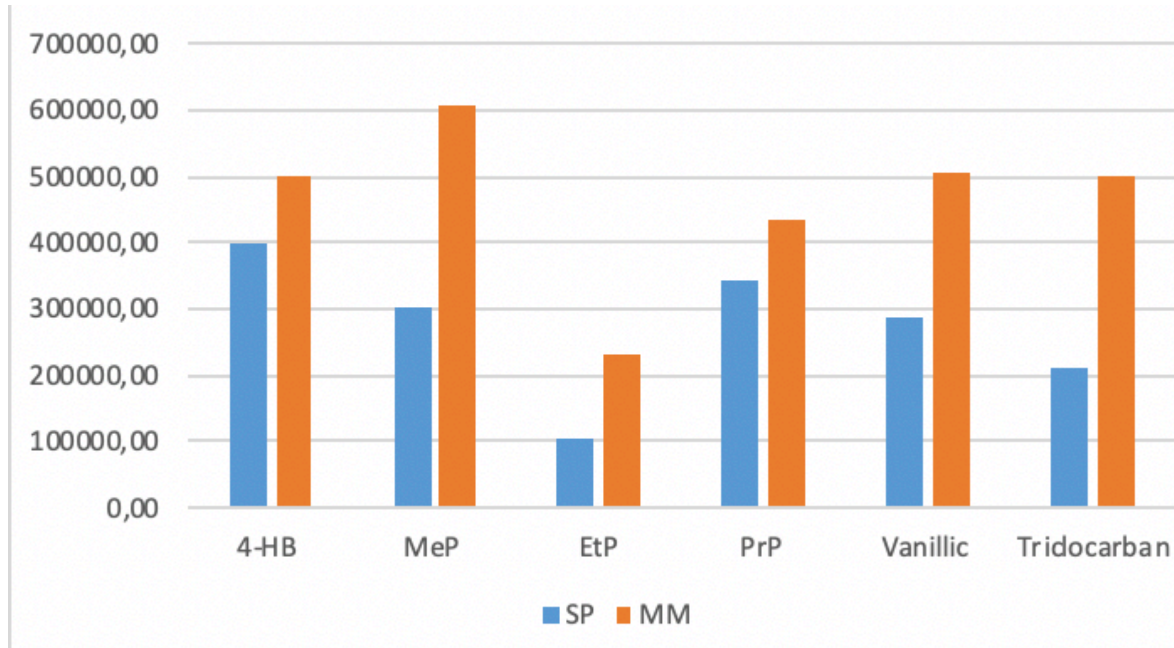


FIGURE 7a. Demonstrates the comparisons of parabens and triclocarban in LC-MS/MS analysis.

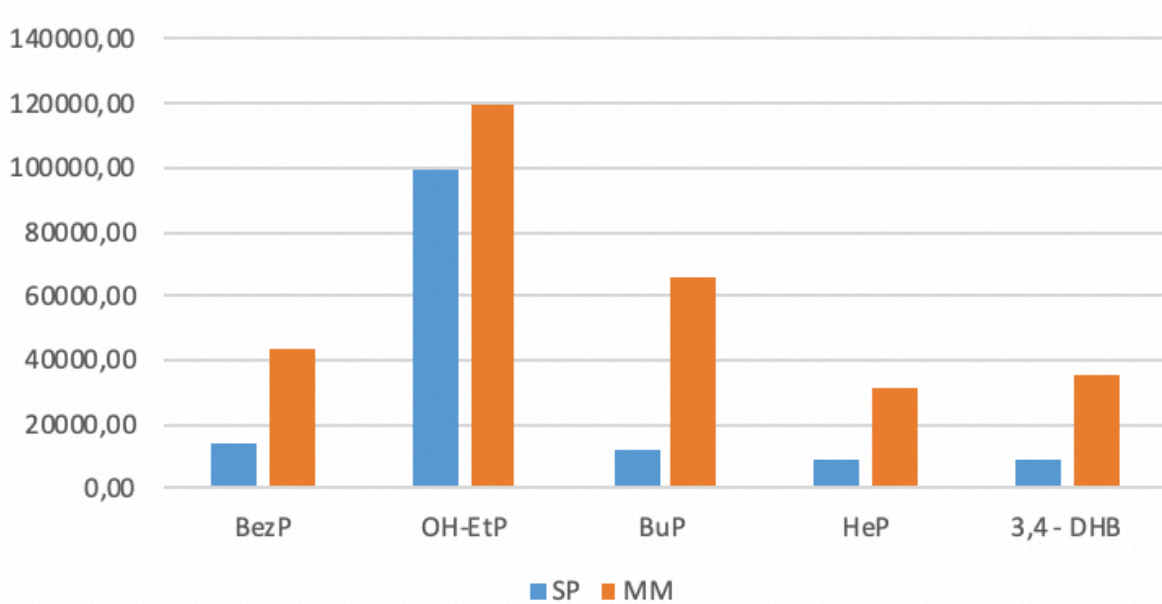


FIGURE 7b. Demonstrates the comparisons of parabens and triclocarban in LC-MS/MS analysis (continue).

PrP and 4-HB are the first and the second highest value among the parabens, EtP and OH-EtP also show high potential of parabens appearance when comparing the areas of matrix match. This contrasts with HeP, 3,4-DHB, BezP and BuP, which are in the very low areas while the triclocarban and vanillic is located relatively in the middle level. Parabens show significantly lower rate compared to the parabens derivatives, which shows that parabens derivatives are created by the parent parabens reacting chemically during the manufacturing of storage processes (Asimakopoulos et al. 2014). There is a possibility that alkyl protocatechuates found in the feed samples were formed from parent parabens by the photo-oxidation process.

The transformation of MeP to OH-EtP as a result of light-induced hydroxylation was studied, also, EtP and OH-EtP are considered to have the same kind of relationship. However, the mechanism of reaction transforming 4-HB and 3,4-DHB from parabens in fish food is still insufficient while the process of transformation 4-HB and 3,4-DHB from parabens in the organism was proved in animal and human studies (Aubert, Nicolas, Thibault, Ameller & Jean 2012). Parabens, including methyl paraben, have been used in the food industry over the course of more than 50 years due to their effective anti-microbial properties and low toxicity. The FDA regulation recognizes methyl paraben as generally safe (GRAS) when it is

used in a role of food preservative, with the usage limit set to 0.1%. So, it is reasonable to presume that the methyl paraben accounts for the large area of the distribution profiles.

One of the major problems in the paraben analysis method is the blank contamination. It is hard to achieve low detection levels because of the high background signals. Also, unstable blank levels can cause underestimations and overestimations in the analysis results. FIGURE 8 shows the problems of blank contamination of 3,4 DHB, BezP, BuP and HeP.

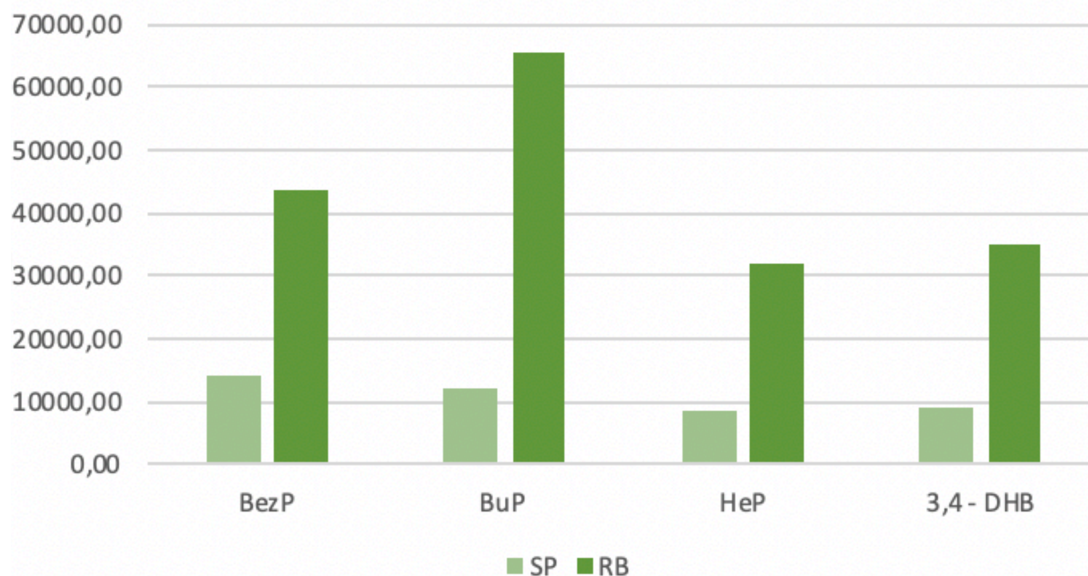


FIGURE 8. Blank contamination

High detection limits can be caused by the blank contamination. When the blanks are not constant, quantification problems can be caused by the subtraction. So, it is highly important to keep the blank contamination as low as possible, or at least below the critical value. In parabens analysis, there are multiple factors that contribute to the presence of blank contamination. The laboratory glassware should be cleaned without detergents, this helps to reduce the blank contamination problems. However, the contaminants can be found even in the air of a typical laboratory (Xie, Selzer, Ebinghaus, Caba, Ruck 2006) and there were reports of an LC-MS vial with fresh MeOH that was left in the auto- sampler of the instrument and within weeks it absorbed contamination from the laboratory air (Loos, Wollgast, Castro-Jimenez, Mariani, Huber, Locoro, Hanke, Umlauf, Bidoglio, Hohenblum, Moche, Weiss, Schmid, Leiendecker,

Ternes, Ortega, Hildebrandt, Barcelo, Lepom, Dimitrova, Nitcheva, Polesello, Valsecchi, Boutrup, Sortkjaer, Boer & Staeb 2008).

Blank contamination can be also caused by solvents used as mobile phases and blank vials (Wererniuk, Gerstmann, Frank & Sep 2006). Another important source of contamination can be mobile phase connectors and tubes (Lloyd Bailey, Hird, Routledge & Clarke 2009). Sampling, samples storage, and treatment are another sources of blanks contamination. The most widely used methods of extracting parabens in water samples are liquid-liquid extraction (LLE) (UNE-EN ISO 18857-1) and solid-phase extraction (SPE) (Martı´nez, Gans, Weber & Scharf 2004) but it can also cause contamination because the SPE cartridges are made of plastic (Loos el at. 2008), relatively high volumes of solvents and multiple processing steps. These days other techniques are replacing SPE, like and stir bar sorptive extraction (SBSE) (P. Richter, Leiva, Choque, Giordano & Sepulveda 2009), solid-phase microextraction (SPME) (Lopez-Darias, Pino, Meng, Anderson & Afonso 2010) and dispersive liquid liquid microextraction (DLLME). They have the advantage of simplified extraction process with fewer steps and minimalization of the organic solvents waste in accordance with the principles of Green Analytical Chemistry (Namiesnik & Sep 2001). This also results in reducing the sources of contamination.

The liquid-chromatography mass spectrometry (LC-MS) often produces chromatograms that are highly complex and have a ‘noisy’ appearance, especially when using an electrospray ionization source. Matrix match together with spike samples and unknown impurity peak collected from different controls were used to assess the selectivity. The selected samples are presented in a form of chromatogram in FIGURE 9.

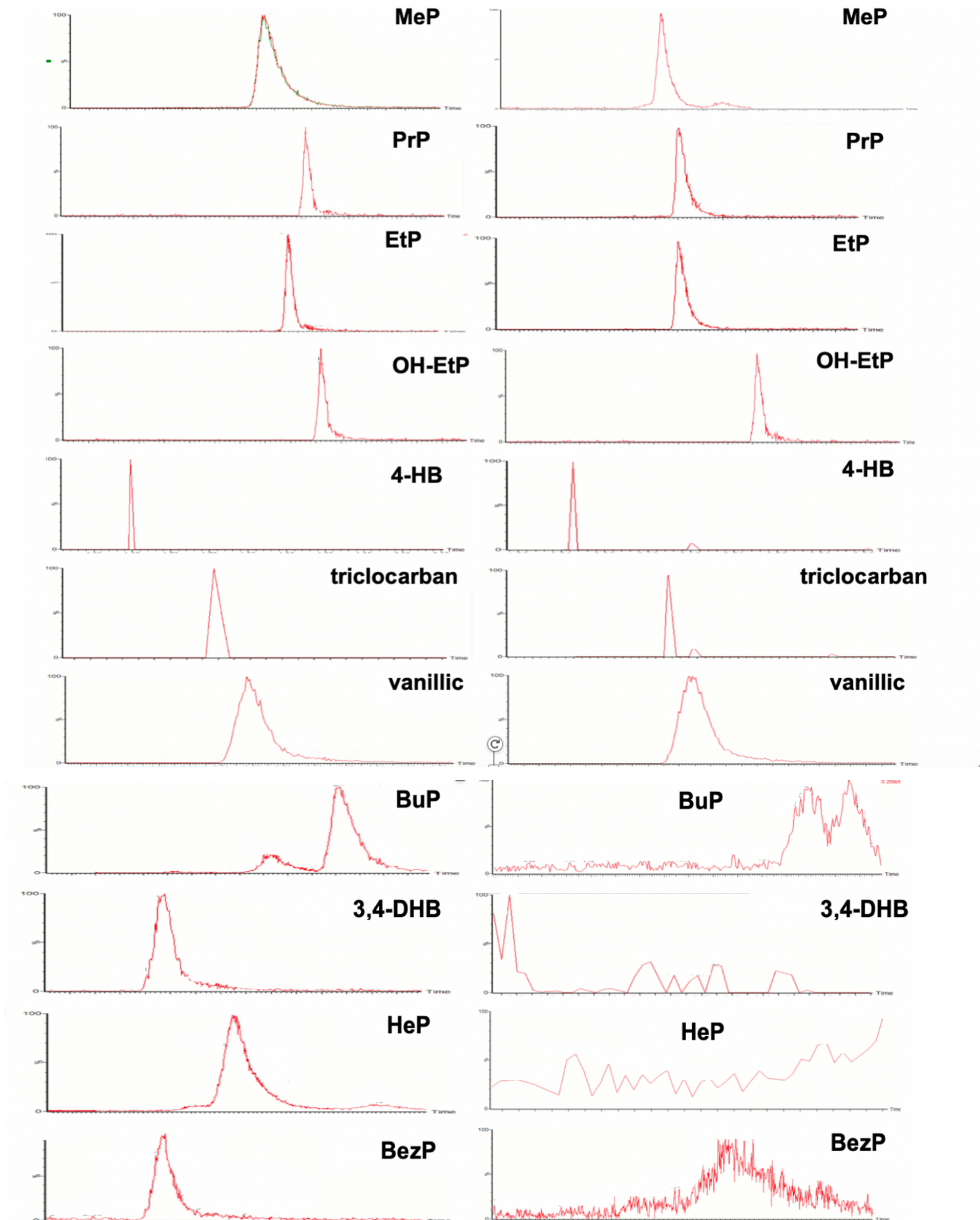


FIGURE 9. Selected chromatogram of MM and SP

The analytic method was evaluated for the applicability by the determination of the target compounds in the feed samples. Chromatograms, corresponding to a spike sample and to a standard solution are shown in FIGURE 9. As shown in the TABLE 4., all the samples detected in the analyzed spike samples contained parabens, apart from 3,4-DHB, BezP, HeP and BuP . Good peaks were presented by compounds PrP and EtP with a perfect standard retention time. Triclocarban and 4-HB were detected in the spike samples with their peaks following an unknown compound peak. Besides, the presence of parabens and 4-HB in the marine fish can be linked to natural sources. There were reports about marine bacterium producing HeP and BuP (Peng, Adacji, Chen, Kasai, Kanoh, Shizuri & Misawa 2006), but these parabens were not detected in feed sample over the course of this study. The most frequent detection of the six checked parent parabens was MeP, because it showed the highest potential of these compounds. This is reportedly associated with alkyl chain length (Byford, Shaw, Drew, Pope, Sauer & Darbre 2002; Darbre & Harvey 2008). Another clear peak was observed for OH-EtP and frequencies of vanillic detection levels were close to what other authors observed in food samples (Sayavongsa, Cooper, Jackson, Harris, Ziegler & Hibbert 2007).

It is possible that LC/MS-MS results have a small amount of noise because of impurities in the compounds. But it is impossible to exactly identify this impurity and its amount without its synthetic standard for measurement and comparison. The instability of absorbance detection and noises were caused by further extraction times. In situations when many samples must be analyzed one of the best choices is the usage of centrifugal separation together with LC/MS-MS detection. While it takes LC/MS-MS more time to analyze a single sample it can work automatically. Also, only this method can detect other compounds in the sample. Nevertheless, LC/MS-MS can result in a significant underestimation of the feed quantification.

5 DISCUSSION AND CONCLUSION

This project concentrates on parabens and triclocarban, the group of chemical ordinarily used as additives in beverages, food, cosmetics, personal care and several pharmaceuticals products. Parabens and triclocarban have been considered as contaminants because of some of their properties. The aim of this study was to determine feed samples from a recirculation aqua system for presence of five parabens derivatives, six parabens and antimicrobial.

It is critically important that the analysis determination methods are accurate for both the estimation of the extraction process efficiency and the research aimed at improving the feed quality. Encouraging and positive results were used for comparison in order to achieve the goals of this study. 11 organic parabens compounds were identified in the feed samples, collected at the RAS farming Atlantic salmon (Salmo salar). Very high areas were demonstrated by parabens derivatives MeP and 4-HB. Triclocarban (used as antimicrobial) and another parabens derivative, OH-EtP, also demonstrated clear peaks. Each one of the organic target analytes were found in the feed. However, HeP and BuP demonstrated a very low detection rate. Previous studies also demonstrated very low presence of those compounds, so the extraction method used in this study can be considered reliable for deriving analyte from the feed.

In general, the paraben derivatives, namely OH-EtP and 4-HB, have higher detection than their parent parabens. This result is justifiable because OH-EtP and 4-HB are paraben metabolites. So, the derivatives could be a result of chemical reactions during the storage period between feed production and usage. However, this study did not analyze processes and reactions that happen in feed during its storage period. The most noticeable result for parabens is related to BezP, HeP and BuP. These compounds have long alkyl chain which corresponds to stronger antimicrobial property than the found organic analytes with shorter alkyl chains and they showed very high contamination areas as well as low detection rate. Fish feed often includes EtP, MeP and PrP which are popular parabens and were detected in a variety of fish species in different locations. This can indicate that these compounds in the environment have anthropogenic sources (Khadem & Marles 2010). Usually the simplest parabens with relatively weak antimicrobial properties are used in the fish feed for long term preservation. HeP or BuP and BezP, despite having high antimicrobial activity was not often used in the feed.

Instead, a combination of short alkyl chain parabens is usually used in the feed because such combination is expected to have higher antimicrobial effect than a single paraben.

Blank contamination problems were encountered during several steps needed for analyzing parabens in the feed samples. Analyzing 4 out of 11 compounds, BezP, HeP, 3,4 –DHB and BuP, yielded no detection at all. Checking those analytes showed only some compounds introduced by blank contamination. Multiple factors can contribute to the observed blank contamination problem, such as sampling procedure, samples storage, filtration and treatment of the samples. One of the ways to reduce the blank contamination problem is to avoid detergents while cleaning the laboratory glassware. Low levels of parabens detected in the sample matrices made it necessary to precede the determination step by performing extraction and clean-up procedures for improvement of the detection limits. Executing every step of the laboratory work very carefully, strictly following the protocol helps to reduce blank contamination factors for the samples.

To conclude, this is the study that was able to discover 4-HB, OH-EtP, vanillic acid, PrP, MeP and TCC in fish feed. Parabens that are often used in fish feed as preservatives generally had lower detection levels than paraben derivatives. This is most likely caused by parabens transformation processes during storage. Despite having a long alkyl chain and thus strong antimicrobial activity, HeP, BuP or BezP were scarcely used as a feed additive. A wide variety of PrP and MeP was observed. Besides, a number of elements was detected which implied that different providers use slightly different ingredients in their feed. However, all the feed samples contained essential elements, some in lower and some might be in higher concentrations, which means that all of them still can negatively affect human health in an indirect way, when the parabens from the fish feed get accumulated in the salmon over the course of its growth.

Recent studies shown that exposure to parabens can cause harmful consequences for humans, and parabens are one of the most often used antimicrobial preservatives. This is why parabens analysis in human food sources (direct or indirect) became necessary. This study places emphases on how valuable is the simultaneous analysis in researching organic analytes, on using multidirectional view to evaluate the results, on studying differences in feed samples characteristics, and on the possibility of making comments on health effects, positive or negative

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