DEPARTMENT OF CHEMISTRY

LILIANA BARRETO DE OLIM

BSc in Chemical and Biochemical Engineering Sciences

Development and Validation of Methodology for Simultaneous Determination of BAC, Chlorate, DDAC and Histamine in fish by LC-MS/MS

MASTER'S IN CHEMICAL AND BIOCHEMICAL ENGINEERING

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Abstract

Fish is a valuable source of nutrition and in many places, it is a staple food for survival. Histamine is a biogenic amine that, in large quantities, can lead to histamine fish poisoning. In addition, the amino acid that gives rise to histamine is naturally present in fish, which can cause serious problems for consumers as it is a toxic compound. Due to the benefits of fish in the consumer's diet, they must fulfil food safety requirements so as not to cause harm to the consumer, safeguarding public health.

Thus, the aim of this dissertation is to validate an analytical method for benzalkonium chloride (BACs), chlorate, didecyldimethylammonium chloride (DDACs), and histamine and to develop a method for quantifying histamine, using liquid chromatography combined with mass spectrometer in various species of fish. For BACs and DDACs, the QuEChERS method was used. A different method, QuPPe, was used for chlorate and histamine due to their high polarity.

During method validation, the linearity of the method was assessed in the working range of 0.01 to 0.20 mg/kg. Rikilt's method proved the linearity of all the pesticides in the working range considered. The limits of quantification obtained are between 0.0008 and 0.0193. The accuracy and precision of the method were also evaluated using recovery and repeatability methods. Some recoveries were found to be outside the 70-120% range in the accuracy analysis. However, in terms of precision, the relative standard deviations were less than 20%. In this case, only european seabass, bigeye tuna and chub mackerel had recovery values outside the range. The robustness test was carried out the Youden's approximation, where the only significant factor was the mass of the sample. The stability assay at -18ºC proved that the recoveries obtained were within the expected limits and to estimate expanded relative uncertainty, it was found that the maximum value reached is 22.57% for the pesticide DDAC-C-12. The matrix effect in both methods ranged from 2.92 to 468.29% and so the standard addition method had to be applied. The only pesticide that exceeded the limit allowed by regulations was DDAC-C-10.

Keywords: Fish, Histamine, Method Development, Validation of methodology, Pesticides, Liquid Chromatography, LC-MS/MS, Maximum residue limit, QuEChERS, QuPPe

Resumo

O peixe é uma fonte nutritiva valiosa e, em muitos locais, é um alimento básico para a sobrevivência. A histamina é uma amina biogénica que, em grandes quantidades, pode levar ao envenenamento do peixe por histamina. Além disso, o aminoácido que dá origem à histamina está naturalmente presente no peixe, o que pode causar graves problemas aos consumidores, uma vez que se trata de um composto tóxico. Devido aos benefícios do peixe na alimentação do consumidor, este deve cumprir os requisitos de segurança alimentar de forma a não causar danos ao consumidor, salvaguardando a saúde pública.

Assim, o objetivo desta dissertação consiste na validação de um método analítico para o cloreto de benzalcónio (BACs), clorato, cloreto de didecildimetilamónio (DDACs) e para a histamina e desenvolvimento de um método para quantificação da histamina, utilizando a cromatografia líquida associada à espetroscopia de massa, em várias espécies de peixe. Para os BACs e DDACs, foi utilizado o método QuEChERS. Para o clorato e para a histamina, foi utilizado um método diferente, o QuPPe, devido à elevada polaridade destes compostos.

Durante a validação do método, a linearidade deste foi avaliada na gama de trabalho de 0.01 a 0.20 mg/kg. O método de Rikilt provou a linearidade de todos os pesticidas na gama de trabalho considerada. Os limites de quantificação situam-se entre 0.0008 e 0.0193. A exatidão e a precisão do método foram também avaliadas através de métodos de recuperação e de repetibilidade. Verificou-se que algumas recuperações se encontravam fora do intervalo de 70-120%, na análise da exatidão. Contudo, em termos de precisão, os desvios-padrão relativos foram inferiores a 20%. Para este caso, apenas o robalo, o atum patudo e a cavala, obtiveram valores de recuperação fora do intervalo. O ensaio da robustez foi realizado com base na aproximação de Youden, onde o único fator significativo foi a massa da amostra. O teste da estabilidade a -18ºC provou que as recuperações obtidas estavam dentro dos limites esperados e, para estimar a incerteza relativa expandida, verificou-se que o valor máximo que atinge é 22.57% no pesticida DDAC-C-12. O efeito de matriz em ambos os métodos, variou de 2.92 a 468.29%, pelo que teve de ser aplicado o método de adição padrão. O único pesticida que excedeu o limite permitido pelas regulações foi o DDAC-C-10.

Palavras-chave: Peixe, Histamina, Desenvolvimento de Métodos, Validação de Métodos, Pesticidas, Cromatografia Líquida e Espetroscopia de Massa, Limite Máximo de Resíduo, QuEChERS, QuPPe

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List of Abbreviations and Acronyms

ACTN – Acetonitrile

APCI – Atmospheric Pressure Chemical Ionisation

BA – Biogenic Amine

BAC – Benzalkonium Chloride

CV – Coefficient of variation

DDAC – Didecyldimethylammonium Chloride

DG – SANTE – Directorate-General for Health and Food Safety

DLLME – Dispersive Liquid-Liquid Microextraction

DOE – Design of Experiment

DS – Difference in Variances

dSPE – Dispersive Solid Phase Extraction

EC – European Commission

EDTA – Ethylenediaminetetraacetic acid

ESI – Electrospray Ionization

EU – European Union

EURL – EU Reference Laboratories for Residues of Pesticides

FDA – Food and Drug Administration

HPLC – High Performance Liquid Chromatography

IEC – International Electrotechnical Commission

IPAC – Portuguese Accreditation Institute

ISO – International Organization for Standardization

IUPAC – International Union of Pure and Applied Chemistry

LC-MS/MS – Liquid Chromatography – Tandem Mass Spectrometer

LOD – Limit of Detection

LLE – Liquid-Liquid Extraction

LPME – Liquid-Phase Microextraction

LOQ – Limit of Quantification

LRVSA – Laboratório Regional de Veterinária e Segurança Alimentar

ME – Matrix Effect

MeOH - Methanol

MRL – Maximum Residue Limit

MS – Mass Spectrometer

OCDE – Organisation for Economic Co-operation and Development

OVAT – One-Variable-At-A-Time

PBD – Placket-Burman Design

QACs – Quaternary Ammonium Compounds

QuEChERS – Quick, Easy, Cheap, Effective, Rugged and Safe

QuPPe – Quick Polar Pesticide

RL – Reporting Limit

RSD – Relative Standard Deviation

SD – Standard Deviation

SPME – Solid-Phase Microextraction

SRM – Single Residues Methods

TQMS – Triple Quadrupole Mass Spectrometer

1. Introduction

1.1. The laboratory

Laboratório Regional de Veterinária e Segurança Alimentar (LRVSA), administered by the Agricultural and Agri-Food Laboratory Services Department, began operating in 2009 with the aim of increasing the analytical capacity due to the rise in production in short time and the inclusion of new dangerous residues on the market. LRVSA belongs to Regional Directorate for Agriculture and Rural Development and includes services from two divisions: Residues and Contaminants Analyses and Veterinary Analysis.

Since 2011, LRVSA is accredited, according to the NP EN ISO/IEC 17025 for pesticide residues, food microbiology and veterinary analysis. In order to obtain greater profitability and to conquer new markets and new costumers, the laboratory is audited, annually, by Portuguese Accreditation Institute (IPAC). With these two accreditations, LRVSA can guarantee food security in line to the needs of customers and public service. Moreover, this laboratory is a National Reference Laboratory in the field of pesticides analyses determined by Single Residues Methods(SRM) and therefore, is always in contact with European Union Reference Laboratory for pesticide residue analysis by SRM.

1.2. Background and Motivation

One of the greatest source of protein in food industry is fish, as it provides several vitamins, nutrients, and minerals in a very low-calorie content [1]. Besides this, fish has many benefits for our health, including brain development, reduced risk of heart illnesses and other chronic diseases and improve our capacity to concentrate and pay attention [2]. Even with these benefits there are also some disadvantages in consuming fish such as contamination in their own habitat.

Contaminants in fish can include veterinary drug residues and inorganic or organic pesticides like insecticides and herbicides [3]. They can occur from water contamination, pesticides implemented in gardens or crops dragged by the rain or environmental issues. Another source can be the use of biocides or hygiene products on processing activities.

For this reason, it’s important to study and control the use of products that contain harmful residues and set an upper limit for the food people consume.

1.2.1. Objectives

This work aims to validate a methodology for the simultaneous determination of Benzalkonium Chloride (BAC), Didecyldimethylammonium Chloride (DDAC), Chlorate and Histamine in fish, by Liquid Chromatography-Tandem Mass Spectrometer (LC-MS/MS). LRVSA has validated methods for determining BAC, DDAC and Chlorate only in matrices of plant and terrestrial animal origin. Furthermore, another objective was to implement a methodology for the determination of histamine and subsequent validation, if possible, using the same methodology used for the determination of other contaminants analysed in routine.

For a method to be considered valid, several analytical parameters must be determined, such as selectivity and specificity, linearity, limit of detection and limit of quantification, accuracy, and precision. It is also necessary to analyse the following evidence: stability of the analytes in the matrix, matrix effect, robustness, and calculation of result uncertainties.

Although there are several studies on the determination of pesticides using chromatography techniques in different matrices, very few studies have been carried out on fish matrix. This work will apply the QuEChERS and QuPPe methods to fish matrices to determine the pesticides mentioned within a limit of 0,01 mg/kg for BAC, Chlorate and DDAC according to the lower limits established by European Union Regulation 396/2005 and the minimum limit of 100 mg/kg permitted for Histamine based on European Union Regulation 1441/2007.

2. State of the Art

2.1. Pesticides

Pesticides consist of chemical substances that are used to control or eradicate insects, germs, fungi, microbes, and undesirable crops. In agriculture, pesticides are mostly used to protect the crops from pests and diseases caused by bacteria or viruses and to have a faster growth in cultivations [4].

Biomagnification is the process where chemicals or toxins, became more concentrated in living tissues or organisms throughout the food chain. This happens because the degradation of these substances is a very slow process [5]. Because of that, it is important to limit the use of pesticide residues in crops. In fact, these compounds represent the main contamination factor in food [6].

During these years, people started to care more about what they eat, not just to be healthier but also to eat organic and pesticide-free food. There are currently more than 1000 pesticides and each one has different effects and properties. The pesticides covered in this work are widely used for cleaning and disinfecting food [7].

2.1.1. Benzalkonium Chloride and Didecyldimethylammonium Chloride

Benzalkonium Chloride (BAC) and Didecyldimethylammonium Chloride (DDAC) belong to the class of Quaternary Ammonium Compounds (QACs). These compounds are commonly used as disinfectants, antimicrobials, and surfactants in bacteria, viruses, and fungi. They have a very particular structure that allows them to bind their positive charges to the negative charge in bacteria, destroying its wall [8,9].

In this way, BAC and DDAC are pesticides that have attractive surface-active characteristics in which, they can help remove, for example, dirt from surfaces with their ability to interact with and reduce the surface tension between different phases. Because of that, both are used as active agents to clean or sanitise food and the surfaces in contact with it [10].

Benzalkonium chloride usually has shorter alkyl chains than didecyldimethylammonium chloride. This key difference between both has a significative impact on their physical properties and efficiency [11]. The number of alkyl chains is represented when referring to these pesticides.

2.1.2. Chlorate

Chlorate is obtained from chlorinated substances like sodium chlorate, as a by-product. These chlorinated substances can include disinfectants, fertilizers, and plant protection products [12].

This pesticide can be found in food due to the use of herbicides or biocides in its agriculture production, to water treatment processes or during its cleaning or disinfection [13]. At high levels, chlorate leads to iodine inhibition and can cause serious health problems related to thyroid [14].

2.1.3. Histamine

Biogenic amines (BAs) are nitrogenous molecules, formed by the decarboxylation of amino acids [15]. These compounds can be found in living cells through metabolic processes. In this way, many biogenic amines are formed in food, due to environmental conditions such as pH and temperature, bacterial growth, and availability of free amino acids [16].

In fish, BAs can be formed as a result of incorrect storage and refrigeration or inappropriate temperature. Histamine, the most common BA in food, is produced from the decarboxylation of histidine, amino acid that is naturally present in high amounts in fish [17]. Also, histamine can be considered a metric of spoilage as it is stable at high temperatures and its level cannot be undone, after its formation [18].

In low levels, BAs are important for several physiological mechanisms like immune responses, but when consumed excessively can have harmful effects due to its toxicity. A consequence of biogenic amines in food is scombrotoxin fish poisoning. In particular case, histamine fish poisoning, which consists of eating fish species that have high levels of the toxin mentioned. Additionally, histamine, in elevated quantities can cause headaches, dizziness, blood pressure problems and breathing difficulties [19]. Examples of marine species that contain high levels of histamine are: tuna, sardine and mackerel [20].

2.2. Legislative Framework for Pesticides

According to World Health Organization, pesticides are toxic and harmful for other organisms and also for humans, particularly those who work directly with these substances [6]. Due to this, it became necessary define some rules in European Union related to the use of

pesticides in food and the approval of some of them [4]. It was also necessary to control the maximum residue levels (MRLs) legally permitted in food, based on scientific information to protect public health and environment. These upper limits are fixed by Regulation (CE) 396/2005 and can be found in European Union Pesticides database [21]. Nowadays, no MRL is established for BACs, DDACs and chlorate in fish.

Benzalkonium chloride, unlike DDAC, has never granted approval in European Union in plant preservation, but, according to Directive 98/8/EC both are used as biocides to clean and disinfect tools, spaces and food [22].

Current European Union maximum residue level for BAC, DDAC and Chlorate in food can be observed in Table 2.1 [23]. Like for other pesticides or biocides, when no value is defined,the default value to be considered is 0.01 mg/kg.

Table 2.1: MRL for BAC, DDAC and chlorate.

Pesticide	Maximum Residue Level (mg/kg)
BAC	0.01
Chlorate	0.01
DDAC	0.01

According to U.S. Food & Drug Administration, a limit of 50 mg/kg for histamine in fish was established [24]. In line with European Union Regulation 1441/2007, the maximum legal limit for that contaminant in some species of fish is between 100 and 400 mg/kg [25].

2.3. Fish

The increase in the world's population over the years has led to an increase demand for food [26]. In addition to this, people have become more and more concerned about their health and well-being and prefer foods that are as nutritious as possible. Fish and seafood provide highly beneficial nutrients in an easy way. As a result, people are looking for food with the best possible quality and the least amount of harmful residues [27].

Fish contamination is a serious problem that occurs when fish are exposed to pollution and toxins in the environment or to biomagnification. Most of these pollutants are accumulated at the beginning of the fish food chain, in fish feeding or they can be found in process of storing or processing the fish [3]. Throughout the life cycle of a fish, these contaminants increase in concentration. Therefore, when consuming it, it is essential to be aware of the MRL allowed in

these and eat this kind of food in moderation. In addition, their acute and chronic toxicity should be considered.

2.4. Methods for Histamine Determination

Identifying histamine in a sample can be a complicated process due to interfering substances that can affect the analysis. There are several studies of methods used for the determination and quantification of histamine [24].

In compliance with RSC Analytical Methods Committee [28], the first step in developing a method is to research the chemical structure of the analyte and important related information like polarity which is a critical parameter since it influences the behaviour of the analyte during the analytical steps including chromatographic separation. Another important parameter that can be found in surveys is whether or not the analyte will ionise in mass spectrometer. Afterwards, it is necessary to select the chromatographic conditions and only after that, proceed to optimize these parameters.

Table 2.2 shows LC-MS/MS chromatographic conditions for fish matrix in reference methods, for the determination of histamine. The characteristics of each chromatographic column are listed in Table 2.3.

Table 2.2: Chromatographic conditions used in the histamine identification reference methods.

Column	Mobile phase	Flow (µL/min)	Injection volume (µL)	Strategy	Recovery (%)	Retention Time (min)	Gradient
Hypersil Gold C18 [18]	Methanol + 0.1% formic acid (30:70)	250	25	Ultrasonic radiations	-	-	Isocratic
Zorbax (Eclipse XDB C18) [29]	**A:** Ammonium acetate 0.1M pH 7.9 **B:** Acetonitrile	1000	20	Derivatization	97-103	18.2	Time A(%) B(%) 0 65 35 15 30 70 25 5 95 30 5 95 31 65 35
Cogent Diamond Hydride [25]	**A:** Water + 0.1% formic acid **B:** Acetonitrile + 0.1% formic acid	400	0,5	-	> 96	-	Time A(%) B(%) 0 30 70 2 35 65 6 90 10 8 90 10 9 30 70
Shodex HILICpak V-50 2D [30]	250 mM formic acid aq./acetonitrile = 70/30	300	20	-	-	-	-
HILIC Silica, Atlantis [31]	**A:** Acetonitrile 100 mM + formic acid **B:** DI + 0.5% formic acid	300	5	-	95-107	3.68	Time A(%) B(%) 0 90 10 4.2 40 60 4.3 90 10 9.5 90 10

Table 2.3: Characteristics of each column.

Column	Diameter (mm)	Length (mm)	Maximum pressure (bar)	pH range	Particle size (µm)	Surface Area	Column type
Hypersil Gold C18 [32]	4.6	250	400	1-11	5	220	Reversed Phase
Zorbax (Eclipse XDB C18) [33]	21.2	100	400	2-9	5	-	Reversed Phase
Cogent Diamond Hydride [34]	2.1	150	-	2.5-7.5	4	-	Normal Phase
Shodex HILICpak V-50 2D [35]	2	150	-	-	5	-	Reversed Phase
HILIC Silica, Atlantis [36]	2.1	50	415	1-5	3	330	Reversed Phase

As we can see from the Table 2.2, the development of a method is not a linear process, it depends on the conditions of each laboratory, each equipment and the specific matrices used.

The most important factors to consider when developing a method include the mobile phase content and its flow, the chromatographic column, the gradient, the retention time, and the sample injection volume [37]. All mobile phases in Table 2.2 contains acetonitrile or methanol. Both compounds are the extraction solvents of choice, and the fact that they are polar solvents [38] facilitates retention and separation in reverse phase columns, due to the strong polarity of histamine. In addition, with the exception of reference [29], all mobile phases contain formic acid. This compound is very widely used in mobile phases, as it helps to improve the shape of the peaks, to control the pH of the solution and evaporates easily at the equipment interface [39]. The flow, injection volume and retention time factors have a relatively wide range of values. Flow and retention time are concepts that can be related. If the flow is low, the retention time will be long, which means the compound will take longer to elute and, consequently, the peaks will be broader. In this case, the results will be less accurate and precise. In the opposite case, a rapid flow could cause ion suppression and possibly, co-elution, making it difficult to identify the compounds of interest [28]. Changing the gradient facilitates the separation of compounds and allows peaks with similar retention times to be moved.

Some approaches use strategies to facilitate the extraction of the analyte from the sample prior to its analysis. *A. Ali et al* [18] uses ultrasonic radiations during thirty minutes, after adding the extraction solvent to the sample, to improve the homogenization of the mixture through the use of high wave frequencies. *I. Altieri et al,* [29] on the other hand, uses the sample derivatization which consists of modifying the compound adding substances to make the sample easier to analyse or separate by changing its properties.

The achieved signal in detector is analysed using the most appropriate precursor ion and product ions for the analyte [28]. Table 2.4 represents these two parameters as well as the collision energy found in previous surveys.

Table 2.4: Percursor ion for histamine and its product ion and energy collision in LC-MS/MS.

Reference	Percursor ion	Product ion	CE (V)
3	112	68.1	24
	112	95.1	16
	112	54.1	48
	116	99.1	12
	116	85.1	16
4	112	95	-
5	112.2	68.2	23
	112.2	83.2	16
	112.2	95.1	15

The ion precursor is related to mass-to-charge ratio (m/z). After ionizing the sample, the defined precursor ions fragment through a collision energy, to form smaller fractions of the molecule, the product ions. These fragments allow the mass detector to identify properties of the molecular structure of the precursor ion [40,41]. Thus, these parameters are essential in the development of a method in LC-MS/MS. It is possible to predict, based on Table 2.4, that an important precursor ion in the progress of the method is 112.

Optimizing the chromatographic conditions and parameters mentioned is a crucial step, in the analyte determination procedure. Furthermore, it is also important to obtain the best possible signal in the mass detector, taking into consideration the selectivity of the method, which can be measured through the signal-to-noise ratio. The optimization of the method allows for a more efficient and reliable determination and quantification of the analyte under study, in addition to its applicability in other laboratories or even in other analytes with similar properties.

2.5. Quantification of Pesticides by Different Techniques

Today, there are about 800 active substances with different chemical structures and mechanisms available on the market and easily accessible [42].

National and European Union Control Programmes includes analysis of BAC, chlorate and DDAC in food [22], since all these pesticides are used in the disinfection and cleaning of surfaces in contact or even in food [10,43]. Histamine, being formed in the food itself, is subject of different control programmes. As both programmes are compulsory, simultaneous analysis of all components increases the laboratory's efficiency and productivity, avoiding the unnecessary use of reagents.

Chlorate and especially histamine are very polar compounds [44,45]. In contrast, because of the existence of benzene rings in its chemical structure, benzalkonium chloride has a low polarity [46] just like didecyldimethylammonium chloride [47]. These differences in polarity influence the identification of pesticides in the compounds, mainly in relation to the extraction method. Therefore, the polarity of the extraction solvent must be related to the polarity of the analyte to be analysed in order to achieve an effective extraction.

Table 2.5: Quantification methods for less polar pesticides using LC-MS.

Matrix	Pesticide	Column	Extraction Method	RSDR (%)	LOQ (mg/kg)	LOD	Recovery (%)
Food and water [48]	Pesticides (Table B.1)	-	QuEChERS	-	0.0001-0.0478* µg/L	-	93-96
Asparagus [11]	BAC8 BAC10 BAC12 BAC14 BAC16 BAC18 DDAC-C-10	Phenomenex Aqua 5µL C18 125A	QuEChERS	13 12 13 11 14 18 13	0.01 0.01 0.02 0.02 0.01 0.01 0.01	-	102 102 101 99 93 90 98
Cucumbers [11]	BAC8 BAC10 BAC12 BAC14 BAC16 BAC18 DDAC-C-10	Phenomenex Aqua 5µL C18 125A	QuEChERS	8 7 10 9 8 9 8	0.01 0.01 0.02 0.02 0.01 0.01 0.01	-	100 100 100 97 95 91 97
Fish [49]	Pesticides (Table B.1)	Zorbax Eclipse Plus C18, 2.1 mm × 150 mm, 3.5 µm	QuEChERS	3-54	0.001-0.01	-	81-157
Fish [50]	Pesticides (Table B.1)	XSELECT HSS C18, 2.1 mm × 150 mm, 3.5 µm	dSPE	< 20	< 0.01	< 0.005	70-125
Fish muscle [51]	Pesticides (Table B.1)	ZORBAX Eclipse XDB-C18, 150 mm x 4.6 mm, 5 µm	QuEChERS	1-17	0.001-0.01	-	70-108

Table 2.6: Quantification methods for polar pesticides using LC-MS.

Matrix	Pesticide	Column	Extraction Method	RSDR (%)	LOQ (mg/kg)	LOD (mg/kg)	Recovery (%)
Milk [52]	Chlorate	Waters Ion-Pak Anion HR, 4.6 mm × 75 mm	SPE	-	0.5	-	-
Lettuce [53]	Chlorate	HILIC Obelisc R	QuPPe	-	0.001	-	-
Fish [54]	Histamine	Zorbax XDB C18, 150 mm × 3.5 mm, 5um	Potassium dihydrogen phosphate, deionised water, and phosphoric acid	6-7	5	1.5	90-102
Fish [55]	Histamine	ODS Hypersil C18, 150 mm × 4.6 mm	Tri-chloro acetic acid (TCA) + Distilled water	12.5	1	0.2	77-107

Table 2.5 and Table 2.6 show that the extraction methods used for polar analytes and those used for less polar ones are different. Although QuEChERS is the most used extraction method for less polar pesticides, *M. Nasiri et al.* refers to various extraction methods which are usually used [56]. In case of chlorate, extraction is carried out either by SPE or QuPPe, which is an improvement on QuEChERS for very polar compounds [57]. Histamine is usually extracted using liquid-liquid extraction.

Table 2.7 shows the main drawbacks of some extraction methods compared to QuEChERS [58].

Table 2.7: Disadvantages of some extraction methods compared to QuEChERS.

Extraction Method	Main disadvantages
Liquid-Liquid Extraction (LLE)	Time-consuming, expensive due to the use of high volumes of solvent.
Solid-Phase Extraction (SPE)	Expensive and include more steps. Not effective in pesticides analysis.
Dispersive Solid-Phase Extraction (dSPE)	Not recommended for complex matrices.
Liquid-Phase Microextraction (LPME)	Long extraction time.
Solid-Phase Microextraction (SPME)	Expensive. Carefully consider the complexity of the matrix.
Dispersive Liquid-Liquid Microextraction (DLLME)	It may be necessary to use other extraction techniques after this.

R. Perestrelo et al. separate QuEChERS in two parts: extraction and clean-up [58]. In addition, *M. Colazzo et al.* says that in compounds with a high matrix effect, which is the case of fish, the second step is advisable to improve the recoveries. The clean-up step is frequently the dispersive solid-phase extraction, which consists of using a sorbent to attract the target analytes, using the dispersion technique [56].

Based on the recoveries in Table 2.5 and Table 2.6, the most appropriate column to consider would be Phenomenex Aqua 5µL C18 125A for less polar analytes and Zorbax XDB C18, 150 mm × 3.5 mm, 5 µm for more polar ones. Both of these columns are reversed phase columns [59,60].

D. Shin et al. points out that pesticides are usually analysed by gas chromatography (GC) but the fact that pesticides can be unstable makes it difficult for the GC to monitor them. According to *Fu et al,* LC-MS/MS, because it allows analysis of analytes with different polarities, is the most popular used system for analysing pesticides [50].

In compliance with the articles reviewed, the limit of quantification is in the range of 0.01- 0.02 mg/kg for BACs, 0.001-0.5 mg/kg for chlorate and 1-5 mg/kg for histamine. The limit of detection for histamine is between 0.2-5 mg/kg.

2.6. LC-MS/MS

LC-MS/MS, liquid chromatography-tandem mass spectrometer, is a technique that combines liquid chromatography with a pair of mass spectrometry detectors. The fact that this equipment enables a mass spectrum to be obtained that can identify compounds after separation is highly appreciated, especially in the pharmaceutical industry for analysing impurities in drugs and in the food industry for analysing and quantifying contaminants [61].

High performance liquid chromatography (HPLC) is widely used to efficiently separate compounds of interest from a complex mixture. Additionally, it allows compounds to be analysed, purified, and quantified in a quickly way [28]. The basis of HPLC separation involves the degree of affinity of the compounds with the mobile phase and the stationary phase. The function of the mobile phase is to transport the sample containing the analyte into the system and the time that the analyte is kept in the column is called retention time.

The main instrumentation of an HPLC includes a pump that is essential for maintaining the flow of the mobile phase, injectors that ensure that a defined volume of sample is injected into the mobile phase, a column which is the key of the separation and a detector that gives the equipment responses for the compounds separated by the column.

Liquid chromatography can be classified according to its interactions with the compounds [62,63]:

- Reversed Phase Liquid Chromatography – includes a non-polar stationary phase, while the mobile phase is polar. Non-polar molecules will be retained on the stationary phase due to its hydrophobic properties.
- Normal Phase Liquid Chromatography – the opposite of the previous one. Here, the stationary phase is polar, and the mobile phase is non-polar.
- Ion Exchange Chromatography – the separation is influenced by the electrical charges of the packing material, as there is an attraction off opposite charges between the sample and the stationary phase.
- Hydrophilic Interaction Liquid Chromatography – similar to normal phase liquid chromatography, the only difference is that water is added to the organic mobile phase. It is the type of chromatography mops commonly used for polar compounds.

- Size Exclusion Chromatography – the separation occurs through the difference in size of molecules. The smaller molecules are caught in the stationary phase until the larger molecules leave.

The mass spectrometer (MS) is an appliance that quantifies the mass-to-charge ratio and separates the molecules based on it. On its own, MS is not frequently used, since there are some compounds with the same molecular weight and would therefore give origin to the same precursor ion [64]. When there is a combination of two mass analyzers (MS/MS), the first generates precursor ions according to the m/z ratio and the second fragments these ions into product ions, providing more reliability and selectivity to the results [28].

Triple quadrupole mass spectrometers (TQMS) are the most frequently used tandem mass spectrometer. In this configuration there are three quadrupoles, two of which are mass detectors (Q1 and Q3) and one of which consists of a collision cell, where energy is supplied to fragment the ions in the first quadrupole. Once the product ions have been identified by the third quadrupole, they are sent to the detector [65].

These two technologies used together are made up of a liquid chromatography unit, an interface that only separates LC from MS, an ion source, a mass analyzer that is responsible for separating molecules, and a detector which identifies the fragmented ions. Since the LC works at normal pressures and the MS works in vacuum, the ion source is imperative in this system, as it allows the evaporation of the solvent from the LC and consequent vaporization and ionization of the molecules formed [65]. This procedure can be done by electrospray ionization (ESI) where a capillary with a potential difference is used or by atmospheric pressure chemical ionisation (APCI) which also uses a capillary, and the liquid is nebulized but in this case a discharge at high temperature occurs at the end of the capillary [28]. Figure 2.1 shows a schematic representation of LC-MS/MS configuration.

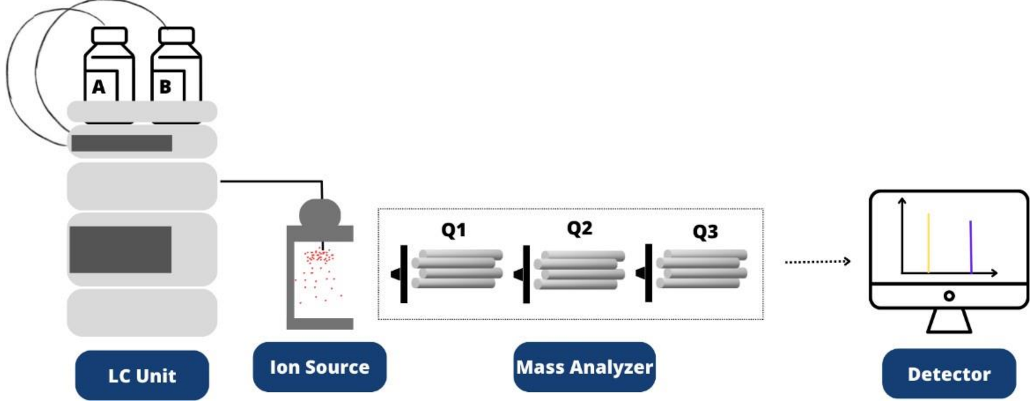

Figure 2.1: LC-MS/MS scheme.

2.7. Extraction Methods

In the determination and detection of pesticides, the extraction of analytes is an essential step since it allows the target compound to be obtained from a complex mixture or matrix. LLE is a conventional extraction method which uses two immiscible solvents, to separate pesticides from matrices, from its dissolution. Although, traditional extraction methods have disadvantages like the high price, time-consuming, its complexity, and the use of large quantities of solvent [66].

Alternative methods such as SPE, which consists of retaining the analytes of interest in a solid phase adsorbent [67], or QuEChERS, were developed to surpass the disadvantages of conventional ones.

2.7.1. QuEChERS

QuEChERS, abbreviation for Quick, Easy, Cheap, Effective, Rugged and Safe, is used to extract residues of pesticides in a simpler, cheaper, and more efficient way. This technique allows us to achieve high recovery rates and covers a wide range of residues, since doesn't use mass transfer steps [56].

This approach, for polar and nonpolar pesticides, was presented by *Anastassiades et al.* and checked by *Lehotay et al.*, in 2003. In the beginning, it was only applied to vegetables and fruit, but with the rapid recognition of the method, and based on its benefits, it became useful in several food matrices and beyond [58].

The method begins by transferring 10-15 g of well-homogenised and previous milled sample into a centrifuge tube. It's important to keep the sample at a low temperature to prevent analyte breakdown and water losses. The next step is to add 10-15 mL of the chosen solvent and shaking vigorously [68,69]. Many food products contain a certain content of water and the fact that acetonitrile is soluble in water and is a very polar solvent, makes it easier to extract polar and non-polar pesticides. Acetonitrile with 1% of acetic acid was used as the extraction solvent. The use of an acidified solvent is required to improve extraction performance, to avoid the degradation of compounds and to adjust pH [70]. The next step is to add 6,5 g of a mixture of salts consisting of 4 g of magnesium sulfate anhydrous, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0,5 g of disodium hydrogencitrate sesquihydrate. This addition of salts works as a buffer solution and helps to control pH and to induce the separation [71]. The acidic pH prevents ionisation of the molecules, keeping them neutrally charged. Being neutral, they are more easily soluble in organic phases. Immediately after the addition of this mixture, shake very well and centrifuge the sample [72]. At the end of this process three layers are obtained: the top layer

contains acetonitrile with the analytes of interest, the second onecontains the solids from the sample and the third one is an aqueous phase which contains the saltsof the buffer solution.

After the extraction process, there is an optional clean-up step, the dispersive solid-phase extraction (d-SPE), which is used to remove components that could affect the analysis like some sugars and fatty acids [58,70]. Filtration on 0.2 µm filters is another way to obtain clear extracts if this step is made with cold extracts.

2.7.2. QuPPe

QuPPe, that stands for Quick Polar Pesticide, was developed to analyse high polar pesticides that are not efficiently extracted using QuEChERS. This technique was a work produced by Anastassiades together with European Union (EU) Reference Laboratories for Residues of Pesticides [57].

As in the method mentioned above, the first step in QuPPe is to transfer 10 g of sample to a centrifuge tube. In this case, it may be necessary to add a certain amount of water to adjust its quantity in the sample to 10 mL. The next step is to add 10 mL of methanol with 1% of formic acid and shake the mixture firmly. Methanol is a more polar solvent which allows pesticides to be solubilised by this solvent. Finally, the centrifuge tube is placed in the freezer at -20ºC for an hour and a half and then centrifuged [73]. Clean-up is also an optional step for this method.

In this approach, it is essential to highlight the addition of ethylenediaminetetraacetic acid (EDTA) and formic acid after the addition of water. EDTA is recommended when we get low recoveries of certain analytes and formic acid, as previously pointed, helps to control pH. The combination of these two compounds is useful to improve extraction efficiency and to stabilise polar pesticides [74]. Furthermore, EDTA and formic acid contribute to denature proteins, which can produce precipitates in samples of animal origin in the period of time between centrifugation and analysis of the sample by chromatography.

2.8. Sampling Representativeness

Sample representativeness is necessary when we are trying to validate a method, because it implies that the characteristics of a sample reflect the characteristics of a larger population of which the sample was included. It is important to take a few things into account such as random sampling, environmental conditions, and sampling size to obtain the smallest possible variability

and error. We can only statistically validate the data obtained after determining the representativeness of the sample [75].

According to Commission Regulation (EU) 2017/644, the quantity of the overall sample of each species must be at least 1 kg and for an elementary sample must be at least 100 g. If the lot weight is less than 50 kg, which is the case, a minimum of three incremental samples must be taken. It's important to note that, depending on the size of the fish there are areas of the fish that are more favourable to consider as elementary samples. For small fish, we consider the whole fish as an elementary sample, in the case of larger fish, we consider the middle part of the fish between the backbone and the belly. Samples must be well homogenised to obtain more reliable results. Although this regulation is specific to analysing dioxin and PCB levels, it was taken into account in this work [76].

Based on analysis in fish of several pesticides, carried out by European Union Reference Laboratory, 10 g of sample are used, using SPE method, in GC-MS/MS [77,78]. In another report, considering QuEChERS method and using LC-MS/MS, 5 g of sample was used [49].

2.9. Analytical Method Validation

Analytical Method Validation is a very critical process in pesticide analysis. This procedure does not improve or change the method, but it is important to create reliable, accurate and secure data for regulatory authorities, as European Commission, which require that these data to be within the maximum residue limits. Validation of a method is an indispensable parameter when using a method in a laboratory, as it allows to provide analytical quality control, in this particular case for analytes detection, in order for them to be reported and thereby improve food safety for consumers [79].

DG SANTE, or Commission's Directorate-General for Health and Food Safety is in charge of health and safety policies related to food, in European Union, including set MRLs for residues and developing regulations. Besides this, provides guidelines for the validation of food methods [80].

The document No. SANTE/11312/2021 outlines the criteria for validating methods and ensuring analytical quality control [81]. The guidelines in this document were used as the main orientation for this validation.

This process of validation involves two approaches to evaluate the achievement of a method, direct evaluation, and indirect evaluation. The last-mentioned approach refers to the evaluation of the parameters by means other than direct measures. Direct evaluation is clearly

related to the accuracy of the method [82]. All the parameters analysed to complete the method validation process are shown in the Table 2.8.

Table 2.8: Parameters for Method Validation according to SANTE.

Indirect Evaluation	Direct Evaluation
Specificity and Selectivity	Accuracy
Linearity	
Limit of Detection and Limit of Quantification	
Precision	
Stability of Analytes in Matrix	
Robustness	

2.9.1. Selectivity and Specificity

Selectivity and specificity are the first parameters to be analysed when validating a method, as they make it possible to reliably assess the identification and measurement of the analyte to be analysed in relation to other compounds in the matrix [83].

Selectivity is related to the method's ability, from the extraction process to the mass detector, to identify the analyte under study in the presence of other possible interfering compounds that can affect identification.

Specificity consists of the ability of the method to provide a signal or response exactly and exclusively of the target analyte through the detector, in the presence of potential interferents.

These two terms are similar, but selectivity is a broader concept since it implies discriminating the analyte of interest from interfering compounds, whereas specificity only considers the target analyte. In this regard, we can conclude that a method is selective and specific if it is possible to clearly distinguish the analyte under study in the matrix from the chromatogram [84].

2.9.2. Linearity and Linear Ranges

Linearity is a crucial parameter when we talk about validating a method. It provides the relationship of proportionality between the concentration of a given analyte and its measured response, using the method in question. Furthermore, linearity allows us to define a range of work for which the method under study is acceptable and valid. In accordance with ISO 8466-2,

whenever a new working range is defined, a Fisher's F-test must be performed to check that the limits are properly applied [85].

$$F_{calculated} = \frac{s_1^2}{s_{last}^2}, \text{ for } s_1^2 > s_{last}^2 \quad (2.1)$$

$$F_{calculated} = \frac{s_{last}^2}{s_1^2}, \text{ for } s_{last}^2 > s_1^2 \quad (2.2)$$

s_1^2 – variance calculated for the lower limit of the working range

s_{last}^2 – variance calculated for the upper limit of the working range

- If $F_{calculated} \leq F_{critical}$, the working range is well applied.
- If $F_{calculated} > F_{critical}$, there are significant differences in the variances and the working range has to be changed.

The obtained value is compared with a critical value, $F_{99\%,f_1,f_2}$, where f_1 and f_2 are the degrees of freedom of the numerator and denominator, respectively.

Calibration curves are graphical representations that make it possible to evaluate the linearity of the method and consists, in this case, of a proportional relationship between the x coordinate that represents the concentration of the analyte, and the y coordinate that represents the area of the analyte peak for each concentration. Calibration lines must be carried out at a minimum of three different concentrations, in line with SANTE. The determination coefficient, R^2, evaluates the effectiveness of a model, in predicting a result. This value can range from 0 to 1 and the higher it is, the closer the observed values are to the predicted values. A coefficient greater than 0,9 is accepted [86].

Pearson correlation coefficient (r) is a statistical tool widely used to check the linearity between two different variables, since it measures the direction and intensity of the linear relationship between them. This coefficient varies within the range -1 to 1, including 0. A value close to 1 means that when one variable undergoes a change, the other variable also changes in the same way, thus reflecting a positive correlation. On the other hand, a value closes to -1 indicates an opposite change in the direction of the second variable in relation to the first. A value of 0 indicates that there is no correlation among the variables [87,88].

$$r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]}} \quad (2.3)$$

According to the LRVSA's internal method and based on SANTE's guidelines, linearity can be demonstrated by:

- A Pearson correlation coefficient of at least 0,98 of a line with 5 single points or 3 double points;
- Or a Pearson coefficient of at least 0,95 on a residual plot based on data at four concentration levels that are randomly distributed around the regression line.

The study of this parameter is a complex process, and the calculation of the correlation coefficient is not sufficient to confirm linearity in a given range of work. It is therefore necessary to use statistical tests to obtain a more reliable decision [89,90].

2.9.2.1. Mandel's Test

Norm ISO-8466 proposes that linearity be checked by the Mandel test. This standard is divided in two parts, the first relating to linear calibration functions and the second to second-order calibration functions.

The first step in applying this test is to calculate the residual standard deviations of the linear calibration function (ISO 8466-1) and the non-linear function (ISO 8466-2).

$$S_{\text{lin}} = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \widehat{y_1})^2}{N-2}}, \text{ where } \widehat{y_1} = mx + b \qquad (2.4)$$

$$S_{\text{non-lin}} = \sqrt{\frac{\sum_{i=1}^{N}\left(y_j - \widehat{y_2}\right)^2}{N-3}}, \text{ where } \widehat{y_2} = ax^2 + bx + c \qquad (2.5)$$

S_{lin} – standard deviations of the linear calibration function

$S_{\text{non-lin}}$ – standard deviations of the non-linear calibration function

y_i, y_j – signal obtained from the detector for a given concentration

$\widehat{y_1}$, $\widehat{y_2}$ – expected signal by calibration functions (linear and non-linear)

N – number of standard solutions

The next step is to calculate the difference in variances (DS^2) using the equation below.

$$DS^2 = (N-2) \times S_{lin}{}^2 - (N-3) \times S_{non-lin}{}^2 \quad (2.6)$$

In order to verify whether there are significant differences between the variances of the calibration functions, a VT test is carried out using Fisher's tabulated value ($F_{95\%,1,N-3}$) [91,92].

$$VT = \frac{DS^2}{S_{non-lin}{}^2} \quad (2.7)$$

- If VT ≤ $F_{95\%,1,N-3}$, the acquired data are well adjusted using a linear calibration function;
- If VT > $F_{95\%,1,N-3}$, the calibration function is of second order and to obtain a linear function, if possible, the working range must be reduced.

2.9.2.2. *RIKILT's Test*

RIKILT's test allows the study of the linearity of the calibration data obtained based on a response factor at each point on the line [92,93].

$$RF = \frac{y_i/x_i}{\overline{y_i/x_i}} \quad (2.8)$$

y_i – signal obtained from the detector for a given concentration

x_i – concentration of analyte in solution

The reference value for this response factor is 100%. However, for this study a deviation of ±10% is considered. If any value is outside this deviation, the working range should be minimised.

2.9.2.3. *Normality of residues*

In compliance with SANTE guidelines, normality of residues is a general requirement for the study of linearity and establishes a relationship between the experimental value obtained through de detector, y_i, and the theoretical value obtained through the calibration function, $\hat{y}$ which can be linear or non-linear.

$$\text{Residuals (\%)} = \frac{y_i - \hat{y}}{\hat{y}} \times 100 \quad (2.9)$$

J. Miller et al. recognises two important concepts that are related to the normality of residuals. Homoscedastic occurs when the values achieved remain relatively constant with the increasing of the concentration, in other words, the variance is constant, while heteroscedastic occurs, for example, when the obtained residual values increase with analyte concentration [94]. When homoscedasticity is verified, it is possible to conclude the linearity of the function. This can be observed through a graphical representation between the concentration of analyte and the response achieved in the experimental.

The residuals of the calibration lines should not deviate by ±20% from the calculated curve, in pesticide analysis.

2.9.2.4. Homogeneity of variances

The homogeneity of variances confirms the homoscedasticity of the data and consequently its linearity.

There are several statistical tests to assess the homogeneity of variances, including tests by Bartlett, Levene, Hartley and Cochran. *P. Mair and A. Eye* states that the simplicity of the Cochran test and the fact that it considers a greater amount of experimental data, makes it the most widely used test. Cochran's test relates the widest variance of the data set to the sum of the variances of all the concentrations levels.

$$C = \frac{s_{max}^2}{\sum_{i=1}^{k} s_i^2} \quad (2.10)$$

If the value achieved for this calculation is lower than $C_{critical}$ ($C_{95\%,n,k}$, where n is the number of observations for each level and k is the number of concentration levels), it is possible to conclude the homogeneity of variances and therefore the homoscedasticity of the data.

2.9.3. Limit of Detection and Limit of Quantification

The next step in validating a method is to determine the analytical thresholds, as these are very important for the sensitivity and the ability of the method [95].

Limit of detection (LOD) is the minimum level of analyte in a sample that can be accurately identified although not necessarily measured [96]. According to IUPAC, the limit of detection is calculated by multiplying the standard deviation of the response obtained at a low concentration by 3 [97].

$$\mathrm{LOD} = 3 \times \mathrm{s}_0 \quad (2.11)$$

Another way of calculating the value of this limit is to use the signal-to-noise ratio (S/N). The LOD corresponds to the concentration where the S/N is 3:1 [98].

$$\mathrm{LOD} = 1 \times \frac{3}{\mathrm{S/N}} \quad (2.12)$$

Limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be precisely measured [96]. This concentration is obtained by multiplying the standard deviation by 10.

$$\mathrm{LOQ} = 10 \times \mathrm{s}_0 \quad (2.13)$$

As with the limit of detection, the limit of quantification can be calculated using the signal-to-noise ratio. In this case, the LOQ matches to the concentration where the S/N is 10:1 [98].

$$\mathrm{LOQ} = 1 \times \frac{10}{\mathrm{S/N}} \quad (2.14)$$

As an alternative to the LOQ, a reporting limit (RL) can be set, based on the laboratory's internal method.

$$\mathrm{CV} = \frac{\mathrm{s}_0}{\bar{X}} \quad (2.15)$$

If the coefficient of variation (CV) is less than or equal to 10%, this limit is proven to be greater than or equal to LOQ.

2.9.4. Accuracy and Precision

Accuracy indicates how close the observed value is to the reference or acceptable value. The concept of accuracy is often compared to the concept of trueness [96,99]. The difference is that, while accuracy is a parameter that characterises individual values, trueness considers the average value of a wide range of results. Furthermore, when applied to a data set, accuracy is

impacted by the total error. For these reason, accuracy is associated with two elements: precision (random error) and trueness (systematic error) [100].

In method validation, accuracy can be assessed through a recovery test using reference data or, when this is not possible, using spiked matrices and calculate the recovery percentage. At least 5 replicates should be carried out at three different fortification levels, including the reporting limit [81].

$$\text{Recovery (\%)} = \frac{\text{Area of the spiked sample peak}}{\text{Area of the sample calibration peak}} \times 100 \qquad (2.16)$$

According to SANTE guidelines, recoveries must be within the range 70-120%. The closer the recovery is to 100%, the more accurate the method is.

Precision reflects the closeness of independent results obtained in replicates, performed under the same conditions [101]. This parameter has an impact on random errors and is expressed in relation to the relative standard deviation (RSD), meaning that the lower this value, the lower the variability and the greater the precision of the method in question [102].

$$RSD = \frac{s_0}{\overline{X}} \times 100 \qquad (2.17)$$

The precision of a method considers three different levels: intermediate precision, repeatability, and reproducibility. Intermediate precision refers to estimating the precision of a sample analysed using the same method, but defining exactly which conditions, one or more, are being changed like different analysts or different times. Repeatability expresses the proximity of the results achieved under the same working conditions, such as the same analyst, the same laboratory, and the same equipment. Reproducibility is an interlaboratory component that measures the results obtained in the same working conditions, but in different laboratories. This last element will not be covered in the validation since it is not required for a validation in a single laboratory [103].

In the practical precision procedure, at least 10 injections are required to obtain a reliable RSD. For a method to be precise, this value must be less than or equal to 20% [99].

2.9.5. Stability of Analytes in Matrix

The stability of analytes in a matrix is considered by SANTE guidelines and FDA as a validation parameter. The stability of a compound is related to how long this substance remains

effective and stable while stored, until it is analysed. Preferably, samples arriving at the laboratory should be analysed within the first few hours of collection so that the analytes don't degrade, and the results obtained are more reliable, but due to factors both internal and external, this is sometimes not possible. In this way, it is essential to consider factors such as environmental factors, storage conditions, properties of pesticides and their interaction with other compounds [104].

In order to avoid any degradation of the analytes, the samples are kept at low temperatures, usually -18ºC [105], until they are analysed. Laboratories should therefore carry out matrix stability tests over time to see the influence of temperature, even though this is a time-consuming and resource-intensive process [106].

The procedure for the stability test consists of preparing solutions of known concentration, applying the method under study, and keeping them stored until they are analysed. At least 5 solutions should be made, one for each time period studied. On the day of each analysis, a new solution in matrix is made under the same conditions as the stored ones, so that the two can be compared [103,107].

$$\text{Stability} = \frac{\text{Area of the sample calibration peak}}{\text{Area of the stability solution peak}} \times 100 \qquad (2.18)$$

Based on SANTE guidelines, the difference of the response between old and freshly prepared extracts should not differ more than ±10%, otherwise storage time should be adjusted.

A Student's t-test can be used to check whether there are significant differences between the stability values obtained over time.

$$t_{n\text{-}1} = |\overline{X} - \mu|.\frac{\sqrt{n}}{s} \qquad (2.19)$$

μ – expected value of stability recoveries (100%)

$\overline{X}$ – mean of the recoveries achieved

n – number of times the recovery was tested

s – standard deviation of the recoveries achieved

2.9.6. Robustness

Robustness is related to the method's ability to remain constant and stable, even when exposed to variations that could affect its performance. These variations can include small changes during the application of the internal method, such as changing the mobile phase content or variations in environmental factors such as the temperature at which the sample is stored until it is analysed [108]. Such variations make it possible to check which factors may be significant in the results obtained. If it is not possible to verify the robustness in a method, both its selectivity and trueness are questioned, since the limit of quantification of the analyte in the sample may not be the real one.

At an early stage, potential factors to be tested should be identified. Choosing the factors to vary should be a careful process, since through research or information from other laboratories we should try to choose the factors most likely to cause changes in the results. Some of the most commonly used factors are: pH of mobile phase or its flowrate, the temperature of the column or the sample injection time [109-111]. Next, it is necessary to choose different levels to which factor can be exposed. The levels in each factor are represented with the +1 (high level) and –1 (low level) signs.

Having identified the factors to be considered and the levels, it is also necessary to select an approach for the analysis of robustness. The ideal would be to very each independent factor, one at a time, while fixing the others, applying the OVAT (one-variable-at-a-time) principle [111]. However, this is not a practical procedure, and it is also time-consuming. Design of experiments (DoEs) is the most widely used approach for checking this parameter as it allows several factors to be varied at the same time. It is advisable to vary at least seven factors, each one with two levels of variation [112].

DoE is a methodology used in statistical analysis which enables as much information as possible to be extracted using the fewest resources. Screening design is an experimental test that makes it possible to identify the key factors within a wide range of potential ones. In addition, it makes it easy to eliminate factors that don't require further investigation, usually using an appropriate software. Placket-Burman design (PBD) is an example of screening and consists in a fractional factorial experimental design with two levels, a number of experiments (N) multiple of four and a number of factors of N-1 for each experiment [113]. It is highly recommended the use of PBDs to validate the robustness of a method when we are using small variations in the factors related with methods and when we are only interested in linear effects, as it is more practical, efficient, and inexpensive.

Therefore, for eight runs, seven factors will be needed, each with two levels, where level +1 will represent the parameter used in the method and level -1 will represent the parameter that has changed [112].

Youden, following Placket-Burman's approach, questioned the existence of confounding effects and proposed an approximation (Figure 2.2) to the design of experiments that took random order into account [114].

	Factors						
Run	A	B	C	D	E	F	G
1	-1	-1	-1	-1	-1	-1	-1
2	-1	-1	+1	-1	+1	+1	+1
3	-1	+1	-1	+1	-1	+1	+1
4	-1	+1	+1	+1	+1	-1	-1
5	+1	-1	-1	+1	+1	-1	+1
6	+1	-1	+1	+1	-1	+1	-1
7	+1	+1	-1	-1	+1	+1	-1
8	+1	+1	+1	-1	-1	-1	+1

Figure 2.2: Plackett-Burman table with random order.

After all the experiments and the determination of the response of the assays for each factor, the next stage is to calculate the effects to conclude which factors are significant. The effect of each factor is calculated based on the average of the sum of all positive responses, $\sum Y^{+}$, and the average of the sum of all negative responses, $\sum Y^{-}$.

$$\text{Effect} = \frac{\sum Y^{+}}{\frac{N}{2}} + \frac{\sum Y^{-}}{\frac{N}{2}} \qquad (2.20)$$

The effect of the factors should be represented in the form of a confidence interval [115].

$$\text{CI} = \text{Effect} \pm t_{95\%,N\text{-}1} \times s \qquad (2.21)$$

If the confidence interval for each factor does not cover the zero value, the factor is considered significant.

2.9.7. Uncertainty

Uncertainty is related to the reliability and accuracy of a method as it reflects the degree of confidence in the results achieved. In compliance with SANTE guidelines, the definition of uncertainty consists of an interval around the outcome within that the real value can be expected to be situated, usually with a probability of 95%. Laboratories are required by ISO/IEC 17025 to estimate the expanded uncertainty (U') considering several contributions. Furthermore, this value must not exceed 50% to obtain trustworthy results.

There are two approaches to calculating this metric. The bottom-up or component-by-component methodology is an within-laboratory procedure and involves identifying all the sources of uncertainty, in which case a very specific knowledge of the entire method is essential [116]. The top-down methodology, on the other hand, is a inter-laboratory procedure, where the uncertainty comes from the analysis or proficiency tests, reference values or from estimates of inter-laboratory data [117].

Fishbone or Ishikawa diagram is a cause-effect diagram that makes it easier to identify the possible sources of uncertainty in a result [117,119]. An example of this diagram is shown in Figure 2.3.

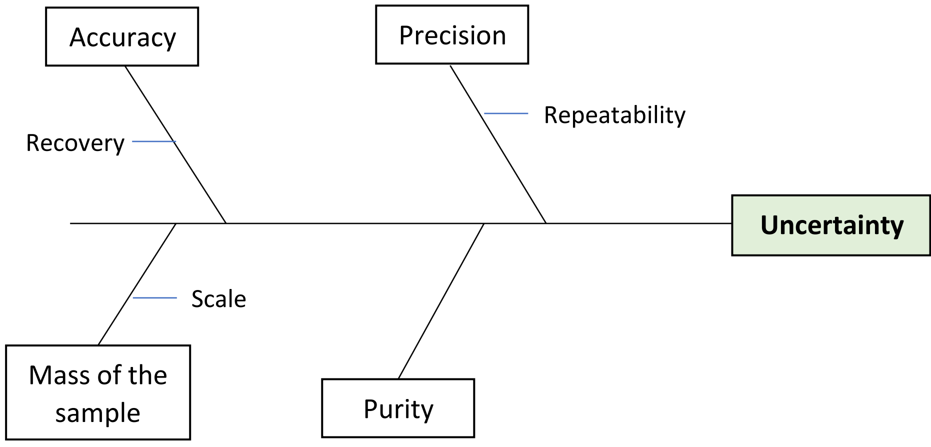

Figure 2.3: Ishikawa diagram example.

The process of identifying sources of uncertainty throughout the validation process can be exhaustive, since there are uncertainties associated with accuracy, precision, the mass of the standard weighed, the purity of the substance and even with the equipment involved.

The determination of the expanded uncertainty will be calculated using the bottom-up method, according to LRVSA method. The following equations represent the terms for each source of uncertainty identified.

i. Accuracy contribution

$$u'_{\overline{Ra}} = \frac{u(\overline{Ra})}{\overline{Ra}} = \sqrt{\frac{s_R^2}{n.\overline{Ra}^2}} \quad (2.21)$$

- $u'_{\overline{Ra}}$, represents the uncertainty associated with the accuracy and it is calculated through the standard deviation (s_R) and the average recovery in within-laboratory conditions ($\overline{Ra}$). The number of trails carried out on accuracy and/or precision is represented by n.

ii. Precision Contribution

$$u'_{precision} = \frac{s_R}{\overline{Ra}}, where\ u_{precision} = s_R \quad (2.22)$$

- $u'_{precision}$ reflects the uncertainty associated with precision of the whole method.

iii. Sample mass contribution

$$u'_{ms} = \frac{u_{ms}}{m}, \text{where } u_{ms} = \sqrt{2.\left(\frac{0{,}1}{\sqrt{3}}\right)^2 + 2.\left(s_{rep}\right)^2} \quad (2.23)$$

- u'_{ms} refers to the uncertainty associated with the mass of the sample. The standard deviation under repeatability conditions is obtained by weighing the sample ten times. The value 0.1 is the acceptance criteria for the calibrated balance.

iv. Contribution of the standard's purity

$$u'_{pur} = \frac{(1 - Pur)/2}{\sqrt{3}} \quad (2.24)$$

- u'_{pur} represents the uncertainty associated with the pattern's purity and uses the purity value of the substance on the certificate.

v. Combined relative uncertainty

The combined relative uncertainty (u') is calculated using the following equation.

$$\frac{u_y}{y} = u' = \sqrt{(u'_{\overline{Ra}})^2 + (u'_{precision})^2 + (u'_{ms})^2 + (u'_{pur})^2} \quad (2.25)$$

vi. Expanded uncertainty

To obtain the expanded relative uncertainty, the combined relative uncertainty is multiplied by the bilateral t-student function.

$$U' = t_{(bilateral;95\%;n-1)} \times u' \qquad (2.26)$$

2.9.8. Matrix Effect

Matrix Effect, ME, reflects the presence of certain components like fatty acids or sugars, in the sample, in addition to the analytes of interest that may interfere with its analysis [119].

$$\text{Matrix Effect}(\%) = \frac{\text{Area of the sample calibration peak}}{\text{Area of the solvent calibration peak}} \times 100 \qquad (2.27)$$

Using the relative standard deviation, it is possible to analyse whether the matrix effect is meaningful in the analysis of a given pesticide. If the RSD is greater than or equal to 15%, this effect must be taken into account [89].

Typically, when using an equipment with a mass detector, it is necessary to take this parameter into consideration in the validation process, because when the components mentioned above interfere with the analyte, thus confirming the ME, there is a suppression or enhancement in the signal, which can be very significant. [89,120].

Rawn et al. states that matrix effect can reach 300% and high values like this can cause problems during the analysis of a sample [120]. In order to avoid an extremely high matrix effect in fish, it is crucial to obtain clean extracts. In the case of fish is very important to precipitate fish proteins that, in extreme case, can lead to clogging of chromatographic column. The method used by EU Reference Laboratories for Residues of Pesticides adds to the sample 1 mL of 10% aqueous EDTA solution and 100 µL of formic acid [74].

2.9.8.1. Standard addition Method

In LC-MS/MS, matrix effect usually brings down the signal and this suppression can often be high. When we are quantifying pesticides mainly in food, it is very important to take this effect into account, as the analytical thresholds in this scenario can be much lower than in reality, which could be a problem for consumers.

The standard addition method is an approach that allows the matrix effect to be corrected, and it is also used when the recoveries obtained are low [121].

Based on EU Reference Laboratories for Residues of Pesticides, this method consists of adding different concentrations of pesticide being analysed to the sample. The first step of the procedure is estimating the concentration of the analyte in the sample from a calibration line previously made using a software screening. One way of checking whether it is necessary to apply the method is through the obtained concentration. If its value is equal to or greater than 0.007 mg/kg, in compliance with internal method of LRVSA, the standard addition method is applied. In the next step of the method, four vials are considered, three of which are spiked (0.5x, 1x and 1.5x) with the pesticide and the remaining one contains only solvent and extract. The concentration of the standard solution of the pesticide is calculated from the concentration obtained on the calibration line, considering the amount of pesticide in a representative volume of the sample. Table 2.9 represents the scheme used when the standard addition method is applied.

Table 2.9: Table representing the standard addition method used in the laboratory.

	Vial 1	Vial 2	Vial 3	Vial 4
Extract volume (µL)	1000	1000	1000	1000
Pesticide volume of concentration of the standard solution (µL)	0	100	200	300
Solvent volume (µL)	300	200	100	0

Then, a straight line must be extrapolated that relates the area of the analyte peak to the mass of analyte added to the extract (Figure 2.4). The absolute amount of pesticide in the sample (µg) is where the y-coordinate is zero.

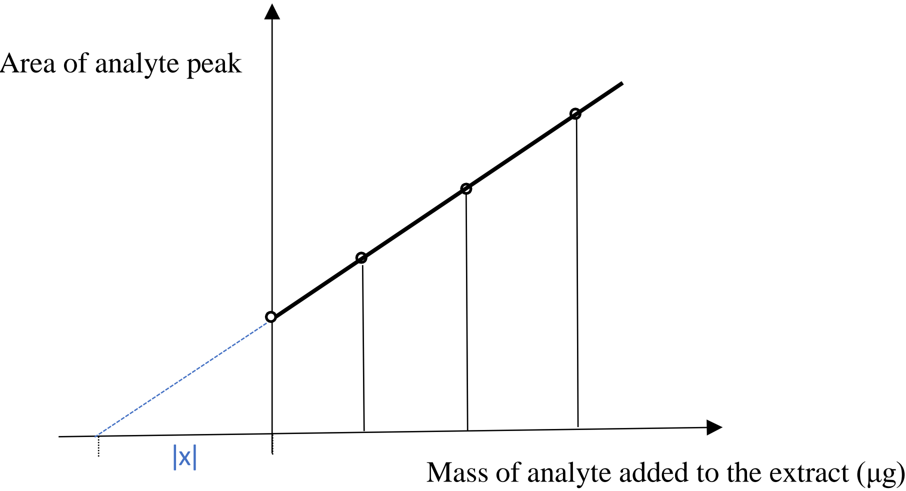

Figure 2.4: Graph used to extrapolate the line.

$$x = \frac{\text{y - interception}}{\text{slope of the curve}} \quad (2.28)$$

Finally, it is possible to calculate the concentration of analyte in the sample.

$$\text{Concentration of analyte in the sample} = \frac{x}{\text{Amount of sample}} \quad (2.29)$$

Using the slope of the last line, it is possible to establish what happens to the signal during separation. If we have a high slope, we have an enhancement of the signal, otherwise we have a suppression. LRVSA assumes that the slope is acceptable if its angle is between 7 and 17 degrees.

2.9.9. Confirmation Method: Decision Limit (CCα)

Screening methods are fast, cheap, and efficient ways of identifying the target analytes in the sample. They can perform identification for many samples and to detect non-compliant outputs. Confirmatory methods are used to confirm, definitely, the presence of the analyte [122].

Decision limit (CCα) refers to the concentration at which it is stated to say that an analyte is present in the sample with a probability of error α. In daily analyses, if the analyte concentration in the matrix exceeds this limit, the product is considered non-compliant. Associated with this concept, error α reflects false positives, which means the probability that the sample analysed is in conformity despite having a non-compliant outcome. In compliance with the Commission Implementing Regulation (EU) 2021/808 of 22 August of 2021, the decision limit is used in confirmatory analysis [123].

The calculation of the decision limit is a metric to be included in the validation, unlike the other pesticides analysed in this report [124], since the sectorial validation guides (DG-SANTE) don't require it.

In this study histamine will be considered a pharmacologically active compound and, as mentioned in chapter 2.2, the MRL deemed was 100 mg/kg.

Based on Commission Implementing Regulation (EU) 2021/808 of 22 March of 2021 for authorised pharmacologically active substances, the determination of the decision limit, with a confidence of 95%, relates the established MRL to the combined uncertainty. This uncertainty must be taken into account the within-laboratory reproducibility.

$$CC_{\alpha} = \text{MRL} + 1{,}64 \times u(\text{combined}) \quad (2.30)$$

Another alternative for this calculation would be to use a calibration curve and fortifications at different levels around the maximum limit, replacing only the uncertainty parameter in the equation with the curve's standard deviation of the results obtained.

3. Materials and Method

3.1. Materials

In the laboratory experiment, various fish matrices, standards and equipment were used.

3.1.1. Fish matrices analysed

The matrices analysed were samples donated by Secretaria Regional das Pescas for the purpose of carrying out this work. Table 3.1 shows the types of fish analysed, as well their scientific name and the condition in which they arrived at the laboratory.

Table 3.1: Fish matrices.

Sample number	Type of fish	Scientific name	Condition	Admission date
1	Bigeye tuna	Thunnus obesus	Fresh	07/02/2023
2	Blue jack mackerel	Trachurus picturatus	Fresh	16/02/2023
3	Black cardinal fish	Epigonus telescopus	Fresh	16/02/2023
4	Phycis phycis	Phycis phycis	Fresh	16/02/2023
5	Bigeye tuna	Thunnus obesus	Fresh	02/03/2023
6	Atlantis bluefin tuna	Thunnus thynnus	Fresh	14/03/2023
7	European parrotfish	Sparisoma cretense	Fresh	14/03/2023
8	Black scabbardfish	Aphanopus carbo	Fresh	14/03/2023
9	European seabass	Dicentrarchus labrax	Frozen	14/03/2023
10	Pink dentex	Dentex gibbosus	Fresh	15/03/2023
11	Blue jack mackerel	Trachurus picturatus	Fresh	18/04/2023
12	Chub mackerel	Scomber colias	Fresh	18/04/2023
13	Black scabbardfish	Aphanopus carbo	Fresh	18/04/2023
14	Black scabbardfish	Aphanopus carbo	Frozen	19/04/2023
15	Bigeye tuna	Thunnus obesus	Frozen	19/04/2023
16	Atlantic salmon	Salmo salar	Frozen	19/04/2023
17	Bigeye tuna	Thunnus obesus	Frozen	16/05/2023
18	Black scabbardfish	Aphanopus carbo	Frozen	31/05/2023
19	Black scabbardfish	Aphanopus carbo	Frozen	27/06/2023
20	Albacore	Thunnus alalunga	Frozen	27/06/2023
21	Black scabbardfish	Aphanopus carbo	Frozen	27/06/2023

3.1.2. Standards used and their properties

The pesticide standards used for the analysis were: BAC8, BAC10, BAC12, BAC14, BAC16, BAC18, Chlorate, DDAC-C-8, DDAC-C-10, DDAC-C-12, and Histamine. The solutions made in solvent for these standards had a concentration of 400 ng/µL. The solvents used were acetonitrile for BACs and DDACs and methanol for chlorate and histamine.

Table 3.1 shows the pesticides analysed with the solvent in solution, along with their CAS number, formula, structure, molecular weight, purity, and melting point.

3.1.3. Solvents, equipment, and other laboratory supplies

The solvents used in the extraction methods were acetonitrile with 1% acetic acid for QuEChERS and methanol with 1% formic acid for the QuPPe method. In addition, an aqueous solution of 10% of ethylenediamine tetraacetic acid (EDTA) and formic acid was used for the polar method. In the mobile phase solutions, acetonitrile, ammonium formate, formic acid, glacial acetic acid and methanol were used.

The main equipment used was the Agilent 1200 Series HPLC System combined with the SCIEX Triple Quad 3500 LC-MS/MS System (Figure A.1). Additional equipment such as Blixer Robot Coupe 3, scale, centrifuge, vortex and ultrasonic were also used.

Other laboratory supplies:

- Centrifuge tube of 15 mL
- Centrifuge tube of 50 mL
- Distilled and deionised water
- Flasks of 10 and 20 mL
- Glass beakers
- Micropipettes
- Polypropylene syringes of 5 mL
- QuEChERS mixture (Table A.3)
- Sample storage containers
- Spatula
- Syringe hydrophilic filters of 0.2 µm
- Vials of 2 mL

Table 3.2: Properties of pesticides.

Pesticides	CAS	Solvent	Formula	Structure	Molecular Weight (g/mol)	Melt. Point (°C)
BAC8	959-55-7	ACTN	C17H30ClN	(n = 8, 10, 12, 14, 16, 18)	283.88	N/A
BAC10	965-32-2	MeOH	C19H34ClN		311.93	56
BAC12	139-07-1	MeOH	C21H38ClN		339.99	60
BAC14	139-08-2	MeOH	C23H42NCl		368.04	56-62
BAC16	122-18-9	MeOH	C25H46ClN		396.09	55-65
BAC18	122-19-0	ACTN	C27H50N.Cl		424.15	54-56
DDAC-C-8	5538-94-3	ACTN	C18H40N.Cl	(DDAC-C-10)	305.97	75
DDAC-C-10	7173-51-5	MeOH	C22H48ClN		362.08	88
DDAC-C-12	3401-74-9	ACTN	C26H56N.Cl		418.18	131
Chlorate	7775-099	H2O/ ACTN (90:10)	NaClO3		106.44	255
Histamine	51-45-6	MeOH	C5H9N3		111.15	84.2

3.2. Method Development for Histamine

In a first stage of developing the method for histamine and after some research, the QuPPe method was used to extract the analyte. This method was chosen as it was already the method used daily at the LRVSA for very polar compounds. The next step was to select some of the chromatographic columns available in the laboratory in order to see which would best separate the histamine from the matrix to be analysed.

Table 3.3: Conditions tried to develop the method.

Column	Mobile phase	Flow (µL/min)	Gradient		
Zorbax XDB C18 (4.6x150mm, 5µm)	**A**: Water + 0.1 mL glacial acetic acid **B**: Acetonitrile + 0,1 mL glacial acetic acid	300	Time 0 20 22 22.1 30	A(%) 80 0 0 80 80	B(%) 20 100 100 20 20
InfinityLab 120 HILIC (2.1x150mm, 2.7µm)	**A**: Water + 10 mM ammonium formate + 0.1% formic acid **B**: Acetonitrile/water (90:10) + 10 mM ammonium formate + 0.1% formic acid	250	Time 0 3 11 12 16 18 19 20	A(%) 2 2 30 40 95 95 2 2	B(%) 98 98 70 60 5 5 98 98
Obelisc R (2.1x150mm, 5µm)	**A**: Water + 50 mM ammonium formate + 3,6 mL formic acid **B**: Acetonitrile	300	Time 0 4 12 12.1 20	A(%) 20 80 80 20 20	B(%) 80 20 20 80 80
Hypercarbe (2.1x100mm, 5µm)	**A**: Water + 1% acetic acid + 5% methanol **B**: Methanol + 1% acetic acid	300	Time 0 10 10.1 15	A(%) 100 70 100 100	B(%) 0 30 0 0
InfinityLab 120 EC-C18 (2.1x150mm, 4 µm)	**A**: Water + 0.1 mL glacial acetic acid/L **B**: Acetonitrile + 0.1 mL glacial acetic acid	250	Time 0 20 22 22.1 30	A(%) 80 0 0 80 80	B(%) 20 100 100 20 20
InfinityLab 120 EC-C18 (2.1x150mm, 4 µm)	**A**: Water + 10 mM ammonium formate + 0.1% formic acid **B**: Acetonitrile/water (90:10) + 10 mM ammonium formate + 0.1% formic acid	250	Time 0 20 22 22.1 30	A(%) 80 0 0 80 80	B(%) 20 100 100 20 20

Table 3.3 shows the columns trialed, as well as the mobile phases, flow, and gradient for the development of the method.

The Hypercarbe column and its associated mobile phase are used in the laboratory's routine analysis of chlorate. Table 2.6 illustrates the use of Zorbax column for an essay with histamine and the use of Obelisc column, which is commonly used for polar compounds such as chlorate. The choice of the InfinityLab column consisted of trying to see if it was possible to add histamine to the BACs and DDACs method, but the results obtained were not what was expected, since the lines were not linear or even close to it. Like the InfinityLab, the Zorbax column also didn't show a very linear behaviour. As a result, the columns that best adapted the results to linearity were Hypercarbe and Obelisc. Among these two, the column that allowed a better separation of the target compounds was the Hypercarbe.

3.3. Experimental Procedure for Chlorate and Histamine

The QuPPe method used to analyse Chlorate and Histamine in foodstuffs of animal origin is based on the internal method of LRVSA – IT.MP.DSLAA.01.62.

Sample preparation

1. Cut the sample into small pieces and place the portions on a clean surface, spaced apart.
2. Protect the portions and store at a temperature of approximately -18ºC for at least 12 hours.
3. Grind the frozen sample in a mill to obtain a fine powder.
4. Analyse the sample. If the sample is not analysed immediately, store it in the freezer at the same temperature.

Sample processing

1. Weigh 10 g of sample into a 50 mL centrifuge tube.
2. Add water to the weighed sample until the mass of water is approximately 10 g. In case of fish, it is made up of 70% of water, so 3 mL is added to the tube.
3. Add 100 µL of formic acid.
4. Add 10 mL of methanol with 1% formic acid using a pipette or a dispenser.
5. Shake vigorously for at least 1 minute using vortex.
6. Place the tubes in the freezer at approximately -18ºC for 2 hours.
7. Centrifuge at a speed of 5000 r.p.m (~4136 RCFxg) for approximately 5 minutes.

8. Transfer the supernatant from the centrifuged extract using a syringe and a filter into a beaker.
9. Pipette about 1000 µL of the filtered content into a plastic vial and proceed with its chromatographic analysis on the LC-MS/MS.

3.4. Experimental Procedure for BACs and DDACs

The QuEChERS method used to analyse BACs and DDACs in foodstuffs of animal origin followed the internal method of LRVSA – IT.MP.DSLAA.01.59. Sample preparation is carried out in the same way as the QuPPe method.

Sample processing

1. Weigh 10 g of sample into a 50 mL centrifuge tube.
2. Add 10 mL of acetonitrile with 1% acetic acid using a pipette or a dispenser.
3. Shake vigorously for at least 1 minute using vortex.
4. Add 6.50 g of the QuEChERS mixture and shake vigorously again (Table A.3).
5. Centrifuge at a speed of 3000 r.p.m for approximately 5 minutes.
6. Transfer the supernatant from the centrifuged extract using a syringe and a filter into a beaker.
7. Pipette about 1000 µL of the filtered content into a vial and proceed with its chromatographic analysis on the LC-MS/MS.

3.5. Study to avoid precipitate formation in vials before analysis in QuPPe

Once the sample has been prepared and processed, it is ready for injection into the LC-MS/MS. In the time between the sample being ready and being injected, a precipitate was noted in the vial in the QuPPe method, as shown in Figure 3.1. This precipitate can lead to clogging of the column and consequently damage to the laboratory.

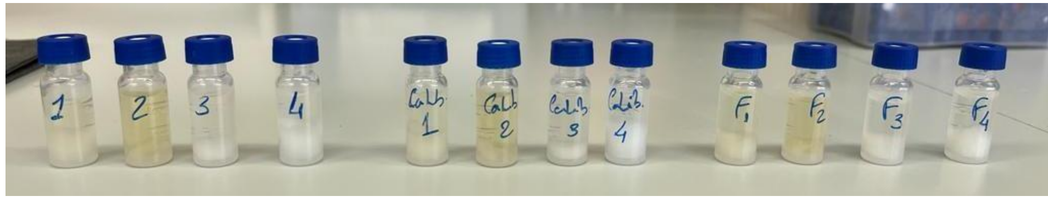

Figure 3.1: Precipitate formation in the vials, using the QuPPe method.

In this way, the aim is to remove or avoid the precipitate formation. 2.9.8, mentions adding 1000 µL of 10% aqueous EDTA solution at the same time as formic acid in the sample processing. Accordingly, a test was carried out with different solutions of EDTA and different concentrations of formic acid in order to check the elimination of the precipitate and establish the best quantity of substances to add. Table 3.4 shows the different tests performed, as well as the volume of water depending on the mass of the sample and whether or not the sample was cooled.

Table 3.4: Quantities of substances added to the sample – Part 1 of 2.

Vial	Sample mass (g)	Volume of water (mL)	Volume of EDTA	Volume of formic acid (µL)	Cold
1	2	0.6	0	0	No
2	2	0.55	1 mL, 5%	0	No
3	2	0.5	1 mL, 10%	0	No
4	2	0.45	1 mL, 15%	0	No
5	2	0.4	1 mL, 20%	0	No
6	2	0.6	0	50	No
7	2	0.6	0	100	No
8	2	0.6	0	150	No
9	2	0.6	0	200	No
10	2	0.55	1 mL, 5%	50	No
11	2	0.5	1 mL, 10%	100	No
12	2	0.45	1 mL, 15%	150	No
13	2	0.4	1 mL, 20%	200	No
14	2	0.6	0	0	Yes
15	2	0.55	1 mL, 5%	0	Yes
16	2	0.5	1 mL, 10%	0	Yes
17	2	0.45	1 mL, 15%	0	Yes
18	2	0.4	1 mL, 20%	0	Yes
19	5	1.5	0	0	No
20	5	1.5	0	50	No
21	5	1.5	0	50	No
22	5	1.5	0	100	No
23	5	1	500 µL, 10%	50	No
24	5	1	500 µL, 10%	50	No
25	5	1	500 µL, 10%	100	No
26	5	1.5	0	0	No
27	5	1.5	0	50	No
28	5	1.5	0	50	No
29	5	1.5	0	100	No
30	5	1	500 µL, 10%	50	No

Table 3.5: Quantities of substances added to the sample – Part 2 of 2.

Vial	Sample mass (g)	Volume of water (mL)	Volume of EDTA	Volume of formic acid (µL)	Cold
31	5	1	500 µL, 10%	50	No
32	5	1	500 µL, 10%	100	No

In the first 25 vials, the sample used was bigeye tuna (sample number 1), while in the rest, the sample used was phycis phycis (sample number 4).

After all the vials had been prepared, they were analysed by direct observation for precipitate formation. The vials were observed immediately after preparation, after 15 minutes, 1 hour, 2 hours, 4 hours, and 24 hours.

This study revealed that the best way to avoid precipitate formation before injection into de equipment was to add 1000 µL of 10% aqueous EDTA solution and 100 µL of acid formic to 10 g of sample, giving a total of 200 µL of formic acid in the method.

3.6. CCα

As explained in the chapter 2.9.9, to calculate the decision limit, the maximum limit of residue established in the laboratory is required, as well as the combined uncertainty at the MRL level. As mentioned in the same chapter, the MRL set for histamine was 100 mg/kg. Thus, to calculate the uncertainty, fortifications must be carried out at the level of the maximum limit, so 10 g of sample were fortified with 100 mg/kg of histamine.

Another important factor to consider when calculating CCα is the within-laboratory reproducibility between the results obtained after injection. Therefore, seven vials were injected with seven different matrices and on different occasions.

4. Presentation and Discussion of the Results

4.1. Specificity

The specificity of the method can be confirmed by observing the solvent chromatograms through the separation of the target analyte into a visible peak, with high resolution and only a single retention time. In this way, different solvent solutions were prepared for each analyte. Solutions of 0.01 mg/kg were made for BAC8, BAC10, BAC12, BAC14, BAC16, BAC18, DDAC-C-8, DDAC-C-10 and DDAC-C-12 in acetonitrile. For chlorate, a solution of 0.01 mg/kg in methanol was made and for histamine, a solution 10 times higher than the previous one, 0.1 mg/kg in methanol, was made. The prepared solutions were then injected into the LC-MS/MS system. The chromatograms obtained in solvent for each pesticide are shown in Figure 4.1 to Figure 4.11.

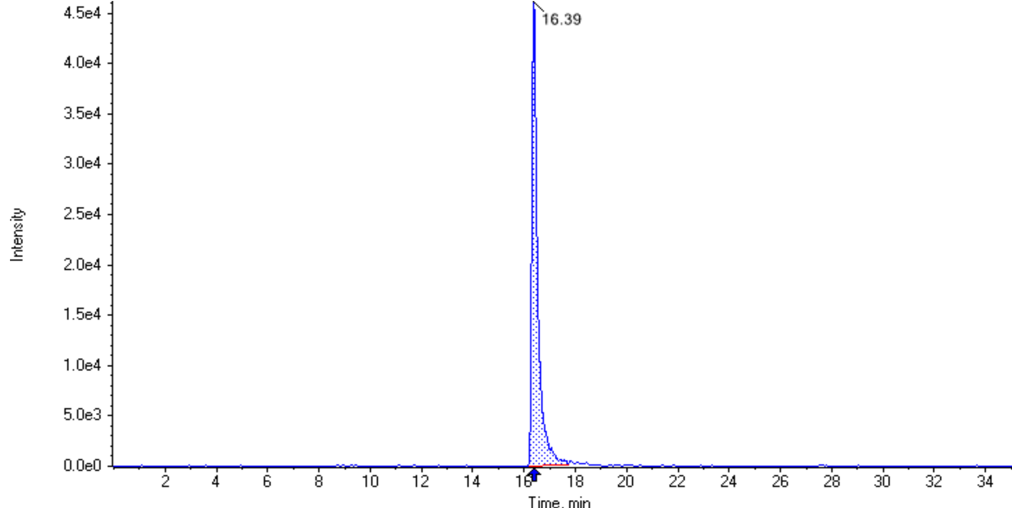

Figure 4.1: Chromatogram of BAC8 in solvent.

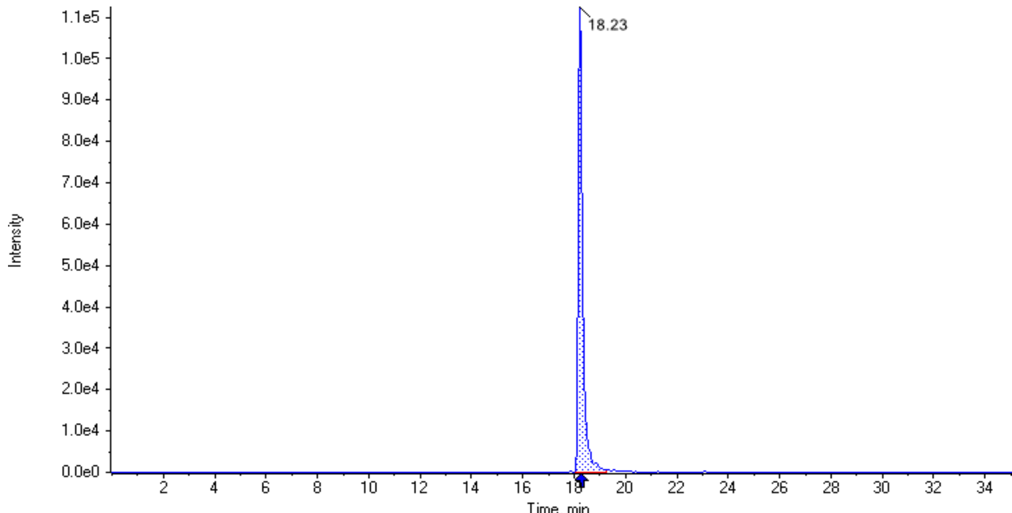

Figure 4.2: Chromatogram of BAC10 in solvent.

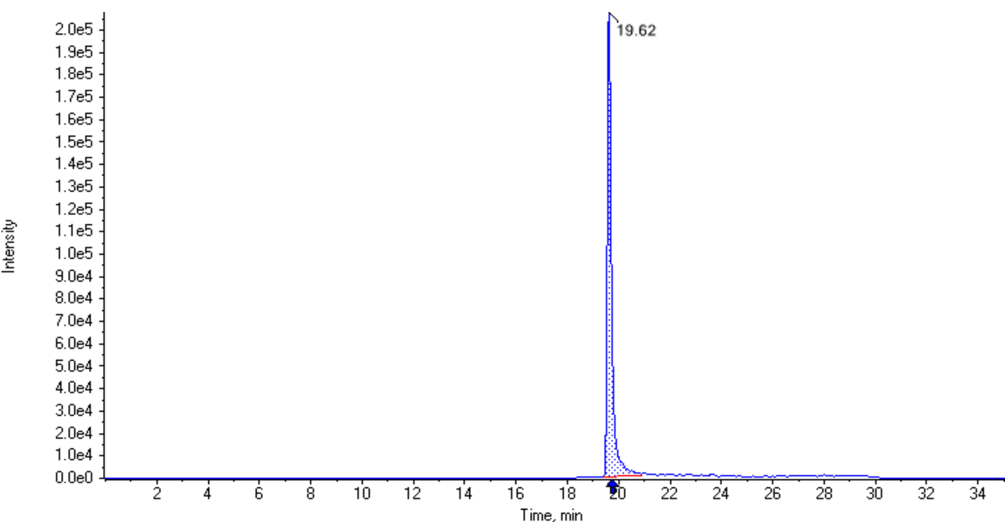

Figure 4.3: Chromatogram of BAC12 in solvent.

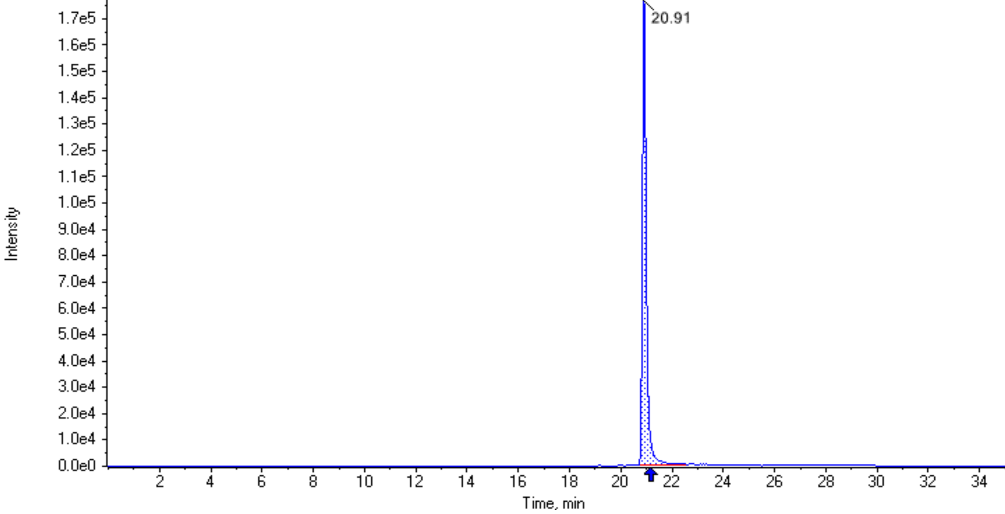

Figure 4.4: Chromatogram of BAC14 in solvent.

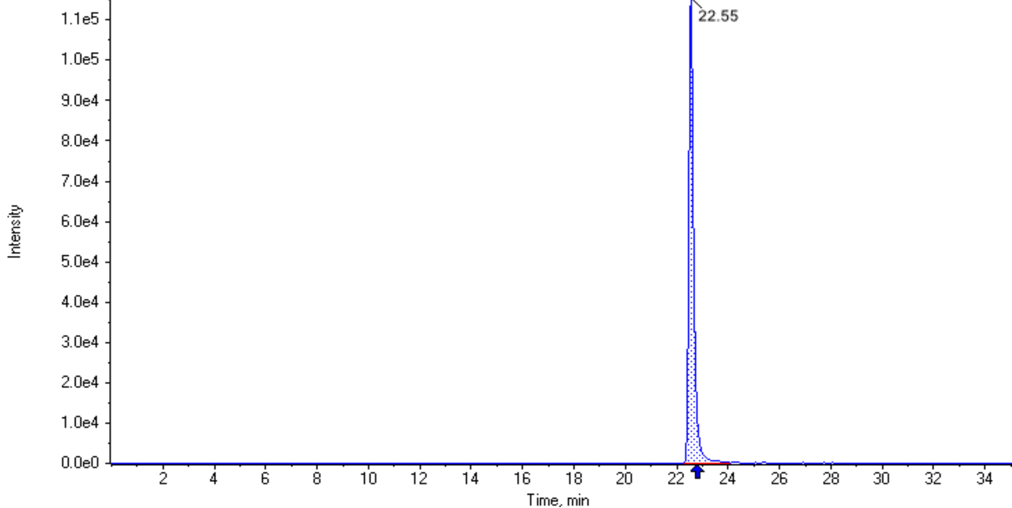

Figure 4.5: Chromatogram of BAC16 in solvent.

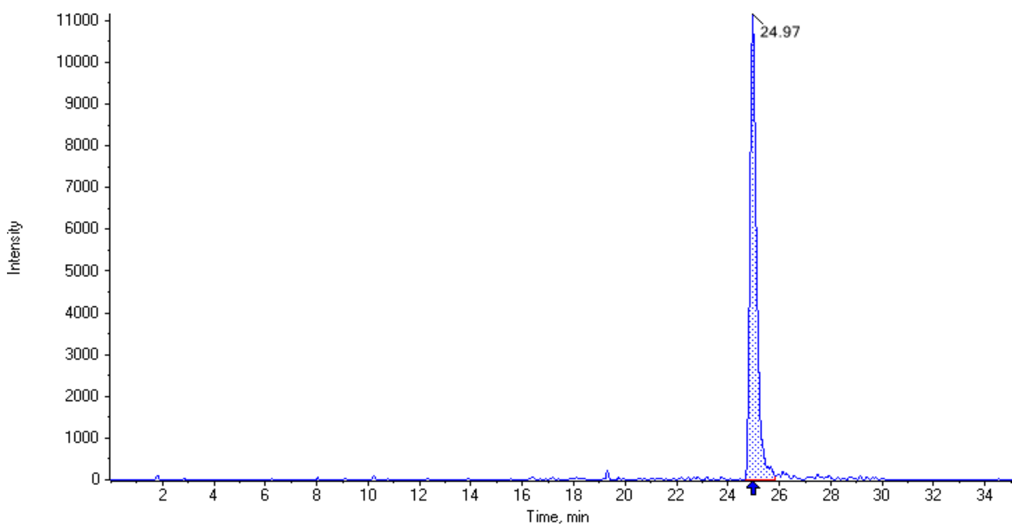

Figure 4.6: Chromatogram of BAC18 in solvent.

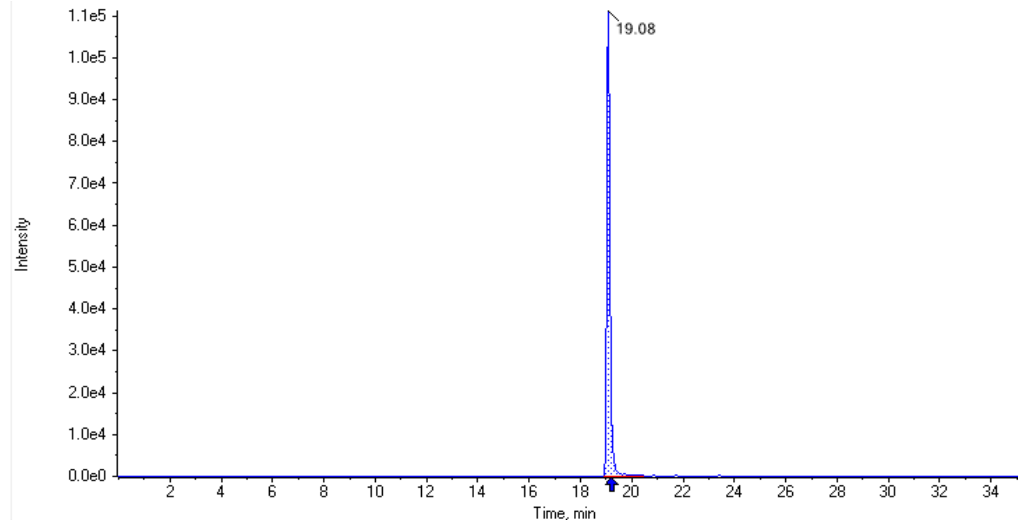

Figure 4.7: Chromatogram of DDAC-C-8 in solvent.

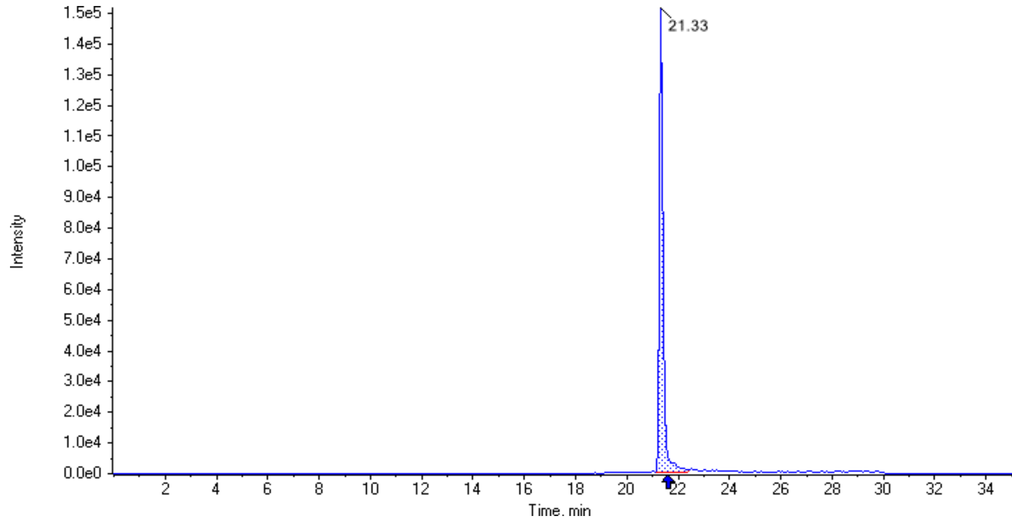

Figure 4.8: Chromatogram of DDAC-C-10 in solvent.

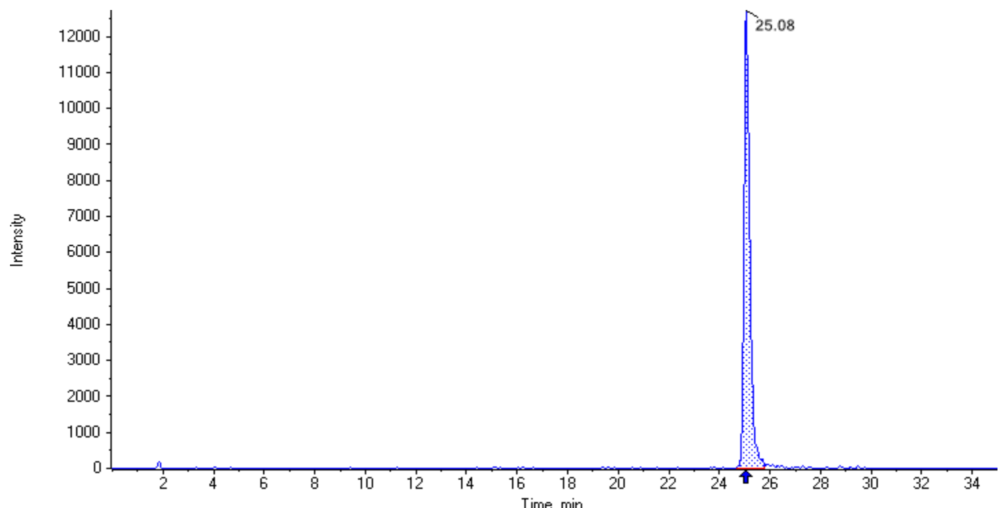

Figure 4.9: Chromatogram of DDAC-C-12 in solvent.

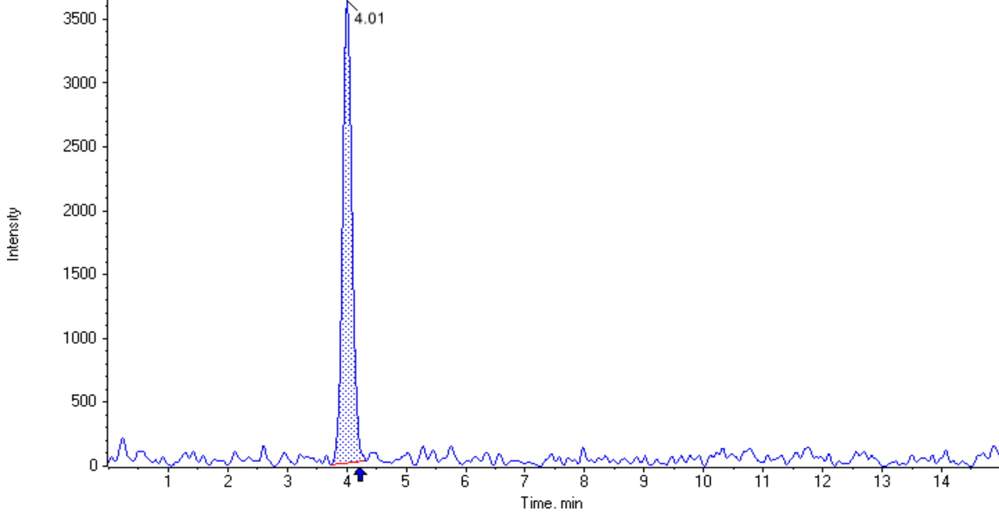

Figure 4.10: Chromatogram of chlorate in solvent.

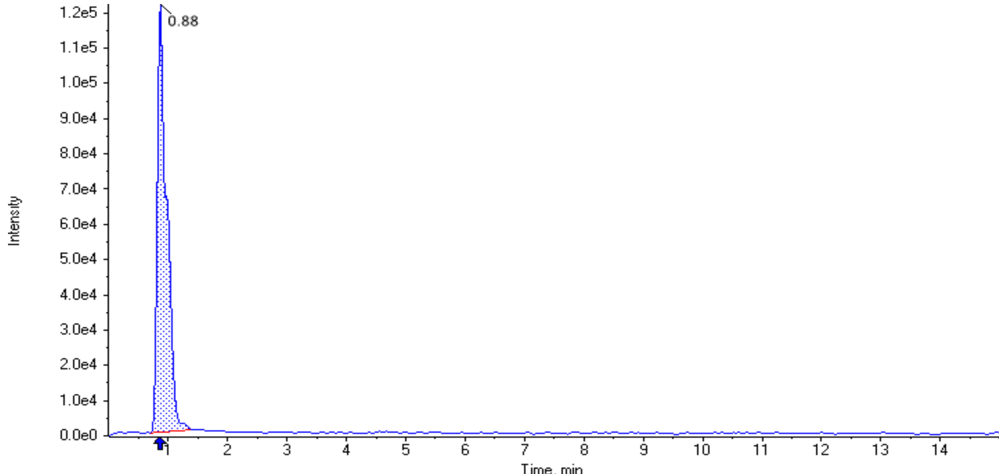

Figure 4.11: Chromatogram of histamine in solvent.

Figure 4.1 to Figure 4.11 represent the chromatograms obtained for each analyte solution in solvent. As can be seen in the chromatograms, the peaks of the individual analytes and their respective retention times are clearly visible. Therefore, since there is no difficulty in identifying the peak of each pesticide, we can say that the QuEChERS and QuPPe methods are specific.

4.2. Selectivity

The selectivity of the method can be confirmed by the chromatogram obtained from the fortification of the matrix with 1 mg/kg of histamine and 0.01 mg/kg of the remaining pesticides. A total of eight matrices were analysed, blue jack mackerel (2), black cardinal fish (3), bigeye tuna (5), atlantis bluefin tuna (6), european parrotfish (7), black scabbardfish (8), european seabass (9) and pink dentex (10). The resulting chromatograms for blue jack mackerel can be seen in Figure 4.12 to Figure 4.22. The remaining chromatograms for the other matrices studied, can be seen in the (Figures D.1 to D.77).

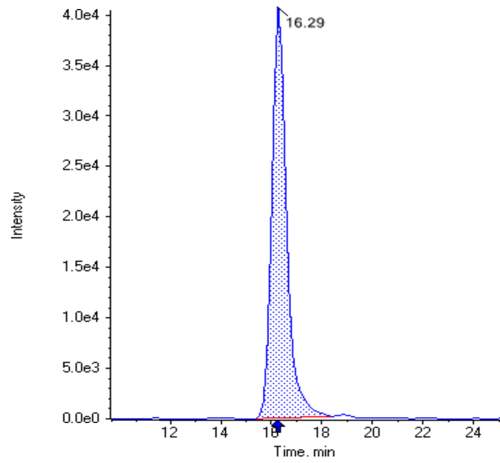

Figure 4.12: BAC8 chromatogram for blue jack mackerel.

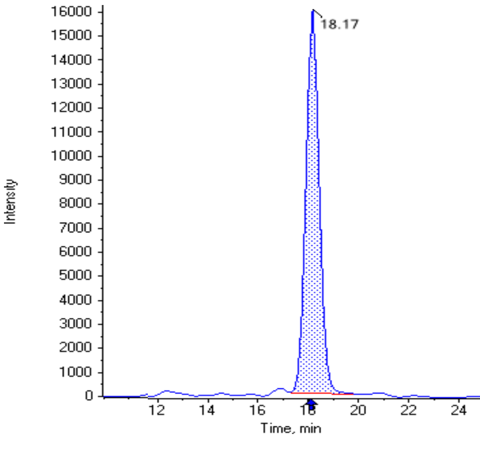

Figure 4.13: BAC10 chromatogram for blue jack mackerel.

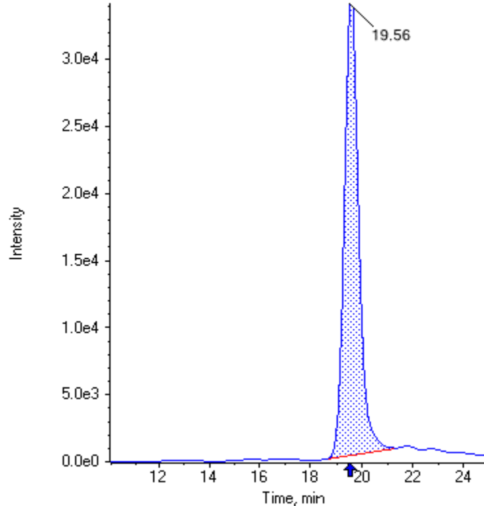

Figure 4.14: BAC12 chromatogram for blue jack mackerel.

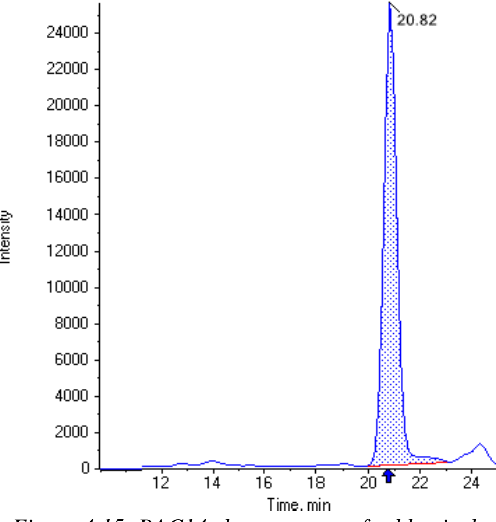

Figure 4.15: BAC14 chromatogram for blue jack mackerel.

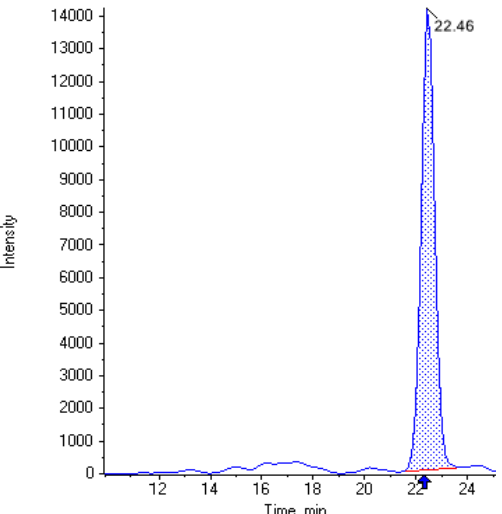

Figure 4.16: BAC16 chromatogram for blue jack mackerel.

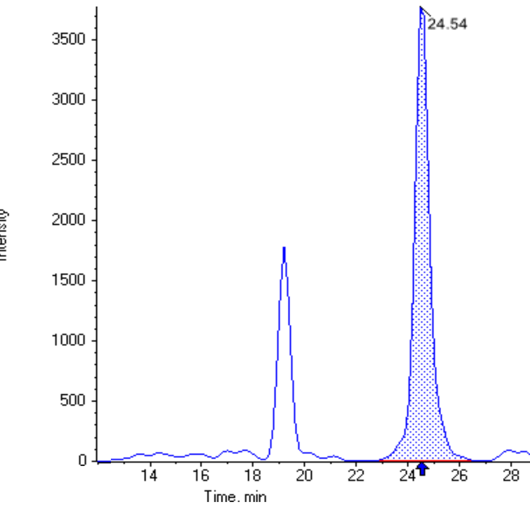

Figure 4.17: BAC18 chromatogram for blue jack mackerel

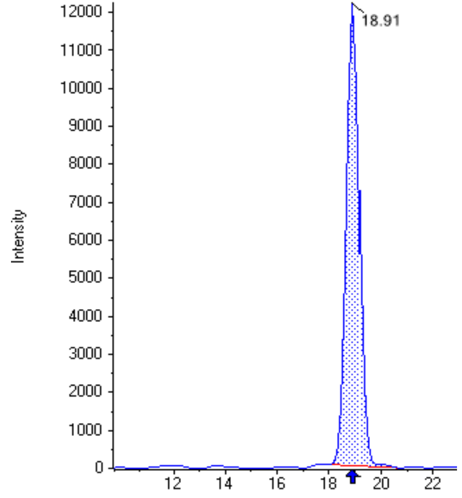

Figure 4.18: DDAC-C-8 chromatogram for blue jack mackerel.

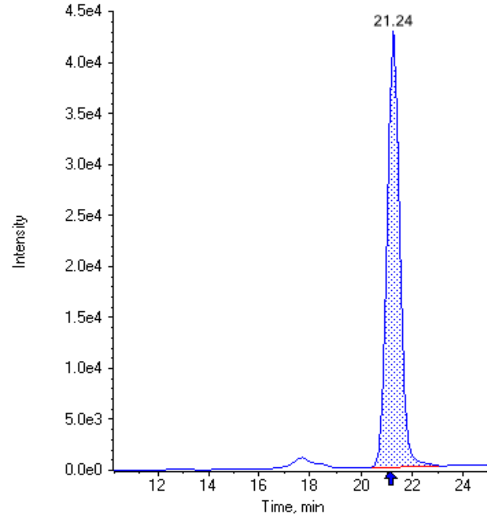

Figure 4.19: DDAC-C-10 chromatogram for blue jack mackerel.

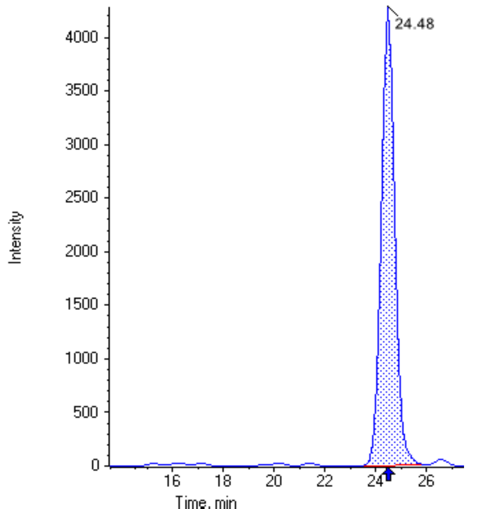

Figure 4.20: DDAC-C-12 chromatogram for blue jack mackerel

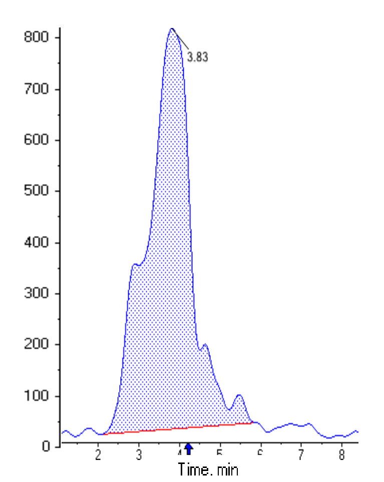

Figure 4.21: Chlorate chromatogram for blue jack mackerel.

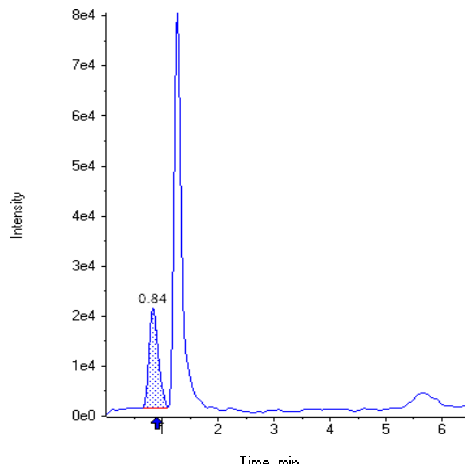

Figure 4.22: Histamine chromatogram for bluejack mackerel.

From looking at Figure 4.12 to Figure 4.22 we can see that each pesticide is independent of the interfering compounds in the matrix, which proves the selectivity of the methods. The difference in the analyte peaks in the different matrices is due to the matrix effect, which in fish is very important and influential.

Some chromatograms show peaks in addition to the pesticide peak. This is due to the existence of other interfering compounds already present in the matrix since it is very complex. The broad peak obtained for chlorate means a high retention time, which can lead to inaccurate results for this compound. One way of improving this result, would be to increase the flow of mobile phase in a controlled manner, as mentioned in chapter 2.4.

4.3. Linearity and Linear Ranges

The first step in obtaining the proportionality relationship between the concentration of the analyte and the response measured by the LC-MS/MS system, consisted of preparing 30 vials for each pesticide. These thirty vials were divided into six different concentrations, 0.01, 0.04, 0.08, 0.12, 0.16, 0.18 and 0.20. For the lowest concentration, ten replicates were performed and for the remaining concentrations, five replicates were prepared. The pesticide solutions used were 400 ng/μL.

4.3.1. Working range

The working range is an essential stage at the beginning of confirming the linearity of the method, as it makes it possible to ascertain whether the method is acceptable within the limits studied. According to *Practical HPLC Method Development* book [125], the working range must contain the maximum residue limit in the middle. In this case, the working range was based on the range used in the LRVSA for other pesticides. Using the variances obtained from the responses for each concentration, Fisher's test was carried out as shown in Table 4.1.

Table 4.1: Fisher's test to confirm the working range.

Pesticide	$F_{calculated}$	$F_{critical}$
BAC8	8.13	10.97
BAC10	7.67	
BAC12	6.99	
BAC14	7.50	
BAC16	7.84	
BAC18	8.11	
DDAC-C-8	7.48	
DDAC-C-10	7.24	
DDAC-C-12	8.00	
Chlorate	8.48	
Histamine	7.60	

It can be seen from Table 4.1 that, since the $F_{calculated}$ is lower than the $F_{critical}$, it was possible to establish that the working range was well adjusted for both methods.

4.3.2. Calibration Curves

Calibration curves that relate the peak area obtained to the known concentration, as well as the coefficient of determination obtained from the curves, allow the linearity of the method to be checked. The graphical representations obtained can be found in the appendix. The curves achieved can be visualised in Table 4.2.

Table 4.2: Calibration curves for pesticides.

Pesticide	Curve	R^2
BAC8	y = 62 413 535.9386x + 303 226.3661	0.9955
BAC10	y = 109 986 752.5205x + 774 560.8810	0.9926
BAC12	y = 124 540 035.0236x + 1 873 620.0436	0.9928
BAC14	y = 144 751 938.5667x + 1 274 750.2874	0.9924
BAC16	y = 127 818 463.1695x + 747 610.6037	0.9939
BAC18	y = 28 438 710.0072x + 117 212.9648	0.9924
DDAC-C-8	y = 69 237 529.3741x + 641 438.6376	0.9911
DDAC-C-10	y = 100 357 954.5860x + 1 159 387.1222	0.9904
DDAC-C-12	y = 26 525 753.5515x + 116 649.7042	0.9925
Chlorate	y = 4 176 113.2709x + 3 629.3939	0.9987
Histamine	y = 98 404 961.6066x + 736 831.7169	0.9955

After calculating the equation of the calibration curves, Table 4.2, make it possible to establish the linear and proportional behaviour of the analysed data. The closeness of the coefficients of determination to 1 also confirms the linearity of the methods. Allthe coefficients observed in the graphical representations are greater than 0.90.

Pearson's coefficient is another additional criteria for confirming linearity. This coefficient was then calculated using the *Pearson* function in *Excel* for each analyte, as shown in Table 4.3.

Table 4.3: Pearson correlation coefficient.

Pesticide	r
BAC8	0.9978
BAC10	0.9963
BAC12	0.9964
BAC14	0.9962
BAC16	0.9969
BAC18	0.9962
DDAC-C-8	0.9955
DDAC-C-10	0.9952
DDAC-C-12	0.9962
Chlorate	0.9993
Histamine	0.9977

The Pearson coefficients displayed in Table 4.2 allow us to affirm that the method is still linear, since its value is greater than 0.98 for all the analytes, as established by the laboratory guidelines.

4.3.3. Mandel's Test

According to chapter 2.9.2, statistical tests are crucial in the study of linearity since the calibration curves and coefficients mentioned are not enough to conclude about this parameter.

For the Mandel linearity test, the areas obtained from four replicates for each concentration in the working range, were considered. The values obtained for this test are presented in Table 4.4.

Table 4.4: Mandel's test for the concentration range 0.01 to 0.20.

Pesticide	S_{lin}	$S_{non\text{-}lin}$	DS^2	VT	$F_{critical}$
BAC8	1.21E+11	8.56E+10	2.26E+11	2.64	10.13
BAC10	5.92E+11	1.39E+11	1.95E+12	14.02	
BAC12	7.52E+11	2.45E+11	2.27E+12	9.28	
BAC14	1.11E+12	2.86E+11	3.57E+12	12.49	
BAC16	6.98E+11	2.00E+11	2.19E+12	10.96	
BAC18	3.55E+10	1.91E+10	8.47E+10	4.43	
DDAC-C-8	2.71E+11	8.71E+10	8.23E+11	9.45	
DDAC-C-10	6.25E+11	1.75E+11	1.98E+12	11.31	
DDAC-C-12	2.74E+10	8.76E+09	8.34E+10	9.53	
Chlorate	6.82E+07	5.13E+07	1.19E+08	2.32	
Histamine	2.62E+11	2.63E+10	9.71E+11	36.89	

In agreement with the Mandel's test conducted on Table 4.4 for the working range 0.01 to 0.20, it can be established that the VT test value is higher than the critical value for BAC10, BAC14, BAC16, DDAC-C-10 and histamine. This means that the linear approximation for these compounds is not adequate. The solution would be to consider non-linear functions or to reduce the working range for them.

Therefore, in Table 4.5 was decided to carry out the Mandel's test again for a lower concentration range.

Table 4.5: Mandel's test for the concentration range 0.04 to 0.20.

Pesticide	S_{lin}	$S_{non\text{-}lin}$	DS^2	VT	$F_{critical}$
BAC8	8.94E+10	1.13E+11	4.20E+10	0.37	18.51
BAC10	3.22E+11	1.77E+11	6.12E+11	3.47	
BAC12	3.64E+11	2.78E+11	5.37E+11	1.94	
BAC14	5.80E+11	3.52E+11	1.04E+12	2.94	
BAC16	3.98E+11	2.62E+11	6.70E+11	2.56	
BAC18	2.17E+10	2.22E+10	2.07E+10	0.94	
DDAC-C-8	1.41E+11	1.05E+11	2.16E+11	2.06	
DDAC-C-10	3.07E+11	2.00E+11	5.19E+11	2.59	
DDAC-C-12	1.60E+10	1.01E+10	2.79E+10	2.77	

Table 4.5 demonstrates that for a working range of 0.04 to 0.20, linearity is proven by the Mandel's test for BACs and DDACs.

For chlorate, following the QuPPe method, linearity is confirmed throughout the working range (Table 4.4), which is not the case for histamine. This latter should therefore be considered a non-linear function.

4.3.4. RIKILT's Test

The Rikilt's test is based on a response factor calculated for each concentration. The factors obtained in Table 4.6 were calculated using the equation 2.8.

Table 4.6: Response factor in % for each analyte at different concentrations – Part 1 of 2.

	Concentration in ppm					
Pesticide	**0.01**	**0.04**	**0.08**	**0.12**	**0.16**	**0.20**
BAC8	99.31	101.89	102.02	100.20	98.53	99.62
	97.85	102.21	101.20	100.05	100.14	99.60
	101.52	99.24	98.44	99.33	102.70	101.63
	101.31	96.66	98.34	100.42	98.64	99.15
BAC10	97.15	99.93	99.63	100.28	102.82	101.10
	98.30	100.98	102.58	98.98	99.14	100.76
	100.29	99.17	99.70	99.88	97.29	97.83
	104.26	99.92	98.10	100.86	100.79	100.31
BAC12	100.42	102.79	97.03	99.20	98.74	98.28
	102.33	101.80	101.58	97.99	100.86	100.45
	97.34	100.18	100.52	100.03	100.29	100.29

Table 4.6: Response factor in % for each analyte at different concentrations – Part 2 of 2.

	Concentration in ppm					
Pesticide	**0.01**	**0.04**	**0.08**	**0.12**	**0.16**	**0.20**
BAC12	99.91	95.24	100.87	102.79	100.11	100.98
BAC14	99.28	102.10	97.83	100.07	99.21	100.85
	99.00	102.46	100.10	98.73	98.20	99.95
	98.23	99.06	100.56	101.49	101.64	100.06
	103.49	96.37	101.51	99.71	100.96	99.14
BAC16	98.79	99.85	99.91	99.21	100.39	100.86
	100.92	98.99	100.86	100.16	97.93	99.87
	99.18	102.41	98.40	100.59	101.37	98.30
	101.10	98.75	100.84	100.04	100.32	100.98
BAC18	100.65	99.79	97.64	100.02	97.83	99.17
	98.52	101.43	99.62	98.98	98.42	100.51
	100.98	98.69	100.14	101.11	101.82	99.32
	99.85	100.10	102.60	99.89	101.93	101.00
DDAC-C-8	99.56	99.71	102.12	100.16	98.07	99.06
	99.80	102.80	96.69	100.95	98.52	102.84
	100.84	100.23	101.12	101.19	101.54	96.75
	99.80	97.26	100.08	97.70	101.87	101.36
DDAC-C-10	100.65	102.18	98.68	101.61	98.56	102.06
	96.56	100.82	104.12	99.72	100.16	101.21
	99.16	99.02	98.09	101.00	98.51	99.14
	103.64	97.99	99.11	97.67	102.78	97.58
DDAC-C-12	97.60	100.69	96.96	99.22	95.38	100.78
	103.45	102.09	102.62	99.38	100.02	100.69
	99.60	97.49	98.28	101.21	102.24	97.41
	99.35	99.74	102.14	100.19	102.37	101.12
Chlorate	101.61	98.57	101.30	103.14	101.01	100.08
	95.86	98.35	96.25	102.60	97.67	100.21
	94.20	100.96	103.38	96.63	98.92	98.23
	108.33	102.12	99.07	97.64	102.40	101.48
Histamine	100.12	101.38	100.55	101.35	100.91	99.30
	96.83	101.97	98.25	101.78	99.67	100.52
	99.06	101.79	99.02	98.95	98.14	99.88
	103.99	94.86	102.18	97.93	101.28	100.29

The acceptance range for these response factors is between 90 and 110%. Table 4.6 shows that all the values are within the range, meaning that linearity of the methods is evident for all the analytes, unlike the Mandel's test.

Since the RIKILT's test considers each point independently, based on its response factor, it was considered that linearity is proven for all the pesticides analysed in both methods. Furthermore, when we apply the standard addition method to a pesticide, we usually check the linearity of the method again (chapter 4.10).

It's important to note that in pesticide analyses the working ranges are very small, so it is much easier to prove linearity.

4.3.5. Normality of Residues

In order to ensure the normality of the residues, graphical representations were made of the normalised residues as a function of concentration for each pesticide studied. The resulting graphs can be found in Figure 4.23 to Figure 4.33.

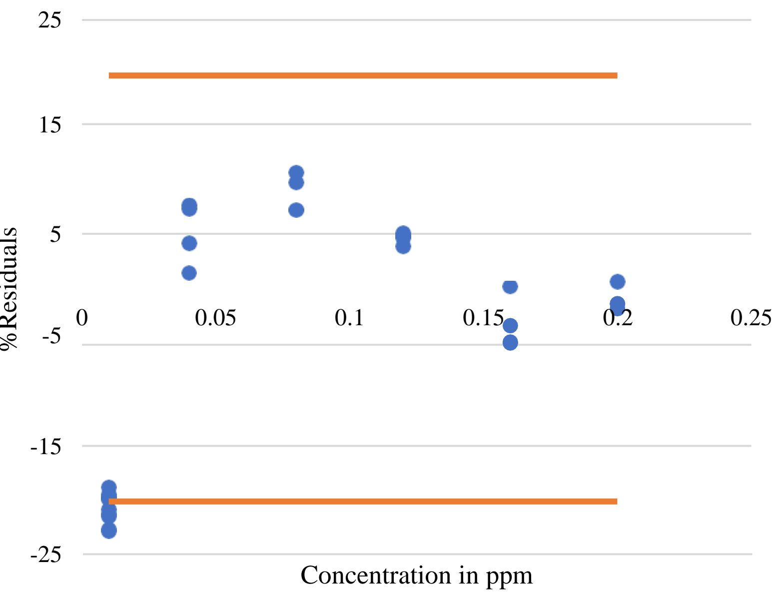

Figure 4.23: Normality of residues for BAC8.

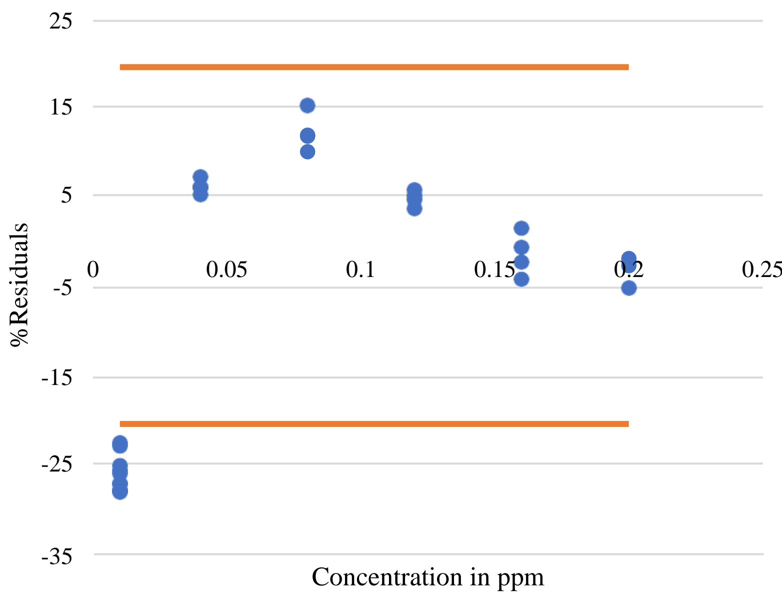

Figure 4.24: Normality of residues for BAC10.

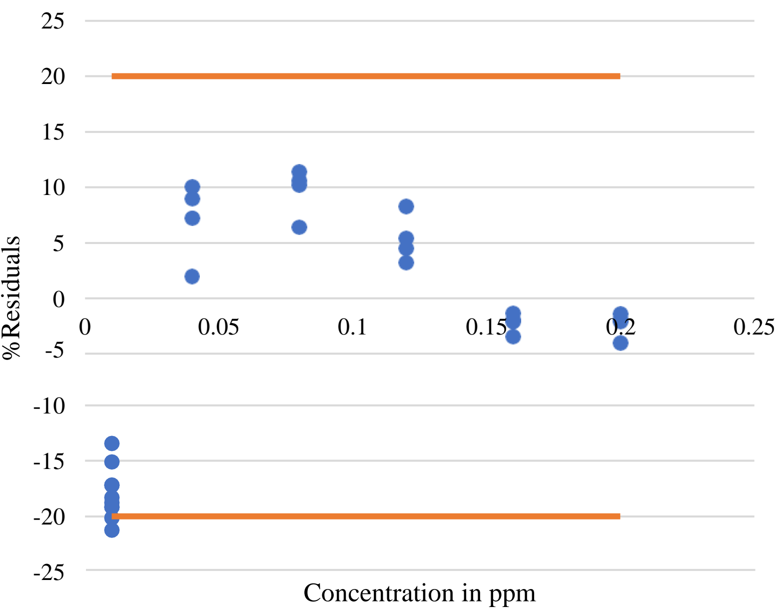

Figure 4.25: Normality of residues for BAC12.

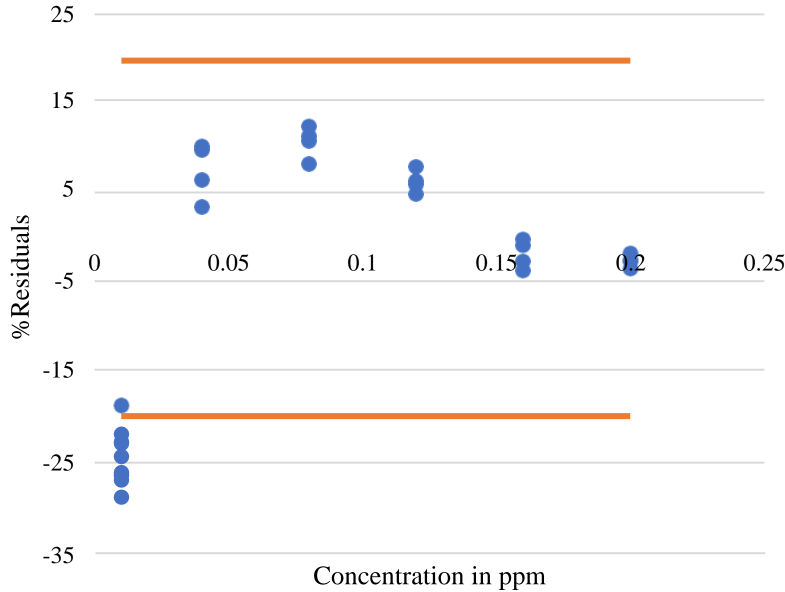

Figure 4.26: Normality of residues for BAC14.

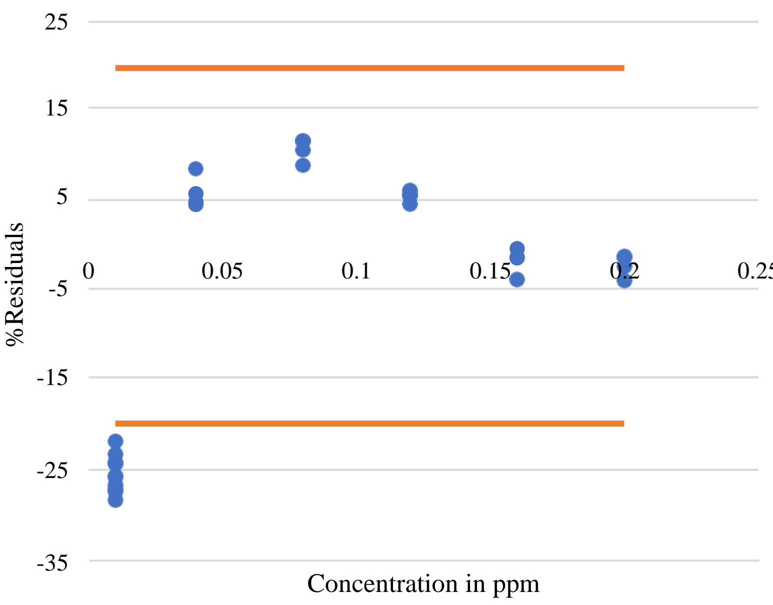

Figure 4.27: Normality of residues for BAC16.

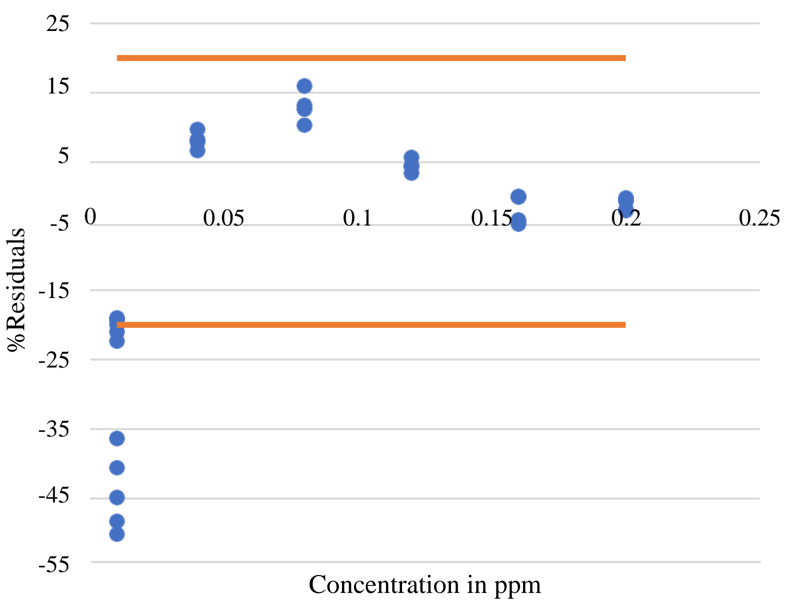

Figure 4.28: Normality of residues for BAC18.

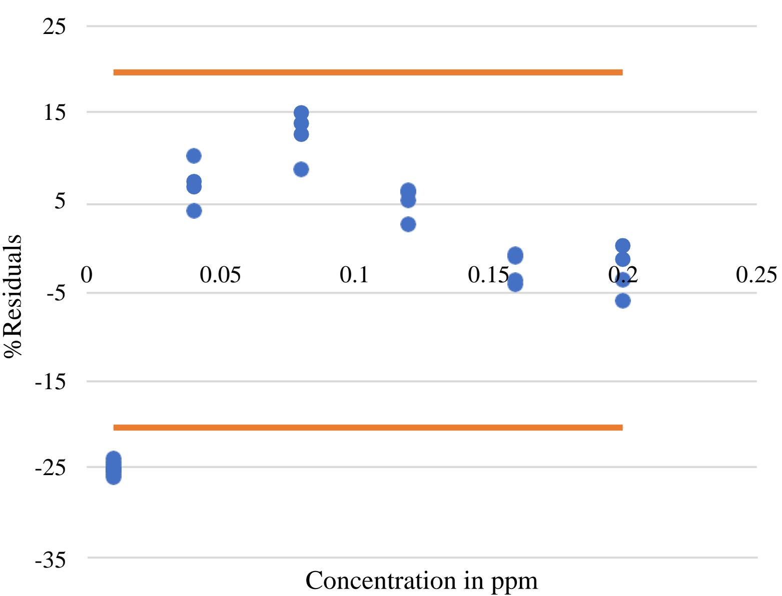

Figure 4.29: Normality of residues for DDAC-C-8.

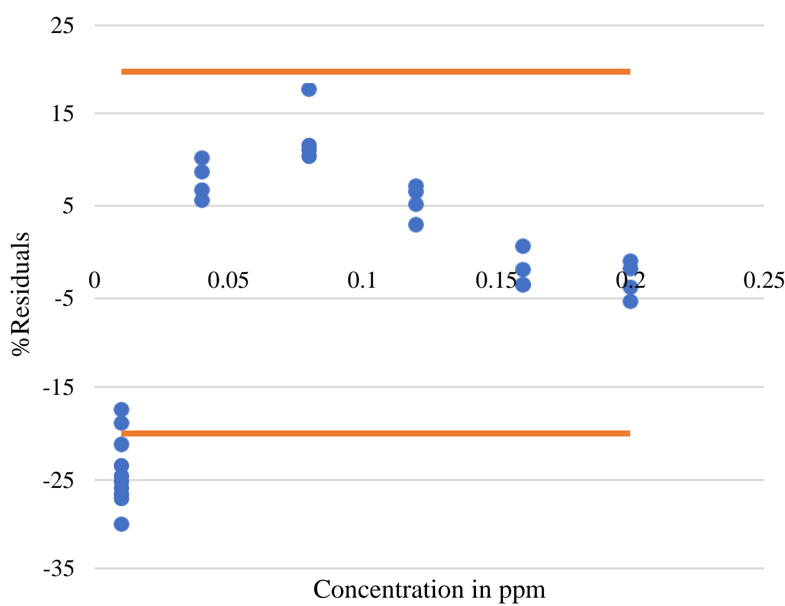

Figure 4.30: Normality of residues for DDAC-C-10.

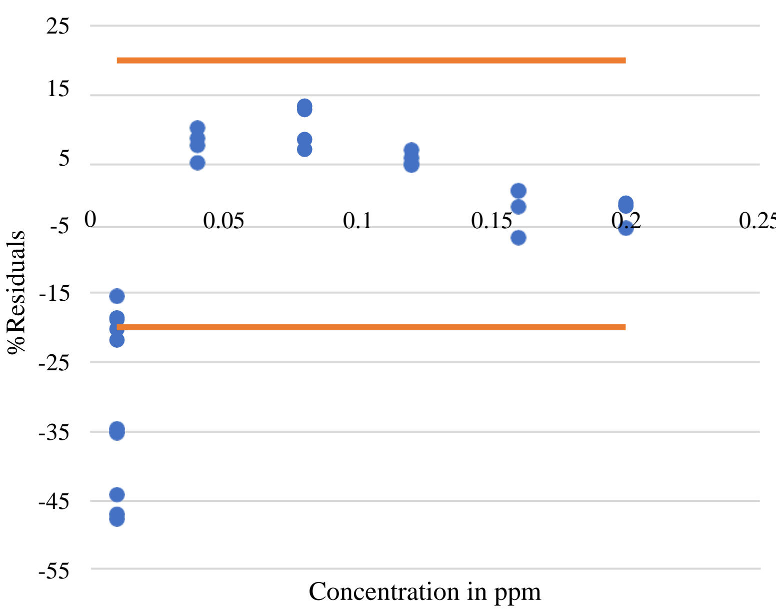

Figure 4.31: Normality of residues for DDAC-C-12.

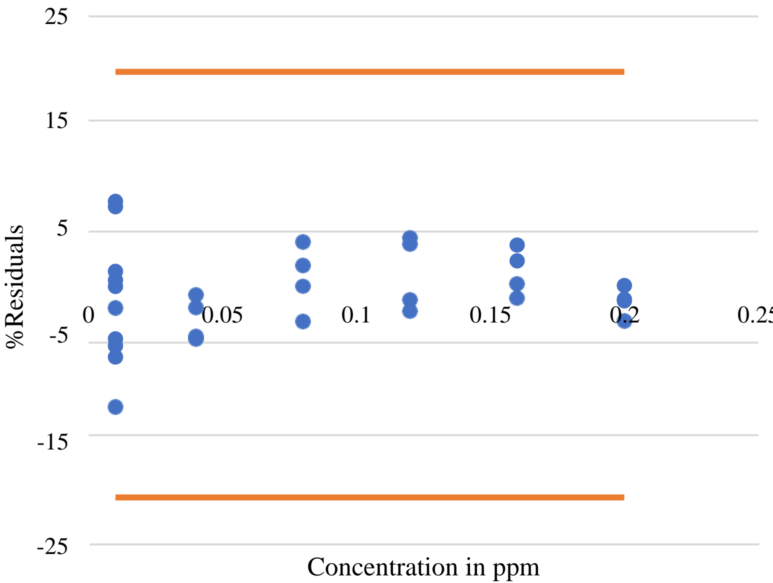

Figure 4.32: Normality of residues for Chlorate.

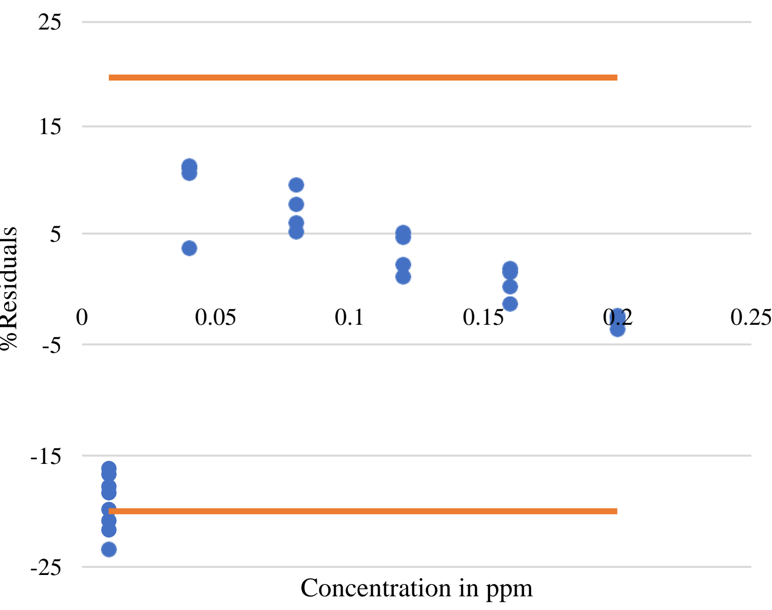

Figure 4.33: Normality of residues for Histamine.

Through the Figure 4.23 to Figure 4.33, it was possible to conclude that only chlorate was within the acceptable limits for deviations (±20%). It was also possible to verify that for the other pesticides, only the lowest concentration, 0.01 ppm, had deviations above 20%, and in the case of BAC18, it could reach approximately 50%. The fact that this happens could be due to the

failure of the Mandel's test, since when the test is carried out without the lower concentration, the test is valid, thus confirming linearity.

4.3.6. Homogeneity of Variances and Cochran's Test

The homogeneity of variances was analysed using the Cochran's test, where the variances were calculated using the equation 2.10. The test performance is shown in Table 4.7.

Table 4.7: Cochran's test for homogeneity of variances.

Pesticide	$C_{calculated}$	$C_{critical}$
BAC8	0.49	
BAC10	0.525	
BAC12	0.33	
BAC14	0.43	
BAC16	0.43	
BAC18	0.51	0.53
DDAC-C-8	0.527	
DDAC-C-10	0.39	
DDAC-C-12	0.52	
Chlorate	0.39	
Histamine	0.32	

The $C_{critical}$ value was taken from the attached table, for an n equal to 4 and a k equal to 6, for a 95% confidence interval. Table 4.7 shows that there is homogeneity of variances for all the analytes studied in the working range analysed, as the calculated C value is lower that the critical one.

4.3.7. Limit of Detection and Limit of Quantification

To determine the limit of quantification and the limit of detection, 10 assays were performed under repeatability conditions for each pesticide, at the RL level of 0.01 mg/kg. Working solutions of 400 ng/µL in solvent were used.

In order to calculate these limits in Table 4.8, the equations 2.11 and 2.13 were used. Furthermore, it was necessary to calculate the mean and the standard deviation of the concentrations of the assayscarried out.

Table 4.8: Quantification and detection limits.

	$\overline{X}$ in mg/kg	**s_0 in mg/kg**	**%RSD**	**LOQ in mg/kg**	**LOD in mg/kg**
BAC8	0.01	0.00017	1,69	0.0017	0.0005
BAC10	0.01	0.00026	2.59	0.0026	0.0008
BAC12	0.01	0.00029	2.85	0.0029	0.0009
BAC14	0.01	0.00039	3.89	0.0039	0.0012
BAC16	0.01	0.00026	2.65	0.0026	0.0008
BAC18	0.01	0.00193	19.31	0.0193	0.0058
DDAC-C-8	0.01	0.00008	0.79	0.0008	0.0002
DDAC-C-10	0.01	0.00051	5.06	0.0051	0.0015
DDAC-C-12	0.01	0.00183	18.30	0.0183	0.0055
Chlorate	0.01	0.00061	6.06	0.0061	0.0018
Histamine	0.01	0.00071	7.15	0.0071	0.0021

Table 4.8 reveals that all the analytes except BAC18 and DDAC-C-12 have quantification limits of less than 0.01 mg/kg. This may be because these compounds are widely used as disinfectants or to clean surfaces or food [9] and these compounds may already be in the device by default. In addition, the coefficients of variation for these two analytes exceed the 10% limit. The detection limits of all the pesticides studied are below the 0.01 mg/kg limit.

4.4. Accuracy

The accuracy of a method can be realised through recovery tests (chapter 2.9.4), using at least 5 replicates with 3 different concentration levels. These tests consider fortified and calibrated samples, the latter being samples that are only fortified at the end of the method's application, already in the vial. The matrices used for this test were sample number 9 (european seabass), 11 (blue jack mackerel), 12 (chub mackerel), 17 (bigeye tuna) and 18 (black scabbardfish).

Table 4.9 indicates the recovery values obtained as well as the relative standard deviation. Most of the recovery values obtained are within the acceptance range (70-120%). Recovery values obtained from BAC18, DDAC-C-12 and chlorate in different matrices and concentrations are below the acceptable value. According to *Guidance Document on Pesticide Residue Analytical Methods* from OCDE [84], when we are working with concentrations of 0.01 mg/kg or less, the acceptable range for recovery values is 60-120%. For the lowest concentration, 0.01 mg/kg, and for matrix 9, chlorate and histamine have a recovery value higher than the upper limit of the range.

Many factors can contribute to recovery values being outside the expected limits, such as poorextraction, insufficient agitation, pH of the mobile phase or problems with the equipment.

In terms of relative standard deviation, all the values obtained comply with the SANTE guidelines, i.e., less than 20%.

Table 4.9: Results obtained for accuracy.

			BAC8	BAC10	BAC12	BAC14	BAC16	BAC18	DDAC-C-8	DDAC-C-10	DDAC-C-12	Chlorate	Histamine
0.01 mg/kg	9	R (%)	97.04	106.10	98.70	99.81	92.62	84.98	109.27	83.44	102.56	121.81	124.11
		RSD	2.33	2.54	1.33	3.00	3.41	2.58	3.64	2.06	9.25	6.68	4.54
	11	R (%)	98.89	101.76	98.76	94.74	93.21	86.38	99.30	98.34	81.88	88.16	110.40
		RSD	1.61	1.75	3.70	3.38	3.92	4.71	2.13	3.83	10.58	2.94	1.94
	12	R (%)	102.32	96.87	98.73	95.90	85.77	81.41	94.87	89.22	72.78	83.11	83.03
		RSD	1.66	3.12	3.78	2.54	3.18	6.73	4.45	2.33	11.39	4.41	3.49
	17	R (%)	98.54	95.00	103.86	98.19	86.90	74.29	105.64	100.21	85.45	58.36	98.61
		RSD	8.24	2.00	2.44	2.83	3.56	5.51	3.77	6.59	7.32	4.12	1.49
	18	R (%)	103.22	99.15	102.34	101.28	95.07	85.67	102.99	96.56	85.84	78.60	104.55
		RSD	4.40	4.81	2.50	3.06	3.06	5.27	3.93	3.97	5.33	1.91	5.31
0.04 mg/kg	9	R (%)	104.00	99.67	100.12	93.09	91.47	79.66	95.35	82.20	72.21	79.56	118.75
		RSD	2.49	1.58	0.97	2.30	1.50	2.69	2.47	1.45	5.07	4.88	4.62
	11	R (%)	97.21	95.49	97.41	71.51	80.72	62.69	98.37	95.38	67.51	79.01	103.83
		RSD	3.25	1.01	1.80	2.05	1.07	2.04	3.06	2.59	5.51	2.51	3.29
	12	R (%)	96.12	96.24	94.46	82.55	76.77	70.38	97.01	89.74	69.90	68.21	98.70
		RSD	1.10	1.62	0.79	1.51	1.18	4.62	0.81	1.82	3.50	2.22	1.72
	17	R (%)	100.52	95.53	98.03	92.27	84.42	88.09	94.93	91.57	81.45	55.22	92.37
		RSD	4.06	8.00	1.32	1.47	2.49	1.08	3.32	4.18	4.93	1.49	3.44
	18	R (%)	99.90	96.71	93.24	90.55	88.69	76.10	93.60	91.42	94.34	67.65	89.48
		RSD	3.59	2.86	2.87	3.01	4.31	4.12	3.59	3.18	4.65	2.37	3.01
0.1 mg/kg	9	R (%)	99.20	95.18	95.42	97.83	86.02	82.23	100.10	82.20	81.56	74.20	117.18
		RSD	2.12	2.03	1.40	0.93	1.65	2.51	2.81	1.51	3.41	2.37	1.96
	11	R (%)	103.31	100.83	100.47	90.93	84.12	73.12	107.14	98.96	84.36	75.05	106.23
		RSD	1.86	2.41	1.52	1.88	1.22	2.32	1.69	2.73	6.89	1.68	1.37
	12	R (%)	91.38	95.95	86.06	78.41	70.30	60.25	89.50	76.31	57.61	70.04	96.73
		RSD	1.22	3.16	2.06	1.21	2.00	3.96	2.01	2.66	3.33	0.86	2.92
	17	R (%)	91.67	101.78	96.28	93.92	86.80	79.50	98.71	94.24	81.25	63.96	97.49
		RSD	4.92	5.96	1.15	1.56	1.48	1.46	1.51	2.19	5.26	1.63	2.05
	18	R (%)	98.36	94.54	92.43	90.99	83.07	87.70	92.50	96.54	96.19	70.62	100.82
		RSD	1.82	1.33	2.11	2.18	3.39	3.18	2.03	2.76	3.22	1.37	1.87

4.5. Precision

In the precision test, experiments were carried out for repeatability and intermediate precision. In the repeatability test, 10 injections were carried out, all under the same conditions, while in the intermediate precision test, the same conditions as the methods were maintained but the injections were performed on two different days. The values achieved are reflected in Table 4.10, Table 4.11 and Table 4.12.

Table 4.10: Recovery values obtained for two different days for BAC8, BAC10, BAC12 and BAC14.

	BAC8		**BAC10**		**BAC12**		**BAC14**	
	Day 1	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**
9	96,71	113,69	108.87	117.01	98.95	113.33	99.70	114.48
11	99,45	112,55	102.60	110.86	99.19	102.55	95.28	107.76
12	103,32	121,64	97.97	106.93	99.51	109.30	96.73	107.33
17	96,08	109,31	96.11	104.50	103.17	105.62	97.46	106.23
18	101,99	104,40	97.47	104.58	100.17	106.07	100.19	104.19
Average	99,51	112,32	100.60	108.76	100.20	107.37	97.87	108.00
Std Deviation	3,17	6,34	5.23	5.28	1.72	4.10	2.05	3.88
RSD in %	3,19	5,64	5.20	4.85	1.72	3.82	2.01	3.59

Table 4.11: Recovery values obtained for two different days for each analyte for BAC16, BAC18, DDAC-C-8 and DDAC-C-10.

	BAC16		**BAC18**		**DDAC-C-8**		**DDAC-C-10**	
	Day 1	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**
9	93.20	103.91	87.91	105.37	109.86	135.10	83.79	147.73
11	93.14	98.52	86.83	115.81	100.77	116.74	99.17	107.85
12	86.60	98.90	82.01	99.13	93.83	115.20	90.56	121.17
17	87.01	100.20	75.76	73.37	106.77	123.13	98.61	115.02
18	94.86	110.62	86.90	108.80	105.06	119.33	94.77	108.68
Average	90.96	102.43	83.88	100.50	103.06	121.90	93.38	119.89
Std Deviation	3.86	5.05	5.09	16.32	6.15	7.97	6.34	16.54
RSD in %	4.24	4.93	6.06	16.24	5.97	6.54	6.83	13.80

Table 4.12: Recovery values obtained for two different days for each analyte for DDAC-C-12, chlorate and histamine.

	DDAC-C-12		Chlorate		Histamine	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
9	106.07	128.98	113.43	67.69	119.67	57.22
11	83.55	108.77	87.08	97.55	110.25	106.16
12	73.59	76.70	83.77	82.59	82.52	80.57
17	79.85	77.49	56.71	66.60	98.70	97.20
18	86.38	98.56	79.73	94.16	101.20	81.18
Average	85.89	98.10	84.14	81.72	102.47	84.47
Std Deviation	12.26	22.08	20.24	14.42	13.88	18.72
RSD in %	14.27	22.51	24.05	17.65	13.54	22.16

Table 4.10, Table 4.11 and Table 4.12 represent the average of the recoveries of the 10 injections done for each of the five samples studied, and their relative standard deviation. As with accuracy, recovery values outside the range may be related to poor extraction, insufficient agitation, pH of the mobile phase or problems with the equipment. Furthermore, in the samples where recoveries achieved were beyond the expected range, lower on the second day, as in the case of histamine and chlorate in sample 9, this may be associated to the degradation of the pesticides over time due to the concentration gradient in the possible formation of precipitate. In addition, the vial used was the same for both days and remained in the equipment until it was injected. On the contrary, where the recovery obtained was greater on the second day can happen as a result of poor practical sample preparation. Only for one of the occasions of DDAC-C-12, chlorate and histamine, the RSD is higher than 20%.

In order to check for significant differences between recovery values, a Fisher's test was carried out, as demonstrated in Table 4.13.

Table 4.13: Fisher's test for the recovery values obtained in the intermediate precision test – Part 1 of 2.

Pesticide	Average	Std Deviation	RSD in %	$F_{calculated}$	$F_{critical}$
BAC8	105.91	5.01	4.73	16.34	5.32
BAC10	104.69	5.25	5.02	6.06	
BAC12	103.79	3.15	3.03	13.01	
BAC14	102.94	3.10	3.01	26.64	
BAC16	96.70	4.49	4.65	16.28	
BAC18	92.19	12.09	13.11	4.72	
DDAC-C-8	112.48	7.12	6.33	17.52	
DDAC-C-10	106.64	12.54	11.76	11.18	
DDAC-C-12	91.99	17.86	19.41	1.17	

Table 4.13: Fisher's test for the recovery values obtained in the intermediate precision test – Part 2 of 2.

Pesticide	Average	Std Deviation	RSD in %	$F_{calculated}$	$F_{critical}$
Chlorate	82.93	17.57	21.19	0.048	5.32
Histamine	93.47	16.48	17.63	2.99	

The Fisher's test displayed in Table 4.13 for the intermediate precision test, shows that, with the exception of BAC18, DDAC-C-12, chlorate and histamine, the calculated F value is higher than the critical one, thus showing that there are significant differences between the recovery values obtained.

4.6. Stability Testing

In the analysis of the stability of the analytes over time, it was necessary to make a 0.1 ng/µL solution with all the BACs and DDACs in acetonitrile, for the QuEChERS method and another solution of the same concentration with chlorate and histamine in methanol for the QuPPe method.

Both prepared solutions were used to fortify the matrices and then, stored the vials at -18ºC for subsequent analysis for different time periods in the LC-MS/MS system. The matrix used for the QuPPe method was sample 6 (atlantis bluefin tuna) and the sample used for the

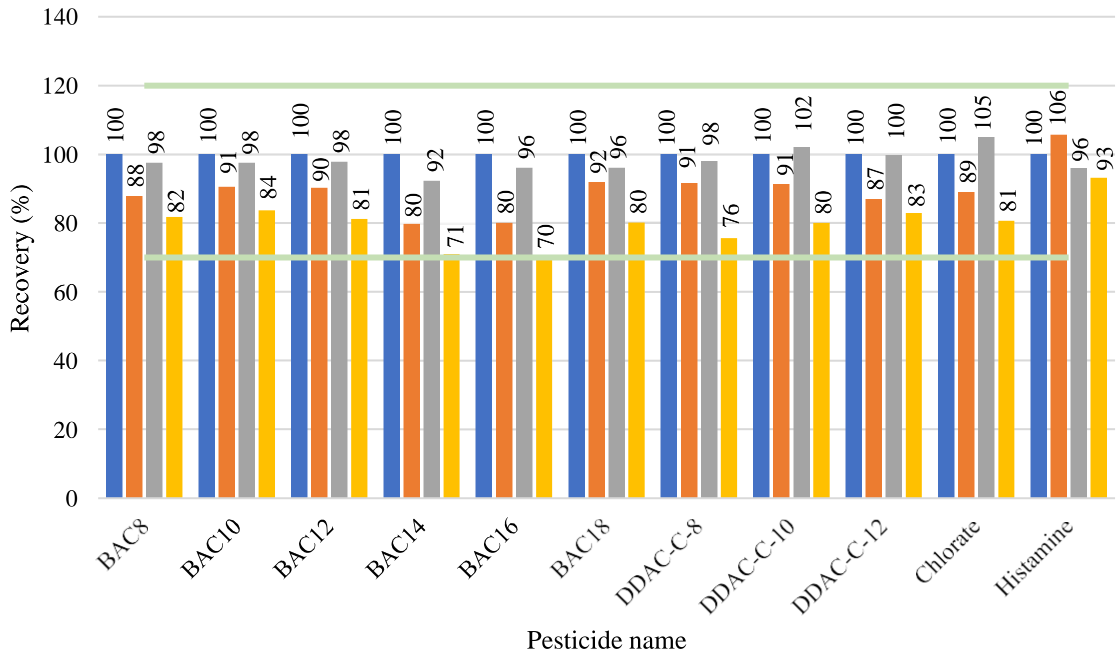

Figure 4.34: Stability of the analytes for 55 days.

QuEChERS method was sample 17 (bigeye tuna). The recovery results achieved during the 4 periods of time studied can be consulted in Figure 4.34.

Based on SANTE guidelines, acceptable recovery values in a stability test should be between 70 and 120%. As we can see from Figure 4.34, all the recoveries obtained are located within the acceptance range.

It is possible to see some fluctuations in the values achieved, especially on day thirty where recovery increases rather than decreasing. This may have been due to measurement errors in the pipetting. In the case of histamine, there is an increase in recovery on day ten, but the rest of the recoveries are as expected.

Checking for significant differences between the values obtained is an essential criteria. Two ANOVA analysis were therefore performed, one for the less polar compounds (BACs and DDACs) in Table 4.14 and the other for the polar compounds (chlorate and histamine) in Table 4.15.

Table 4.14: ANOVA for BAC8, BAC10, BAC12, BAC14, BAC16, BAC18, DDAC-C-8, DDAC-C-10 and DDAC-C-12.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	246.74	8	30.84	0.30	0.96	2.31
Within Groups	2807.85	27	103.99			
Total	3054.58	35				

Table 4.15: ANOVA for Chlorate and Histamine.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	51.07	1	51.07	0.68	0.44	5.99
Within Groups	449.10	6	74.85			
Total	500.17	7				

Since the calculated values of F observed in Table 4.14 and Table 4.15 are lower than the critical value, it can be established that there are no significant differences between the recoveryvalues in this stability test for both methods.

4.7. Robustness

The solutions used for the robustness test were prepared using sample 17 (bigeye tuna) at a concentration of 0.01 mg/kg. For this assay, 8 experiments were performed, considering the following 7 factors for the QuEChERS method.

1. Mass of the sample weighed before applying the extraction method.
2. Analyst conducting the experiments.
3. Mass of the QuEChERS mixture added before centrifuging the sample.
4. Volume of acetonitrile added as extraction solvent.
5. Stirring time after adding the QuEChERS mixture.
6. Centrifugation time.
7. Column temperature in LC-MS/MS system.

For the QuPPe method, the same 8 experiments were carried out, but with a slight change in the choice of the 7 factors since the extraction method is different.

1. Analyst conducting the experiments.
2. Mass of the sample weighed before applying the extraction method.
3. Volume of water added immediately after weighing the sample.
4. Volume of methanol added as extraction solvent.
5. Stirring time after adding the QuEChERS mixture.
6. Centrifugation time.
7. Column temperature in LC-MS/MS system.

Table 4.16: Factors and their levels for the robustness test using the QuEChERS method.

Factor	-	+
Sample mass	10 g	8 g
Analyst	Leonardo	Liliana
QuEChERS mass	6.50 g	3.25 g
Acetonitrile quantity	10 mL	9.7 mL
Stirring time	~1 min	~2 min
Centrifugation time	5 min	4 min
Column temperature	40ºC	36ºC

Table 4.17: Factors and their levels for the robustness test using the QuPPe method.

Factor	-	+
Analyst	Leonardo	Liliana
Sample mass	10 g	9 g
Water quantity	3 mL	2.7 mL
Methanol quantity	10 mL	10.3 mL
Stirring time	~1 min	~2 min
Centrifugation time	5 min	4 min
Column temperature	40ºC	36ºC

Table 4.16 and Table 4.17 shows the factors considered and their two levels defined for the QuEChERS and QuPPe methods, respectively. The (-) level represents the value normally used throughout the validation and the (+) level represents the change made.

In this way, Youden's approximation can be used to analyse robustness. The Table 4.18 provides the values reached using this approximation.

Table 4.18: Results obtained in the robustness test.

Pesticide	Area Average	Std Deviation	Std Deviation of the effect	RSD in %
BAC8	1793441.35	189192.51	143016.10	7.97
BAC10	2641462.74	129745.57	98078.43	3.71
BAC12	3172070.17	610582.49	461556.98	14.55
BAC14	2567077.64	776136.49	586704.04	22.86
BAC16	1946308.91	577493.20	436543.82	22.43
BAC18	236507.68	66625.49	50364.14	21.30
DDAC-C-8	668619.04	129121.39	97606.59	14.60
DDAC-C-10	1616694.67	475728.54	359616.97	22.24
DDAC-C-12	130853.09	38078.74	28784.82	22.00
Chlorate	485680.45	11353.41	8582.37	1.77
Histamine	570802.25	27443.91	20745.65	3.63

Table 4.18 shows the average of the response factor considered, the peak area, as well as its standard deviation and the standard deviation of the effect.

According to the SANTE guidelines, the coefficient of variation must be less than 20% for the method to be considered robust. Although BAC14, BAC16, BAC18, DDAC-C-10 and DDAC-C-12 had coefficients of variation greater than 20%, the deviation was not considered significant.

To make a conclusion about robustness, the same approach was followed as in chapter 2.9.8. The confidence intervals were calculated in Table 4.19 and Table 4.20, based on the calculated percentage effect.

Table 4.19: Confidence intervals obtained for the QuEChERS method.

Pesticide	Sample mass	Analyst	QuEChERS mass	Acetonitrile quantity	Stirring time	Centrifugation time	Column temperature
BAC8	[-128302;413608]	[-325890;216020]	[-192161;349748]	[-509856;32053]	[-340235;201674]	[-91992;449918]	[-228522;313388]
BAC10	[-134727;236906]	[-225762;145872]	[-278382;93252]	[-335340;36294]	[-226612;145022]	[-82721;288913]	[-78604;293029]
BAC12	[-545860;1203051]	[-633423;1115488]	[-511947;1236964]	[-762300;986611]	[-549567;1199344]	[-218147;1530764]	[-197342;1551569]
BAC14	[168906;2392020]	[-847238;1375875]	[-814129;1408983]	[-876947;1346166]	[-841567;1381546]	[-1412940;810172]	[-1414335;808778]
BAC16	[183990;1838123]	[-694148;959984]	[-664177;989955]	[-716096;938036]	[-649534;1004598]	[-1018912;635220]	[-969093;685039]
BAC18	[14808;205646]	[-76134;114703]	[-69311;121525]	[-76432;114405]	[-63452;127384]	[-117071;73766]	[-117363;73474]
DDAC-C-8	[-86544;283302]	[-138824;231021]	[-117535;252310]	[-153973;215873]	[-122687;247158]	[-55825;314021]	[-41706;328140]
DDAC-C-10	[132621;1495266]	[-540355;822289]	[-514414;848230]	[-569341;793303]	[-528534;834110]	[-817938;544706]	[-846638;516006]
DDAC-C-12	[9941;119011]	[-46475;62594]	[-45267;63802]	[-39360;69710]	[-40722;68347]	[-69905;39164]	[-65003;44067]

Table 4.20: Confidence intervals obtained for the QuPPe method.

Pesticide	Analyst	Sample mass	Water quantity	Methanol quantity	Stirring time	Centrifugation time	Column temperature
Chlorate	[-9939;22580]	[-10974;21545]	[-17359;15160]	[-31770;749]	[-10681;21838]	[-24276;8242]	[-23049;9470]
Histamine	[-32210;46397]	[-22344;56263]	[-59116;19491]	[-73751;4857]	[-20173;58435]	[-51808;26799]	[-53327;25280]

Table 4.19 and Table 4.20 represent the confidence intervals obtained for both methods studied. Only the confidence interval for the sample mass does not include the value 0 [115] for BAC14, BAC16, BAC18, DDAC-C-10 and DDAC-C-12. In other words, only this factor is considered significant in the QuEChERS method.

4.8. Uncertainty Estimation

There are several factors to take into account when estimating the uncertainty of a method. In this case, the uncertainties associated with the purity standard, sample mass, precision and accuracy were calculated and represented in Figure 4.35.

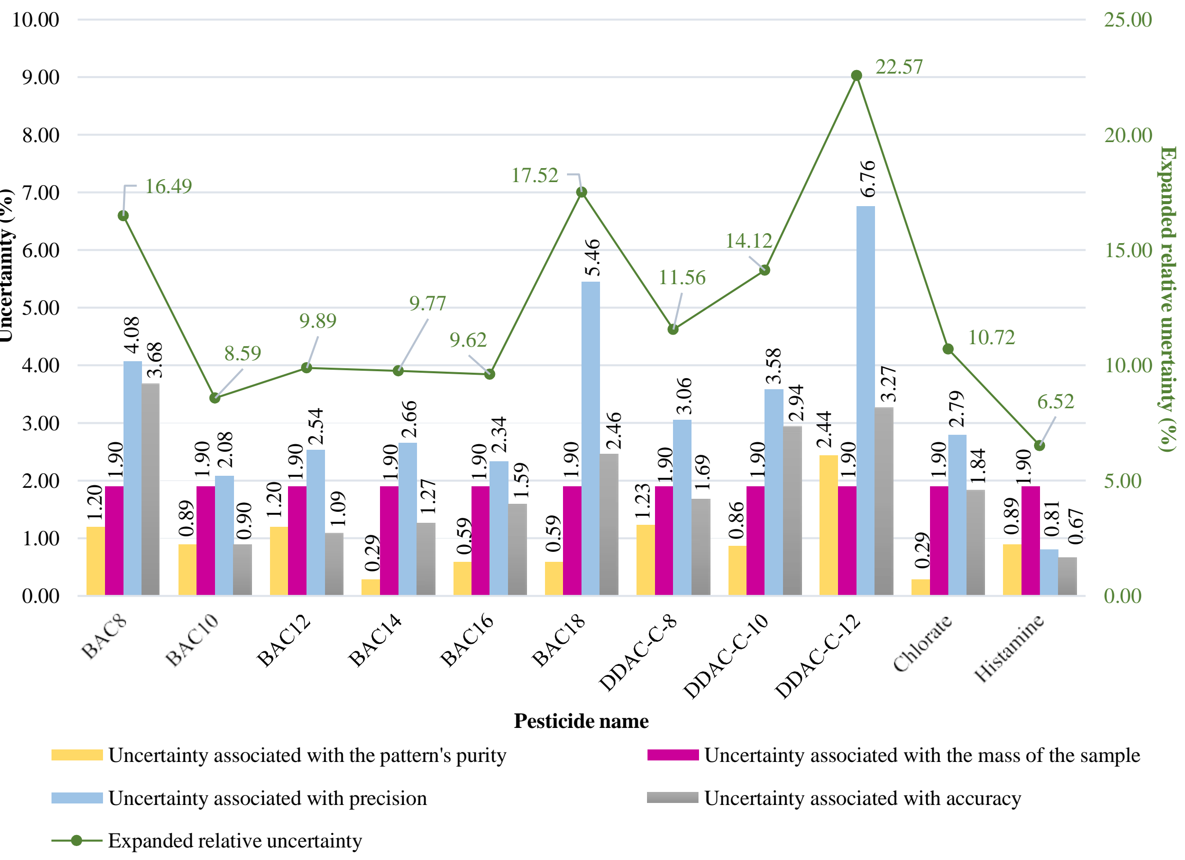

Figure 4.35: Combined uncertainties and expanded relative uncertainty for each analyte.

Figure 4.35 illustrates the combined uncertainty bars for each factor, while the green line shows the expanded relative uncertainty associated with the methods. It was possible to ascertain that the uncertainty associated with precision is the factor with the most influence on the uncertainty estimate. We can see that none of the points relating to expanded uncertainty exceed 50%, thus not exceeding the acceptance criteria.

4.9. Matrix Effect

One of the foods that has a high matrix effect is fish [120]. As mentioned in chapter 2.9.8, the matrix effect can be ascertained through the coefficient of variation of the recovery values. Their values are shown in Table 4.21.

Table 4.21: Matrix effect for pesticides.

Pesticide	Matrix effect in %	RSD in %
BAC8	100.25 – 468.29	37.78
BAC10	42.30 – 96.45	36.67
BAC12	53.32 – 100.13	21.08
BAC14	31.66 – 92.66	38.24
BAC16	33.80 – 79.07	28.64
BAC18	43.81 – 92.41	28.54
DDAC-C-8	21.86 – 83.98	48.74
DDAC-C-10	39.79 – 100.64	25.55
DDAC-C-12	50.92 – 98.25	27.94
Chlorate	114.07 – 343.90	35.78
Histamine	2.92 – 38.36	49.43

The coefficients of variation in Table 4.21, which are higher than 15%, confirm the high matrix effect in the fish matrix. Furthermore, matrix effect values further away from 100% imply a greater matrix effect and, as can be seen from the values above, BAC8 and chlorate reach values of more than 340%.

4.10. Standard Addition Method

The standard addition method enables the high matrix effect to be corrected and, as we can see from Table 4.21, the matrix effect in fish can be very high. This is therefore an essential step to avoid possible errors in the limits of quantification.

The concentration of pesticide obtained in each matrix after applying the method is presented in Table 4.22.

Table 4.22: Pesticide concentration in mg/kg, obtained after the standard addition method.

Sample number	BAC 8	BAC 10	BAC 12	BAC 14	BAC 16	BAC 18	DDAC-C-8	DDAC-C-10	DDAC-C-12	Chlorate	Histamine
1	-	-	-	-	-	-	-	-	-	< 0.01	3.50
2	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.015	< 0.01	< 0.01	3.30
3	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
4	-	-	-	-	-	-	-	-	-	< 0.01	< 0.01
5	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	2.20
6	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	4.20
7	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
8	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	2.00
9	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
10	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.10
11	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	10.60
12	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	5.50
13	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	15.40
14	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.011	< 0.01	< 0.01	< 0.01
15	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	30.30
16	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	1.70
17	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.037	< 0.01	< 0.01	6.20
18	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

From the Table 4.22, we can deduce that after applying the method mentioned above, some samples exceed the limits allowed by legislation for the concentration of DDAC-C-10 and histamine, which means they shouldn't be on the market for consumption.

4.11. CCα

The decision limit for histamine is a metric that is important to really confirm the presence of the analyte. Its value, in Table 4.23, was calculated using the relative standard uncertainty of veracity and the relative standard uncertainty of precision.

Table 4.23: Value obtained for the decision limit.

Relative standard uncertainty of veracity, $u(C_m)$	2.05 mg/kg
Relative standard uncertainty of precision, u_r	7.51 mg/kg
Combined uncertainty, u_c	8.03 mg/kg
CCα	115.60 mg/kg

Based on the value obtained for CCα in Table 4.23, we can realise with 95% confidence that a matrix with a concentration equal to or greater than 115.60 mg/kg of histamine, contains this pesticide in its composition.

Table 4.24 summarises the validation of both methods.

Table 4.24: Summary of method validation.

	Linearity	LOQ (mg/kg)	Mean Recovery (%)	U' (%)	Selectivity/Specificity	Stability	Robustness	ME (%)	Standard Addition Method
BAC8	Linear	0.0017	100.00	16.49	Selective and specific	Stable	Robust	100.25 – 468.29	< 0.01 mg/kg
BAC10	Linear	0.0026	99.78	8.59	Selective and specific	Stable	Robust	42.30 – 96.45	< 0.01 mg/kg
BAC12	Linear	0.0029	100.48	9.89	Selective and specific	Stable	Robust	53.32 – 100.13	< 0.01 mg/kg
BAC14	Linear	0.0039	97.98	9.77	Selective and specific	Stable	Robust	31.66 – 92.66	< 0.01 mg/kg
BAC16	Linear	0.0026	90.71	9.62	Selective and specific	Stable	Robust	33.80 – 79.07	< 0.01 mg/kg
BAC18	Linear	0.0193	82.55	17.52	Selective and specific	Stable	Robust	43.81 – 92.41	< 0.01 mg/kg
DDAC-C-8	Linear	0.0008	102.41	11.56	Selective and specific	Stable	Robust	21.86 – 83.98	< 0.01 mg/kg
DDAC-C-10	Linear	0.0051	93.55	14.12	Selective and specific	Stable	Robust	39.79 – 100.64	0.037 mg/kg
DDAC-C-12	Linear	0.0183	85.70	22.57	Selective and specific	Stable	Robust	50.92 – 98.25	< 0.01 mg/kg
Chlorate	Linear	0.0061	86.01	10.72	Selective and specific	Stable	Robust	114.07 – 343.90	< 0.01 mg/kg
Histamine	Linear	0.0071	103.69	6.52	Selective and specific	Stable	Robust	2.92 – 38.36	30.30 mg/kg

5. Conclusions and Future Work

The validation of the methods for the eleven analytes tested made it possible to establish several conclusions regarding their behaviour in fish matrices. The different polarity between benzalkonium chloride (BAC), chlorate, didecyldimethylammonium chloride (DDAC) and histamine required the use of two different methods, QuEChERS and QuPPe, which were validated together. Therefore, the simultaneous determination for all pesticides was not possible.

The specificity and selectivity of both methods can be checked by looking at the chromatograms obtained, despite the existence of some noise in some analytes due to the matrix effect. It was also possible to calculate the detection limits and quantification limits for all the analytes during method validation. Only BAC18 and DDAC-C-12 showed quantification limits higher than the maximum permitted residue limit and were therefore not quantifiable in the working range considered (0.01-0.2 ppm).

With regard to the linearity of the methods, it was shown that the behaviour of some pesticides was not as expected according to the Mandel's test. BAC10, BAC14, BAC16, DDAC-C-10 and histamine revealed that their behaviour is best reflected by a quadratic curve in the working range studied. The normality of the residuals confirmed what was obtained in the Mandel's test, since the residues observed for the concentration of 0.01 mg/kg exceeded the deviation allowed by the legislation. On the other hand, the RIKILT's method and Pearson's coefficient show the linearity of all the analytes tested.

The recovery tests carried out on accuracy and precision, made it possible to state that some of the recovery values obtained are outside the permitted range. The recovery values are between 60.25 and 106.10% for BACs, between 57.61 and 109.27% for DDACs, between 55.22 and 121.81% for chlorate and between 83.03 and 124.11%. All the values achieved for the relative standard deviation are below 20% for accuracy. Regarding the precision test, under both repeatability and intermediate precision conditions, the recoveries reached were within the acceptable range, except for BAC8, DDAC-C-8, DDAC-C-10, DDAC-C-12 and histamine in the samples of european seabass, bigeye tuna and chub mackerel.

In the stability test of the pesticides at -18ºC, it was verified that in the four time periods considered, the recoveries of all the pesticides were within the expected range. This result was also confirmed by an ANOVA analysis which established that there were no significant differences between the values achieved.

To assess the robustness of both methods, the Youden's approximation according to Plackett-Burman was used, where it was found that only the sample mass factor was significant

in the QuEChERS method. The QuPPe method proved to be robust for all the factors considered. It is important to note that interactions between factors were not taken into account.

The estimates of uncertainty showed that none of the analytes exceed the 50% expanded uncertainty allowed by the regulations. The uncertainties obtained were 16.49% for BAC8, 8.59% for BAC10, 9.89% for BAC12, 9.77% for BAC14, 9.62% for BAC16, 17.52% for BAC18, 11.56% for DDAC-C-8, 14.12% for DDAC-C-10, 22.57% for DDAC-C-12, 10.72% for chlorate and 6.52% for histamine. Furthermore, the source of uncertainty that contributed most to the increase in uncertainty was the one associated with precision.

The matrix effect in fish matrices can affect the results obtained, especially the concentration of pesticide in a matrix. The values achieved for this effect proved that fish, whatever the sample analysed, contains a high matrix effect, which can reach 400%. In this way, the pesticides were quantified using the standard addition method. Even when using this method, DDAC-C-10 and histamine were found to be in higher concentrations than legally permitted.

Finally, the decision limit obtained for histamine of 115.60 mg/kg allowed us to establish that from this concentration onwards, the existence of this analyte in the analysed sample is confirmed.

As a proposal for future work, it is suggested that the method be validated to include original fish matrices that come directly from the environment in which they are found, without having been previously analysed. A new study on the working range is also suggested, since the maximum limit should be included in the middle and not at the beginning of the range.

In addition, it is important to note that, given the limited number of results and following a suggestion from the laboratory, outlier tests were not carried out, but instead the raw data was shown. Eliminating data outside the acceptance range, mainly in accuracy and precision tests, would, at the moment give a false sense of security. As more data is gathered, these results should be eventually disregarded.

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Appendix

A. Chromatographic Conditions

Figure A.1 shows the LC-MS/MS system at the Regional Veterinary and Food Safety Laboratory. Table A.1 and Table A.2 represent the chromatographic conditions used in methods performed and Table A.3 presents the composition of the QuEChERS mixture used in the method.

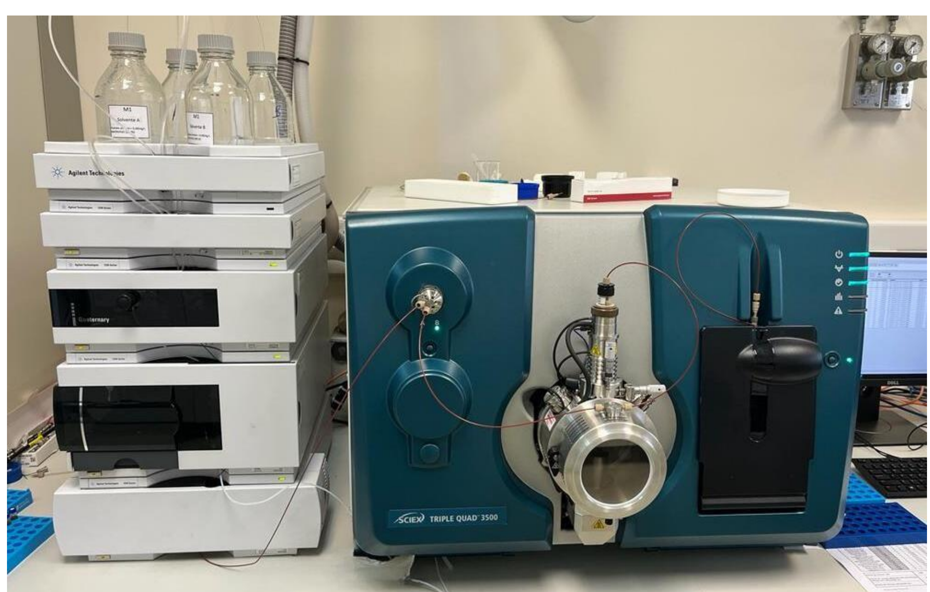

Figure A.1: LC-MS/MS system.

Table A.1: Chromatographic conditions for QuEChERS method.

Equipment	Chromatographic Conditions
LC-MS/MS Agilent 1200	**Column:** Waters Atlantis T3 5 µm, 2,1x150 mm **Flux:** 300 µL/min **Temperature:** 30ºC **Mobile phase A:** Water:Methanol (90:10) + 5mM ammonium acetate **Mobile phase B:** Water:Methanol (10:90) + 5mM ammonium acetate **Gradient:** Time A(%) B(%) 0 100 0 15 0 100 25 0 100 25.1 100 0 30 100 0

Table A.2: Chromatographic conditions for QuPPe method.

Equipment	Chromatographic Conditions
LC-MS/MS Agilent 1200	**Column:** Hypercarbe **Flux:** 300 µL/min **Temperature:** 40ºC **Mobile phase: A**: Water + 1% acetic acid + 5% methanol **Mobile phase B**: Methanol + 1% acetic acid **Gradient:** Time A(%) B(%) 0 100 0 10 70 30 10.1 100 0 15 100 0

Table A.3: Composition of the QuEChERS mixture.

QuEChERS mixture (For 100 g of sample)	→ 40 g of magnesium sulfate → 10 g of sodium chloride → 10 g of trisodium citrate dihydrate → 5 g of sodium hydrogencitrate sesquihydrate

B. Pesticides in quantification methods.

Table B.1 lists the pesticides used in the QuEChERS quantification method.

Table B.1: Pesticides used in quantification methods studied.

Reference	Pesticides
[48]	Methomyl, Methiocarb, Fenipropimorph, Tebuthiuron, Pirimicarb, Thiodicarb, Prochloraz, Trifloxystrobin, Acetamiprid, Thimetoxam, Difenconazole, Pyrimethanil, Ametryn, Boscalid, Butachlor, Cabaryl, Dimethomorph, Hexaconazole, Malathion, Propoxur, Spinosad A, Spinosad D, Spiroxamine, Thiabendazole, Thifensulfuron-methyl, Carbofuran, Dimethoate, Diuron, Ethoprophos, Fenamiphos, Fenbuconazole, Fludioxonil, Metalaxyl, Metsulfuron methyl, Monocrotophos, Pendimethalin, Pyrazosulfuron-ethyl, Triazophos, Azoxystrobin, Bentazon, Bitertanol, Cadusafos, Chlorotoluron, Cymoxanil, Iprodione, Linuron, Oxamyl, Propanil, Tebuconazole, Terbutryn, Tiofanate-methyl, Kresoxim-methyl, Carbendazim, Diazinon, Imidacloprid, Imazalil, Metribuzin, Profenofos, Propiconazole, Pyrachlostrobin, Triadimenol, Terbufos, Thiacloprid, Penconazole, Pirimiphos-methyl, Tebufenozide, Spirodiclofen, Cyflufenamid, Temephos, 2,4-D, Chlorpyrifos, Cyanazine, Terbutylazine, Propazine, Atrazine, Simazine, Isoproturon, Fenoxycarb, Epoxiconazole, Benalaxyl, Hexythiazox
[49]	6-Benzyladenine, Acetamiprid, Azadirachtin, Azinphos-ethyl, Benalaxyl-M, Bixafen, Boscalid, BTS 44595 (prochloraz metabolite), BTS 44596 (prochloraz metabolite), Carbendazim, Carbofuran, Chinomethionat, Chlorpyrifos, Chlorpyrifos-methyl, Clofentezine, Clothianidin, Coumaphos, Cyflumetofen, Cymiazol, Cyproconazole, Diazinon, Difenoconazole, Dimethoate, Epoxiconazole, Etofenprox, Famoxadone, Fenoxycarb, Fenthion, Fenthion-oxon, Fenthion-oxonsulfone, Fenthion-oxonsulfoxide, Fenthion-sulfone, Fenthion-sulfoxide, Fluquinconazole, Flusilazole, Fosthiazate, Furathiocarb, Halosulfuron-methyl, Hydroxy-tebuconazole, Imidacloprid, Indoxacarb, Isoxaben, Lenacil, Malathion, MCPA, MCPB, Methidathion, Metribuzin, N-2,4-(Dimethylphenyl)formamide (amitraz metabolite), N-2,4-Dimethylphenyl-N´-methylformamidine (amitraz metabolite), Oxadiargyl, Oxasulfuron, Paraoxon-methyl, Parathion, Parathion-methyl, Penthiopyrad, Phosalon, Phosmet, Phoxim, Pinoxaden, Pinoxaden metabolite M4, Pirimiphos-methyl, Profenofos, Propoxur, Pyrazophos, Spirodiclofen, Tetraconazole, Thiacloprid, Thiametoxam, Tri-allate, Triazophos, Zoxamide
[50]	Acephate, Acetamiprid, Atrazine, Azoxystrobin, Bendiocarb, Cadusafos, Carbaryl, Carbendazim, Carfentrazone-ethyl, Chlorfenvinphos, Chlorpyrifos, Cyanofenphos, Diazinon, Diflubenzuron, Dimethoate, Dimethomorph, Diuron, Ethion, Ethoprophos, Etoxazole, Ethoxyquin, Fenitrothion, Fenobucarb, Ferimzone, Fipronil, Flufenoxuron, Fluridone, Fonofos, Hexaconazole, Imazapyr, Imidacloprid, Isofenphos, Isoprocarb, Isoprothiolane, Isoxathion, Malathion, Metalaxyl, Methamidophos, Methidathion, Methiocarb, Metolcarb, Mevinphos, Monocrotophos, Omethoate, Phenthoate, Phosalone, Phosmet, Phosphamidone, Pirimicarb, Pirimiphos-methyl, Profenofos, Promecarb, Prometryn, Propiconazole, Propoxur, Pyridaphenthion, Quinalphos, Simazine, Tebuconazole, Teflubenzuron, Thiacloprid, Thiamethoxam, Thiobencarb, Thiophanate-methyl, Triazophos, Trichlorfon
[51]	Acetamiprid, Ametryn, Amitraz, Atrazine, Azinphos methyl, Azoxystrobin, Boscalid, Carbaryl, Carbendazim, Carbofuran, Clomazone, Cyproconazole, Difenoconazole, Dimethoate, Epoxiconazole, Flutriafol, Flusilazole, Hexythiazox, Imazalil, Malaoxon, Malathion, Metalaxyl, Metamidophos, Methidathion, Methiocarb, Metolachlor, Metribuzin, Metsulfuron methyl, Pendimethalin, Penoxulam, Pirimicarb, Pirimiphos methyl, Prochloraz, Propanil, Propiconazole, Pyraclostrobin, Pyrazosulfuron ethyl, Pyrimethanil, Tebuconazole, Thiacloprid, Thiabendazole, Thiamethoxam, Tricyclazole, Trifloxystrobin

C. Non-linear and Linear calibration curves

In addition to the linear calibration curves, non-linear calibration curves were also calculated (Figure C.1 to Figure C.11), which were essential for checking the linearity of the method according to the Mandel's test.

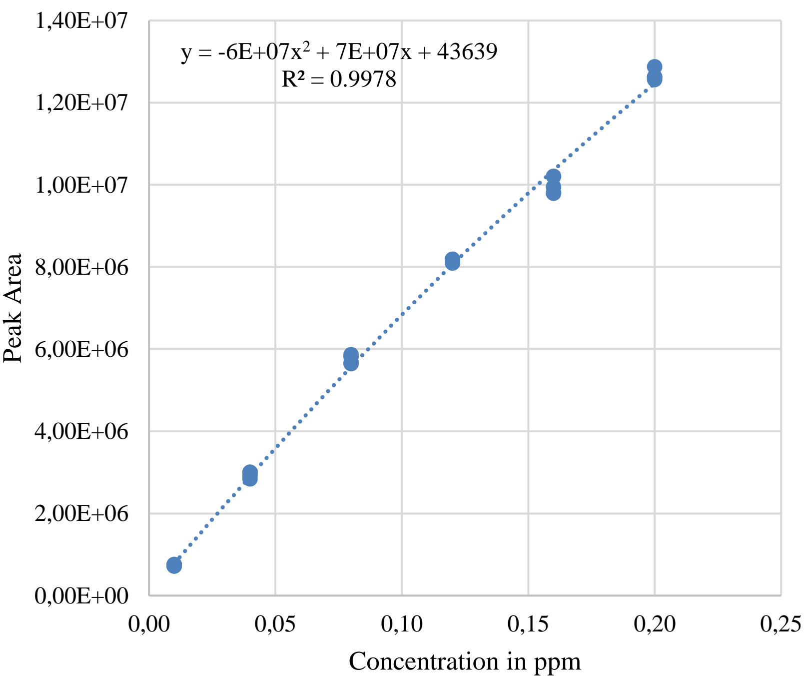

Figure C.1: BAC8 non-linear calibration curve.

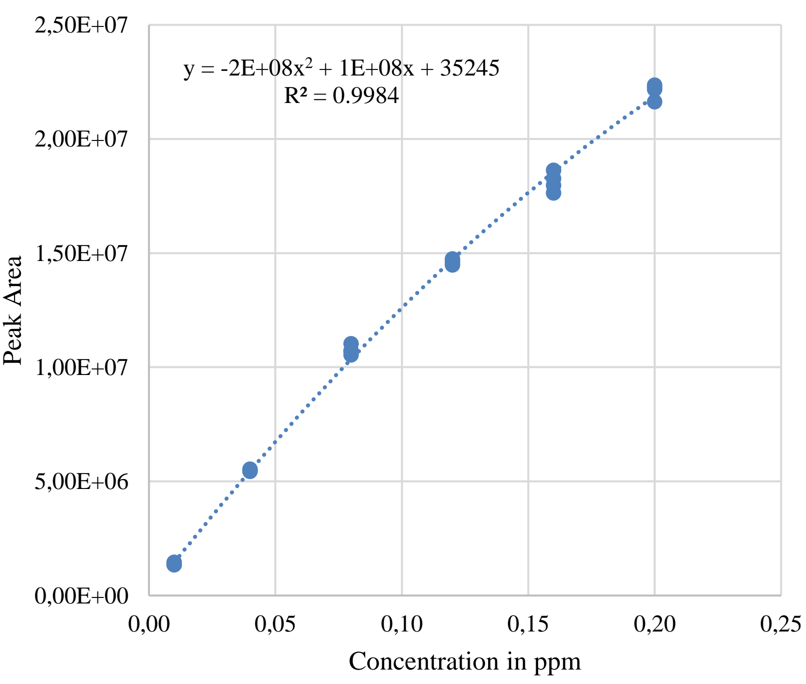

Figure C.2: BAC10 non-linear calibration curve.

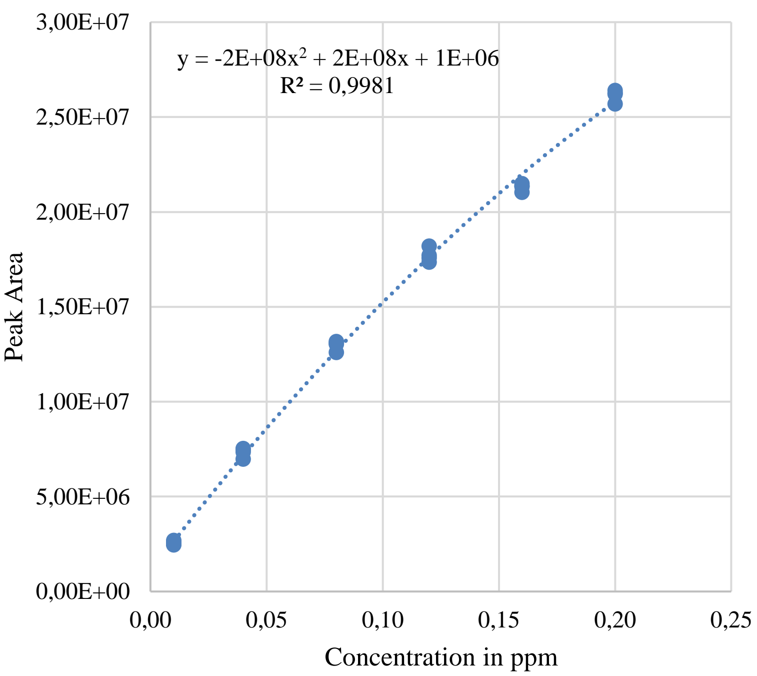

Figure C.3: BAC12 non-linear calibration curve.

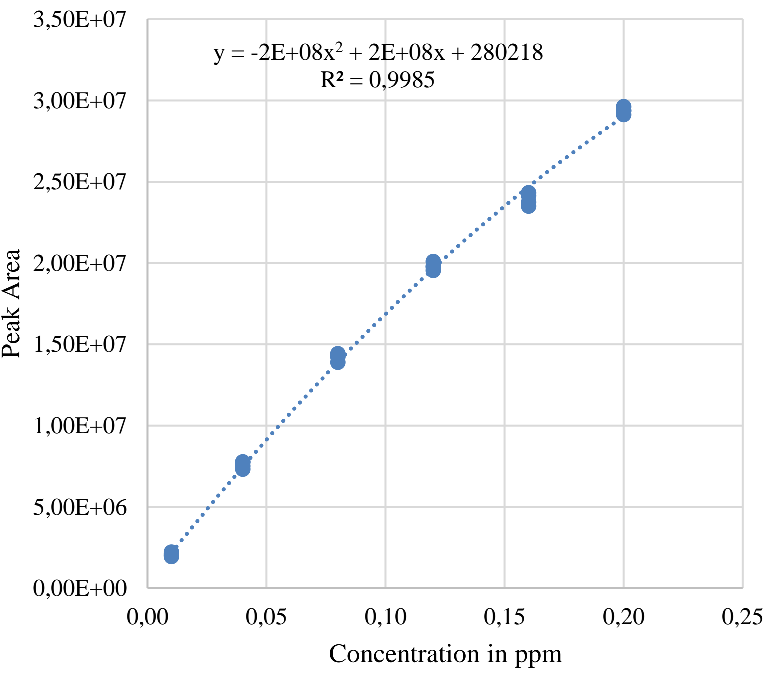

Figure C.4: BAC14 non-linear calibration curve.

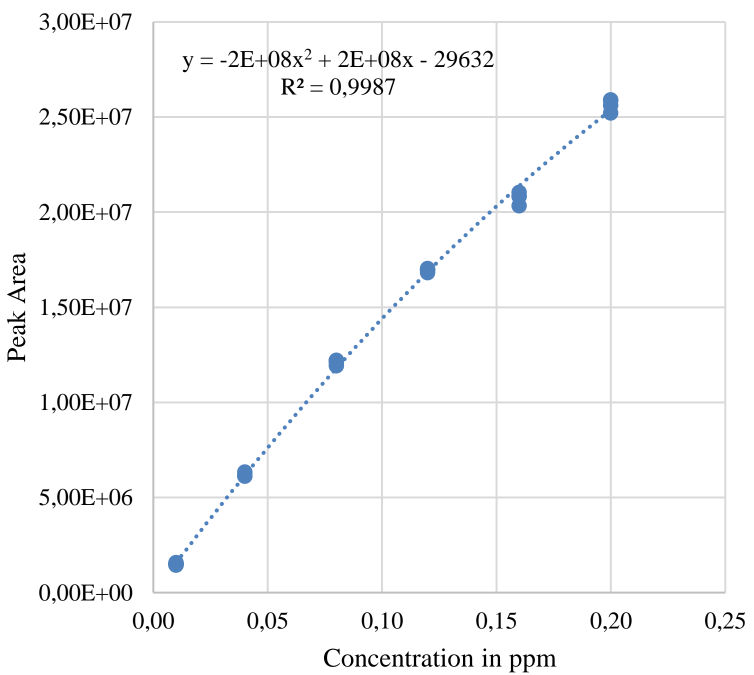

Figure C.5: BAC16 non-linear calibration curve.

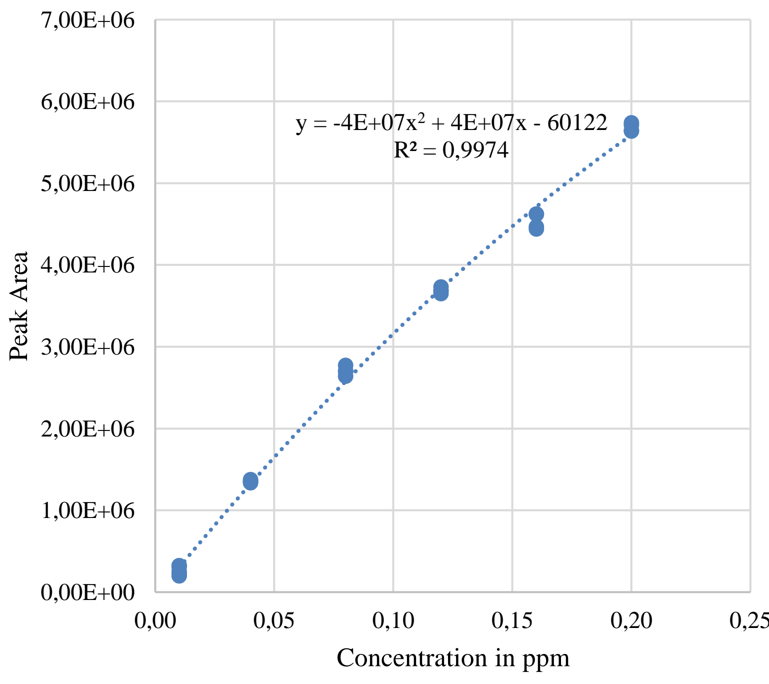

Figure C.6: BAC18 non-linear calibration curve.

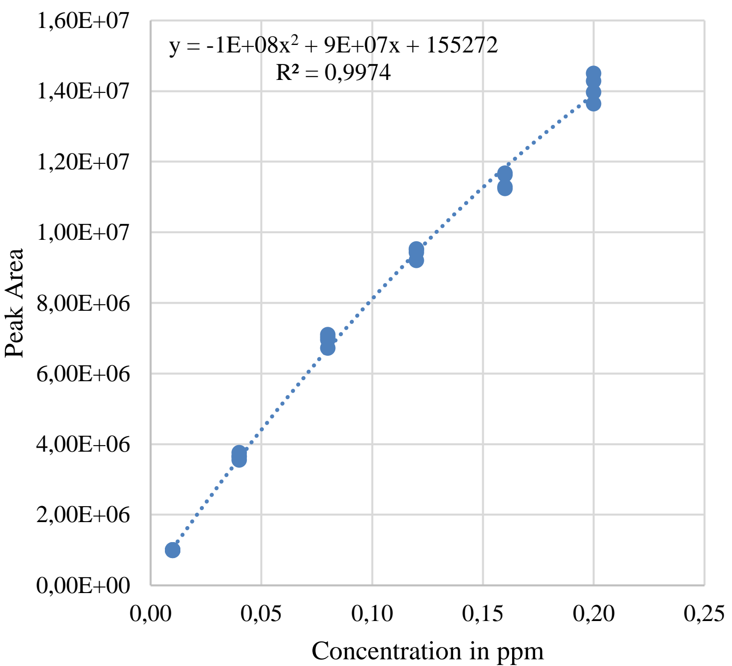

Figure C.7: DDAC-C-8 non-linear calibration curve.

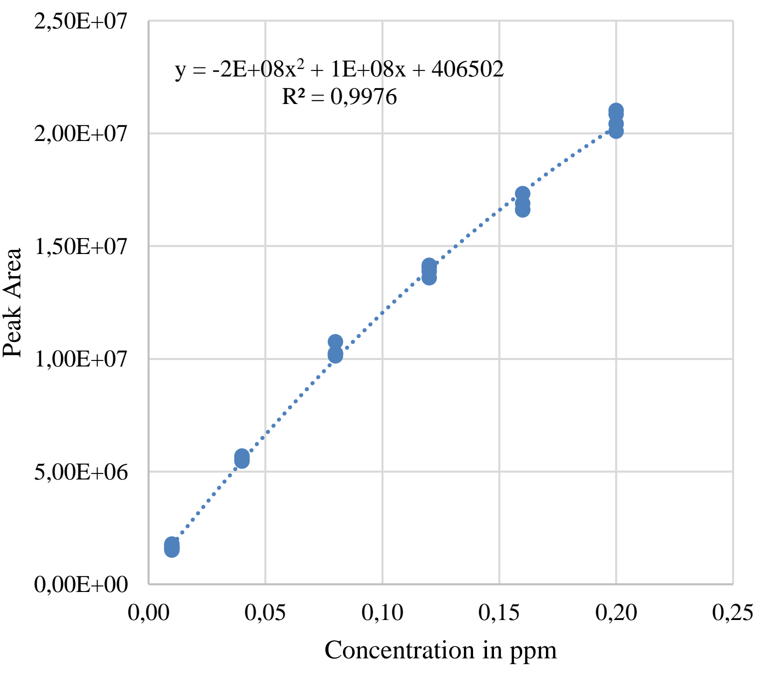

Figure C.8: DDAC-C-10 non-linear calibration curve.

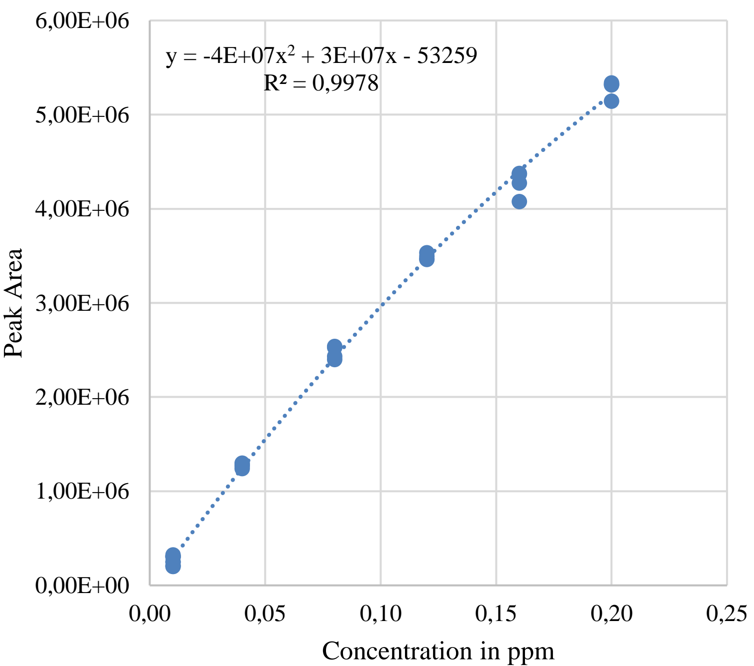

Figure C.9: DDAC-C-12 non-linear calibration curve.

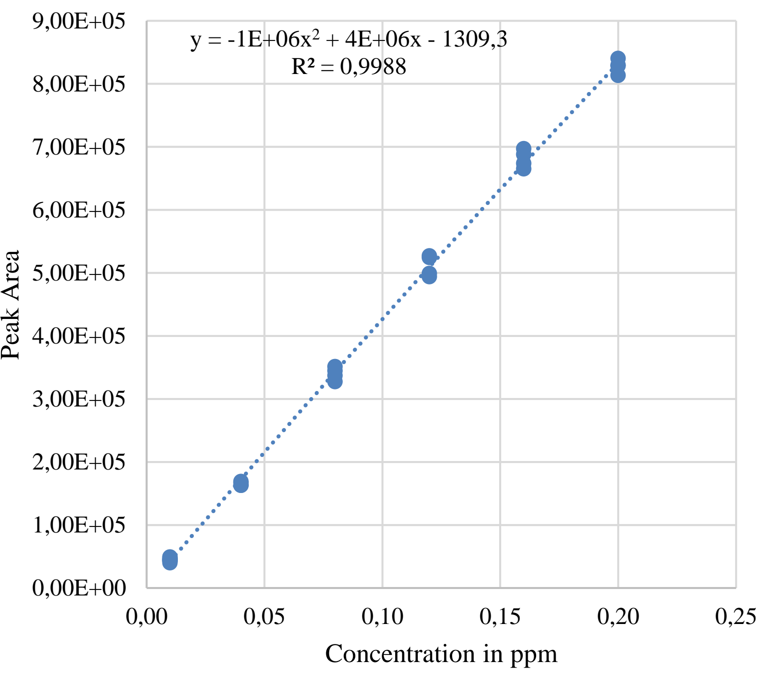

Figure C.10: Chlorate non-linear calibration curve.

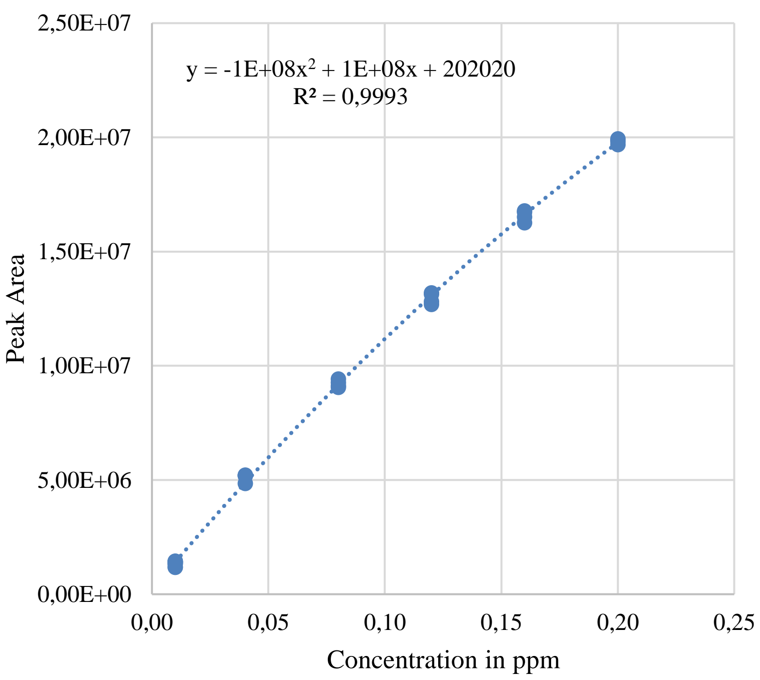

Figure C.11: Histamine non-linear calibration curve.

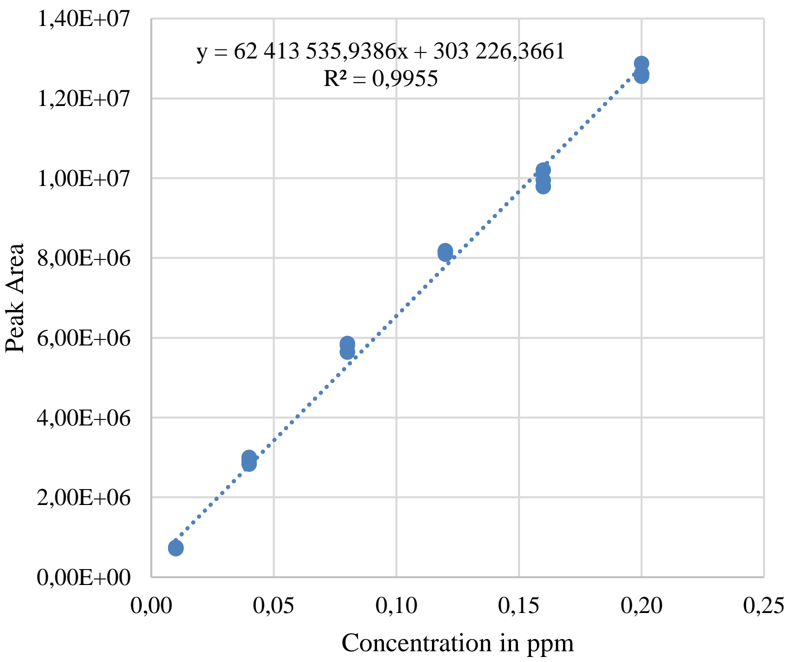

Figure C.12: BAC8 linear calibration curve.

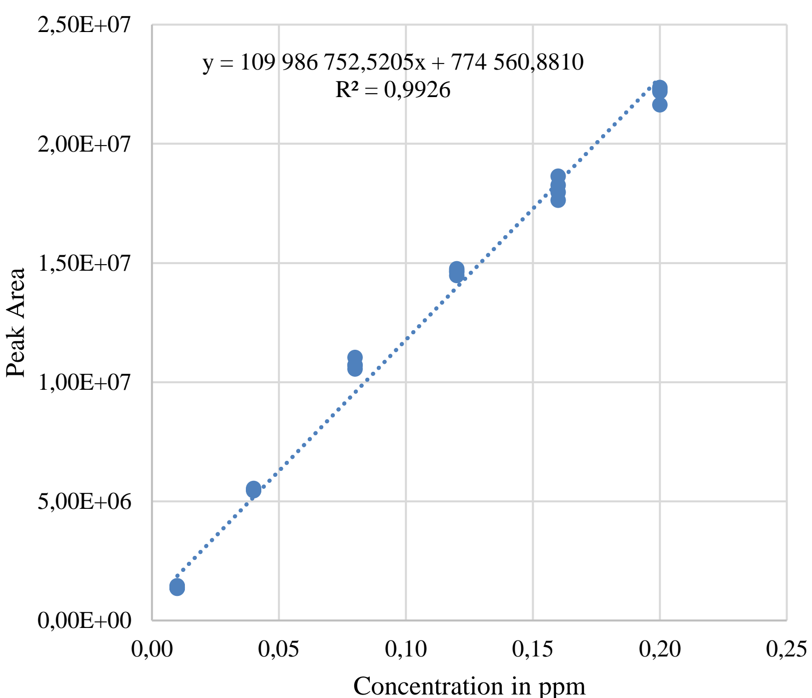

Figure C.13: BAC10 linear calibration curve.

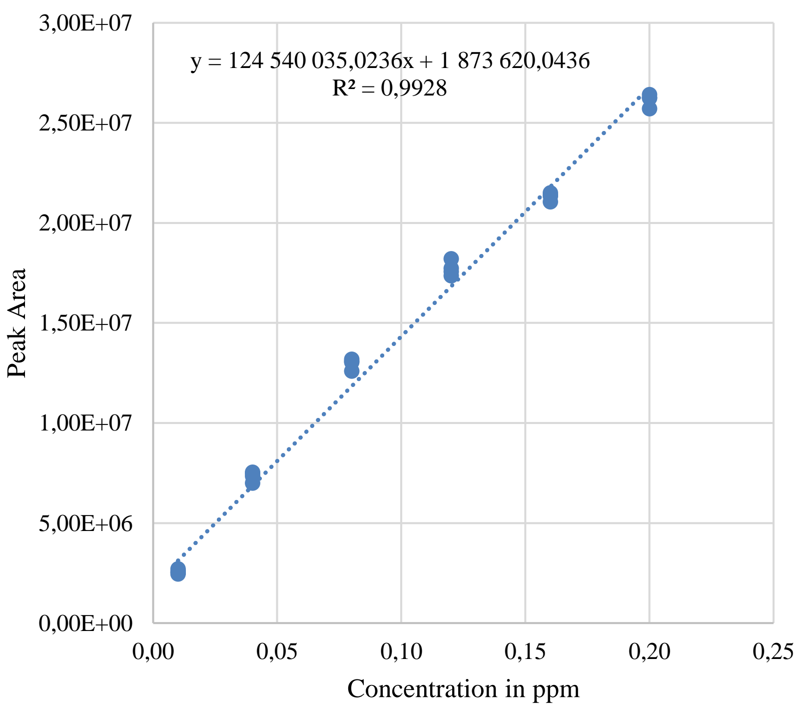

Figure C.14: BAC12 linear calibration curve.

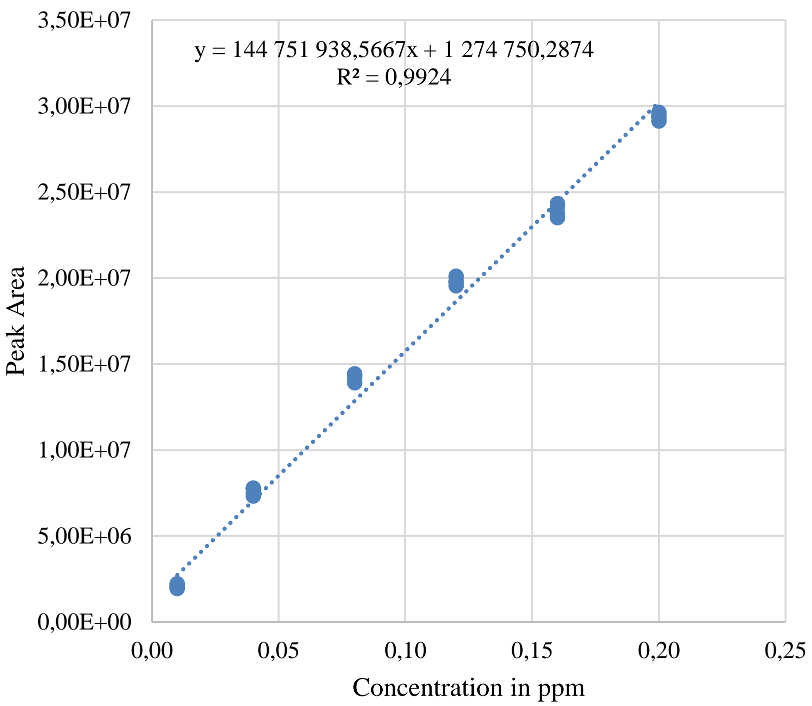

Figure C.15: BAC14 linear calibration curve.

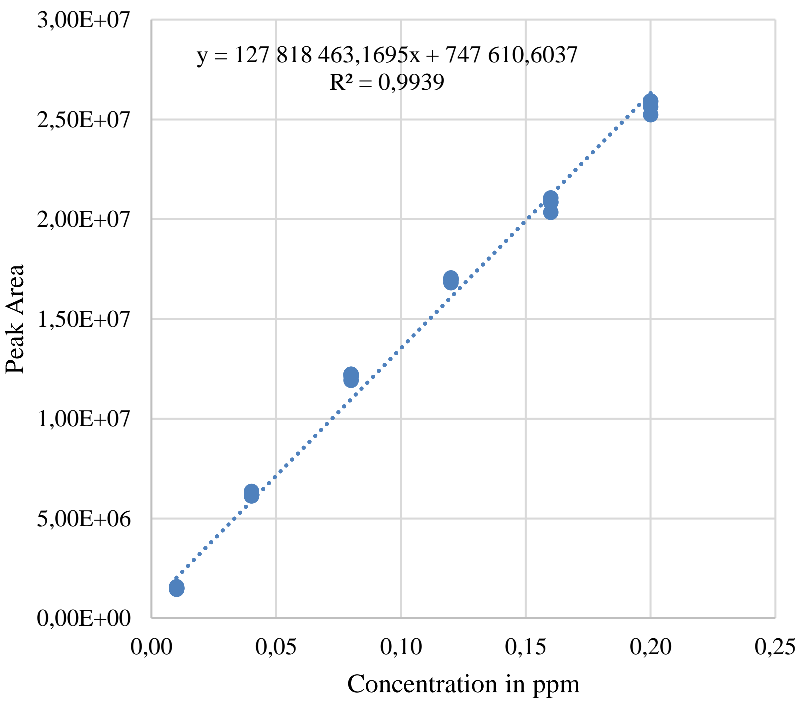

Figure C.16: BAC16 linear calibration curve.

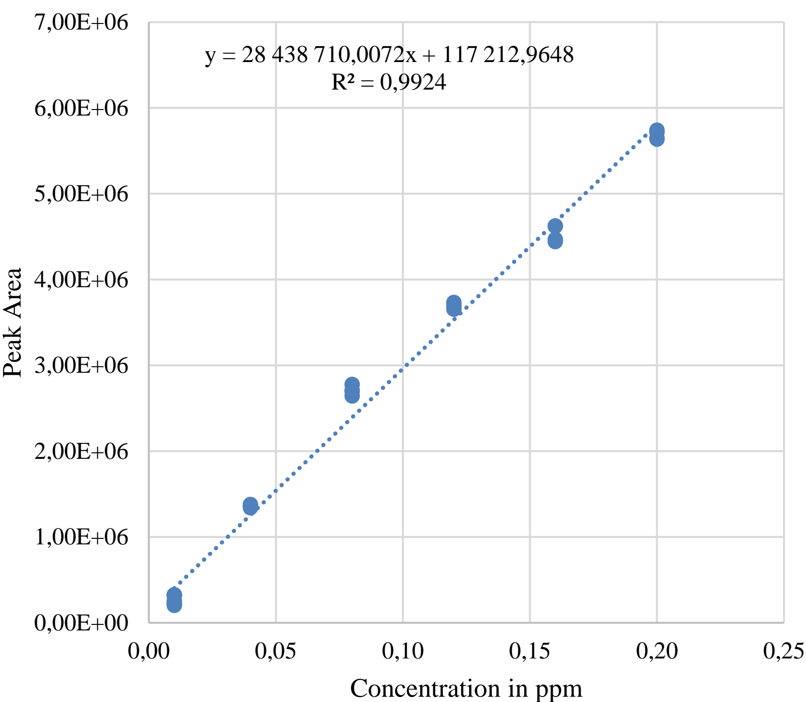

Figure C.17: BAC18 linear calibration curve.

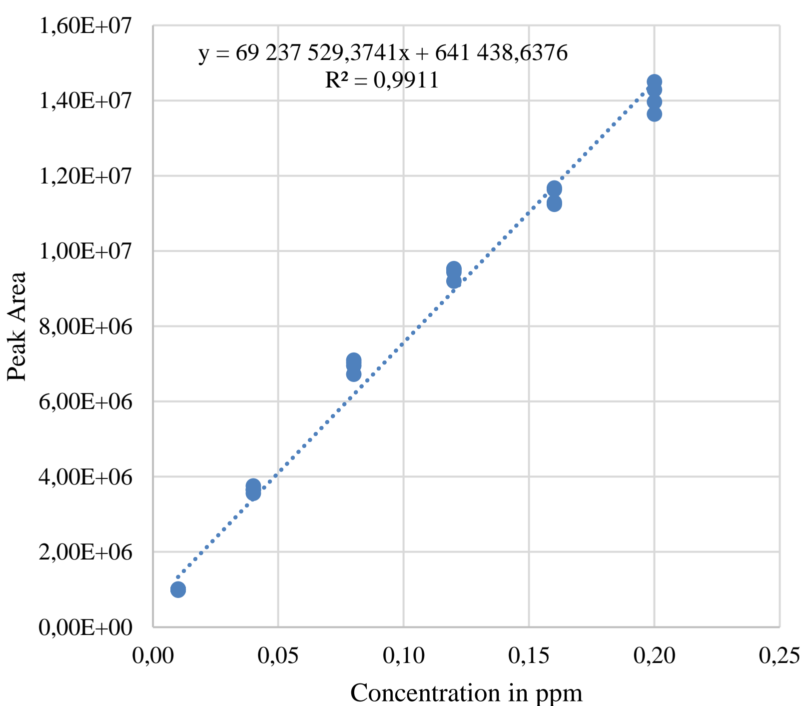

Figure C.18: DDAC-C-8 linear calibration curve.

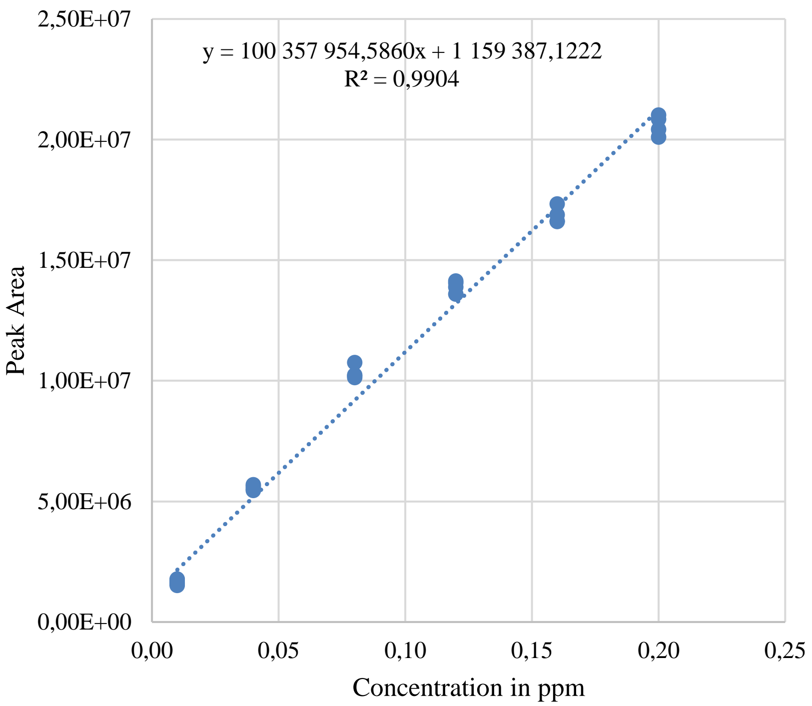

Figure C.19: DDAC-C-10 linear calibration curve.

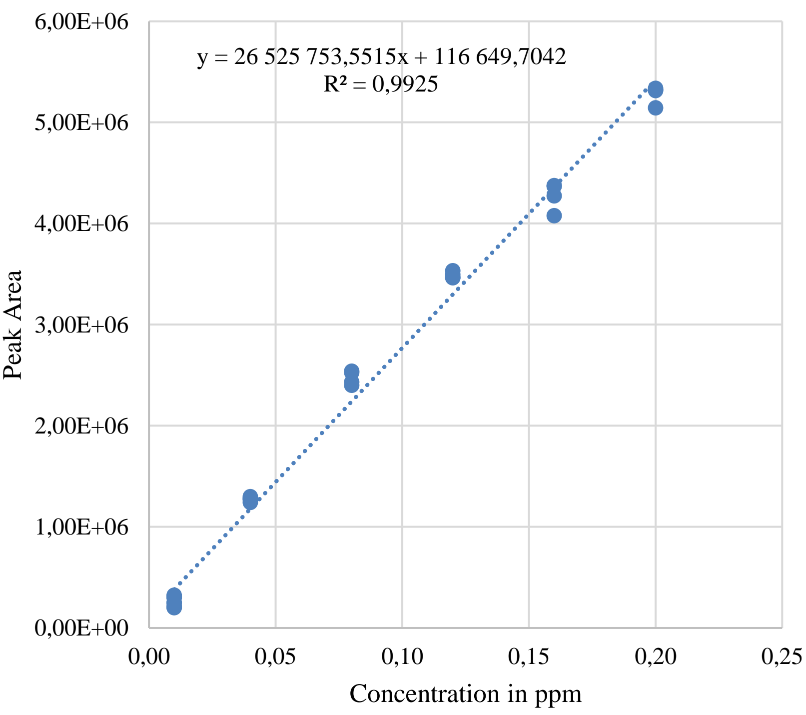

Figure C.20: DDAC-C-12 linear calibration curve.

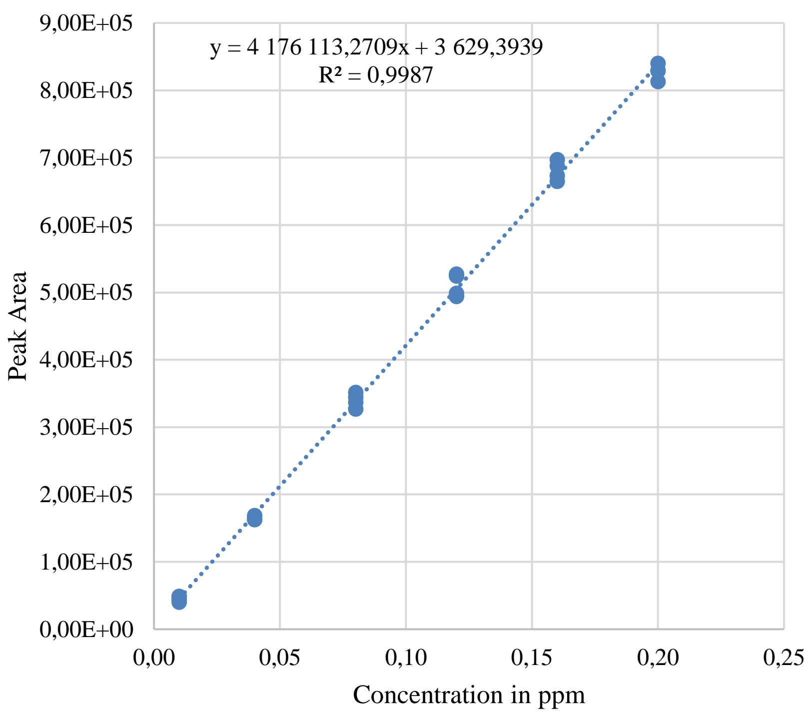

Figure C.21: Chlorate linear calibration curve.

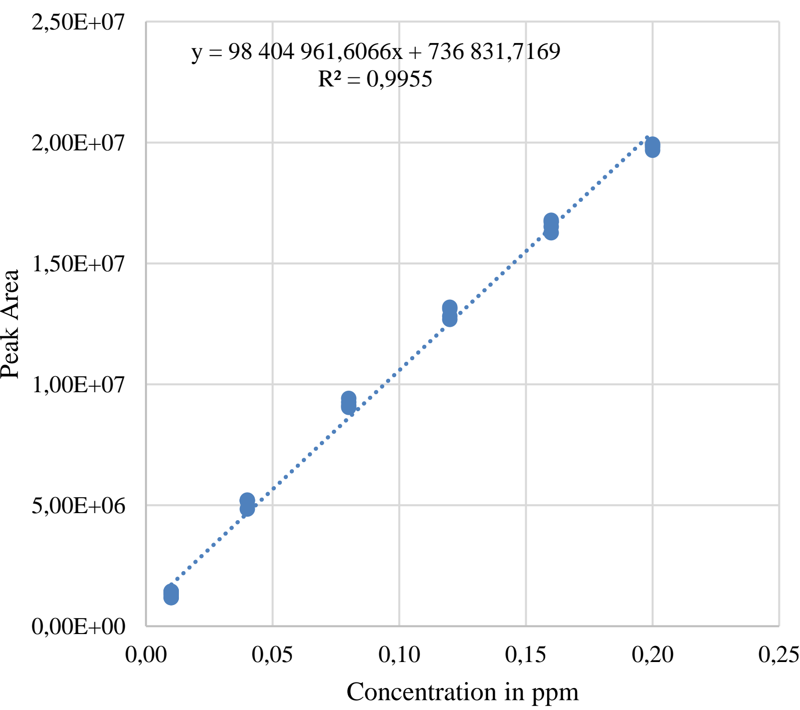

Figure C.22: Histamine linear calibration curve

D. Chromatograms in fish matrices for selectivity

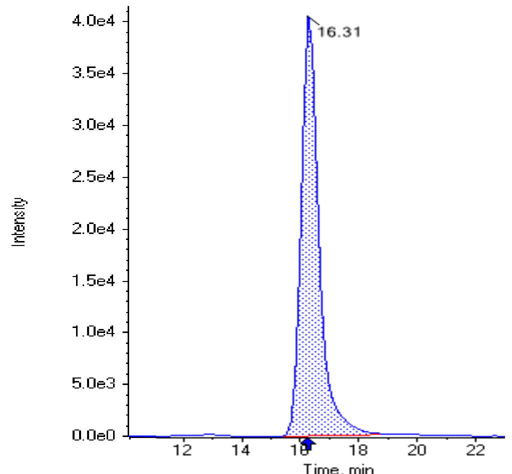

Figure D.1: BAC8 chromatogram for black cardinal fish.

Figure D.2: BAC10 chromatogram for black cardinal fish.

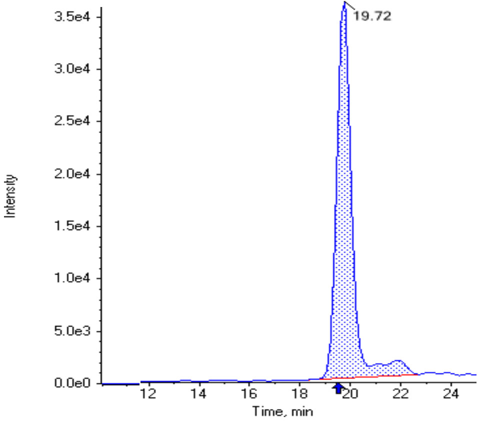

Figure D.3: BAC12 chromatogram for black cardinal fish.

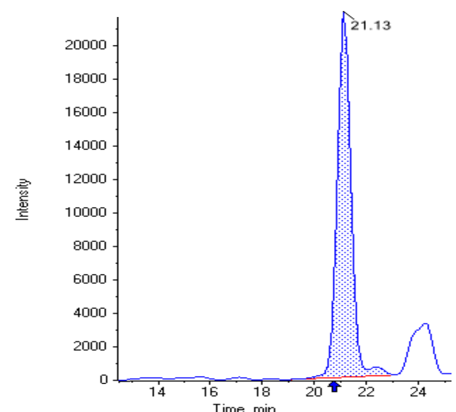

Figure D.4: BAC14 chromatogram for black cardinal fish.

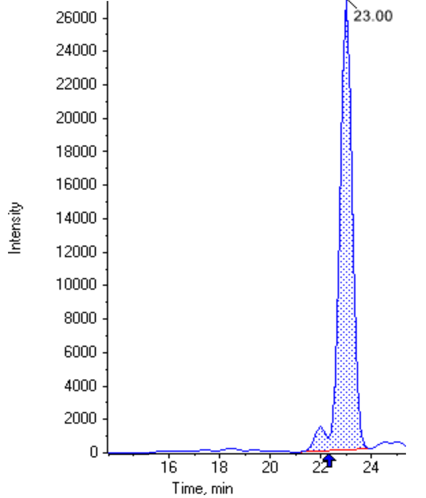

Figure D.5: BAC16 chromatogram for black cardinal fish.

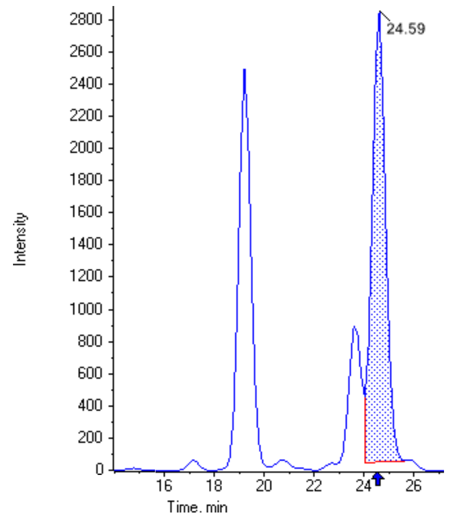

Figure D.6: BAC18 chromatogram for black cardinal fish.

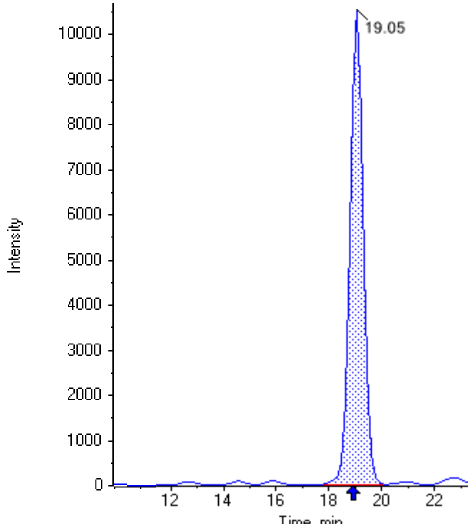

Figure D.7: DDAC-C-8 chromatogram for black cardinal fish.

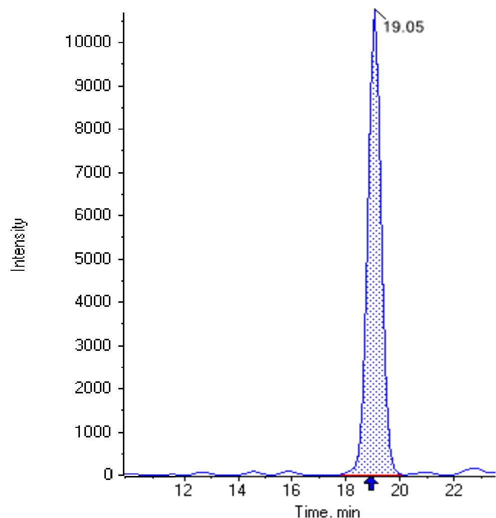

Figure D.8: DDAC-C-10 chromatogram for black cardinal fish.

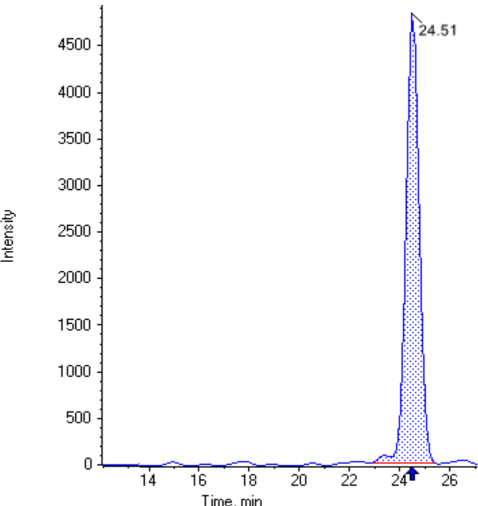

Figure D.9: DDAC-C-12 chromatogram for black cardinal fish.

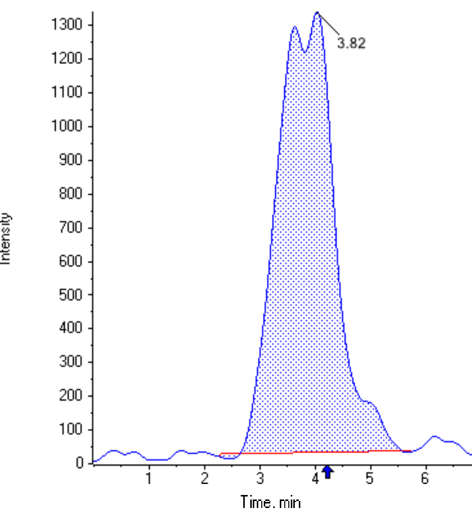

Figure D.10: Chlorate chromatogram for black cardinal fish.

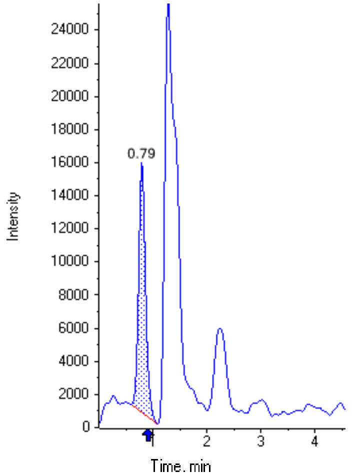

Figure D.11: Histamine chromatogram for black cardinal fish.

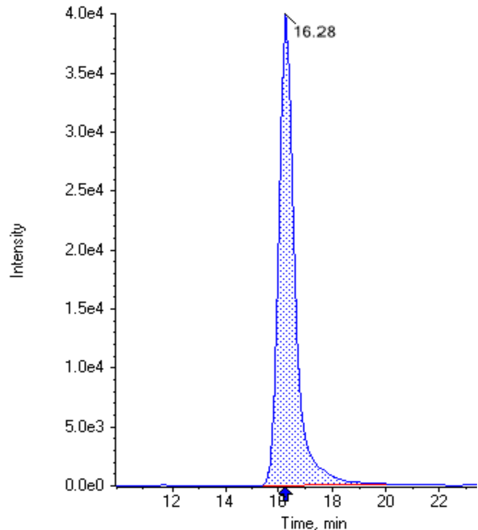

Figure D.12: BAC8 chromatogram for bigeye tuna.

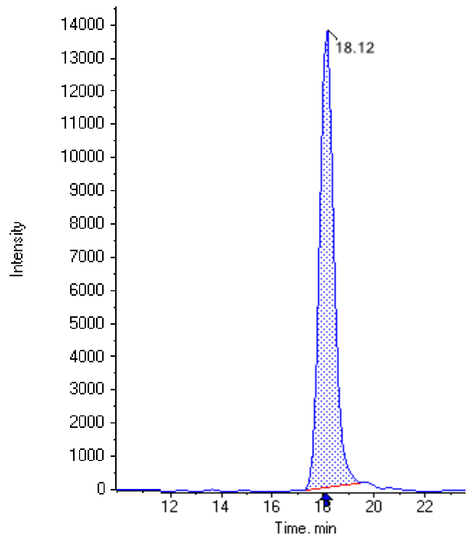

Figure D.13: BAC10 chromatogram for bigeye tuna.

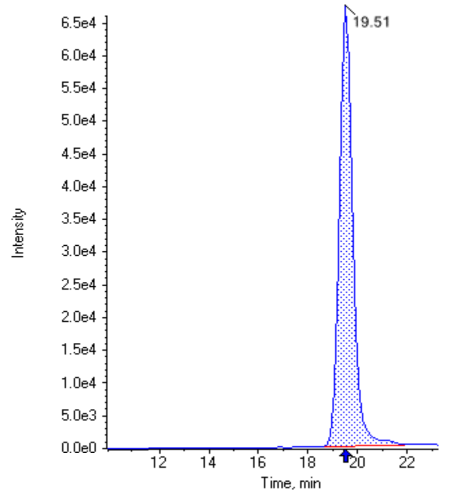

Figure D.14: BAC12 chromatogram for bigeye tuna.

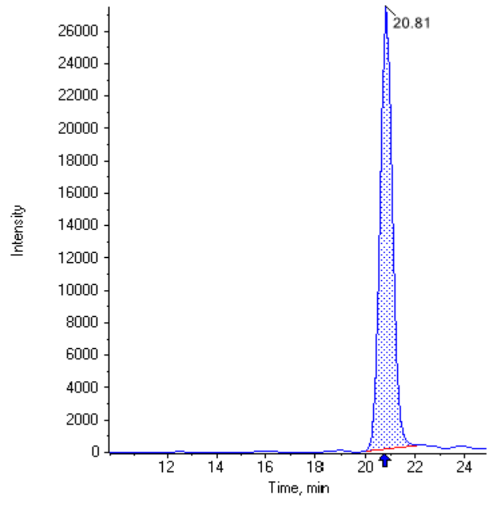

Figure D.15: BAC14 chromatogram for bigeye tuna.

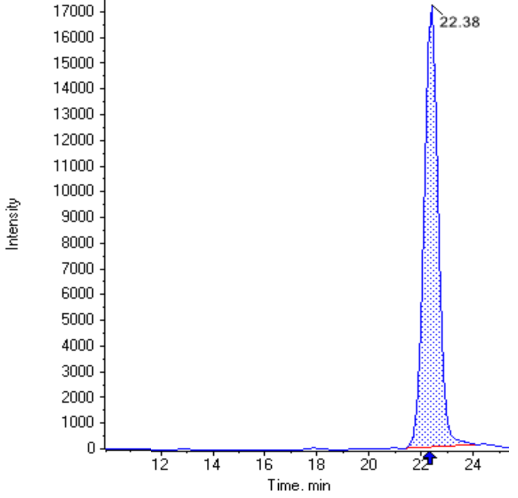

Figure D.16: BAC16 chromatogram for bigeye tuna.

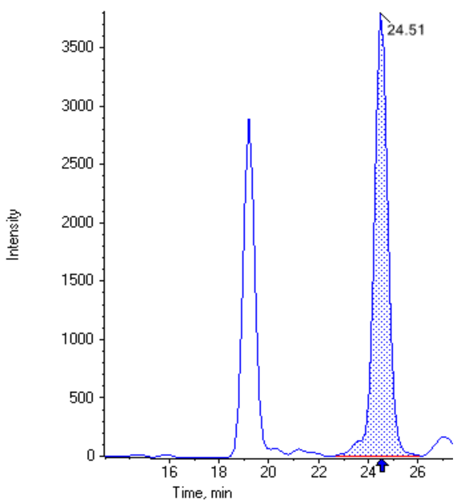

Figure D.17: BAC18 chromatogram for bigeye tuna.

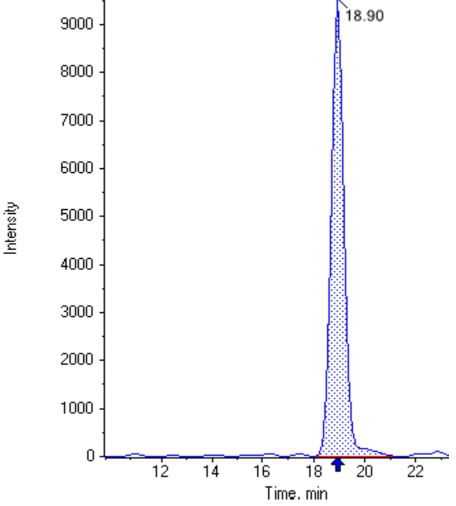

Figure D.18: DDAC-C-8 chromatogram for bigeye tuna.

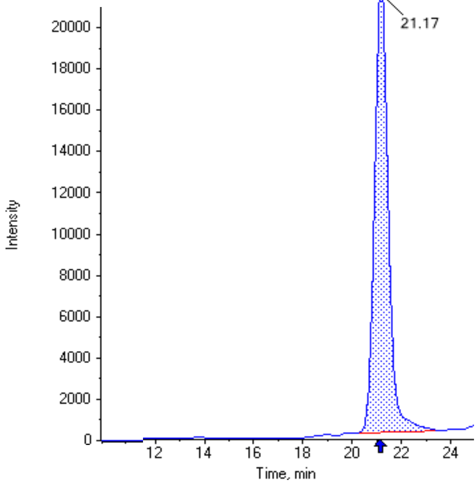

Figure D.19: DDAC-C-10 chromatogram for bigeye tuna.

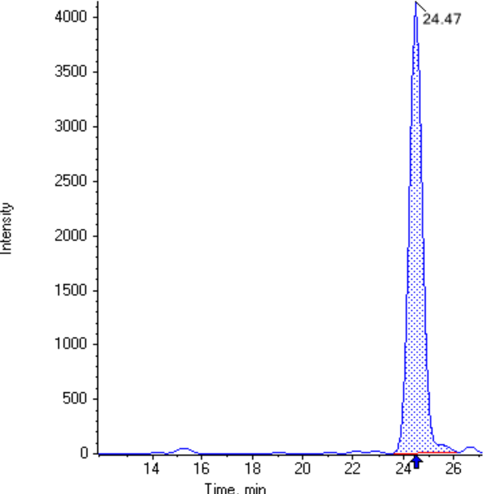

Figure D.20: DDAC-C-12 chromatogram for bigeye tuna.

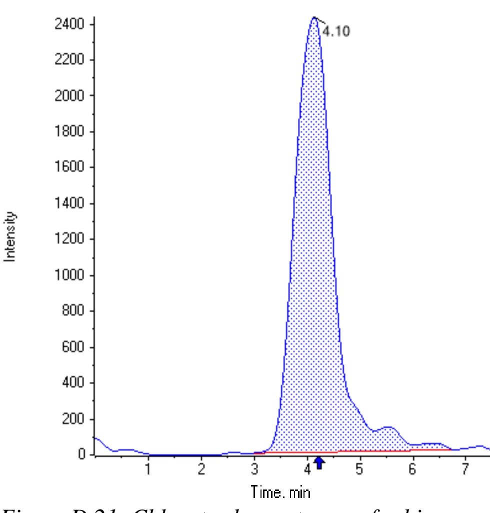

Figure D.21: Chlorate chromatogram for bigeye tuna.

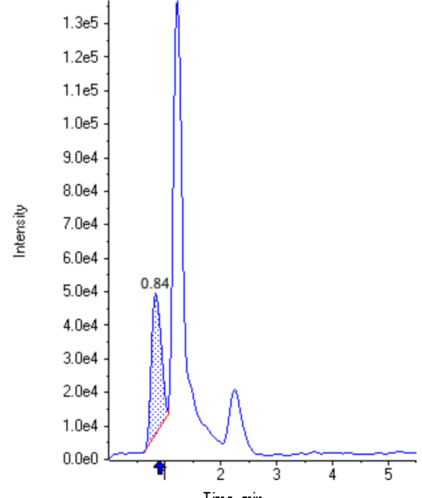

Figure D.22: Histamine chromatogram for bigeye tuna.

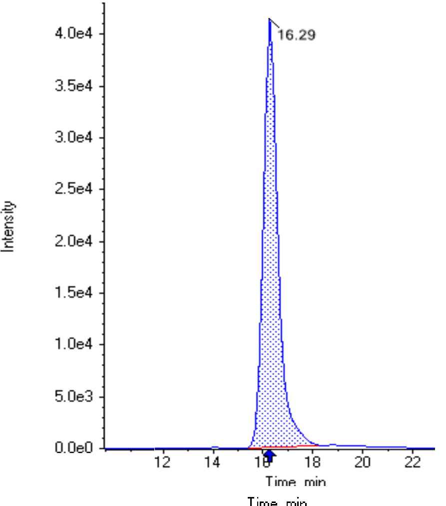

Figure D.23: BAC8 chromatogram for atlantis bluefin tuna.

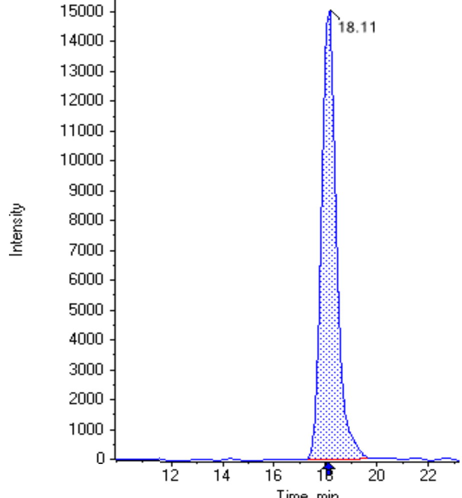

Figure D.24: BAC10 chromatogram for atlantis bluefin tuna.

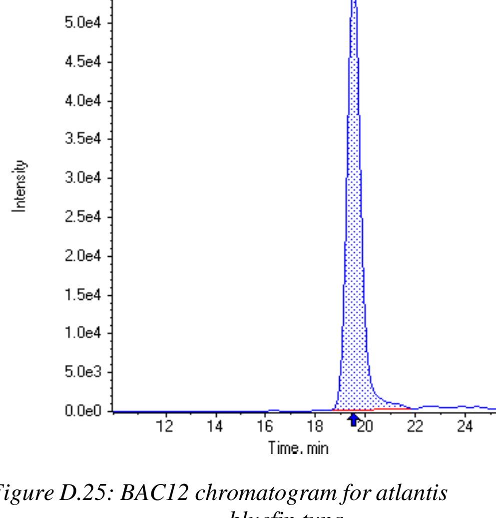

Figure D.25: BAC12 chromatogram for atlantis bluefin tuna.

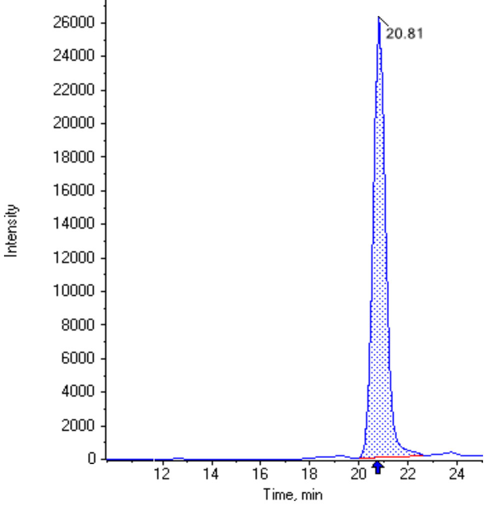

Figure D.26: BAC14 chromatogram for atlantis bluefin tuna.

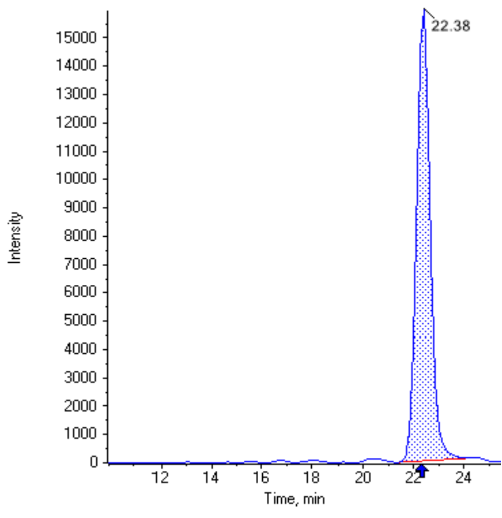

Figure D.27: BAC16 chromatogram for atlantis bluefin tuna.

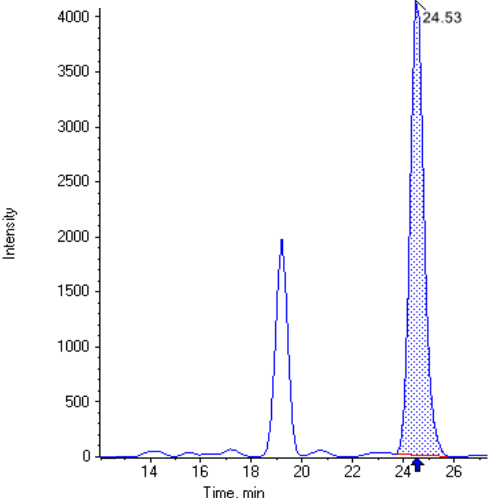

Figure D.28: BAC18 chromatogram for atlantis bluefin tuna.

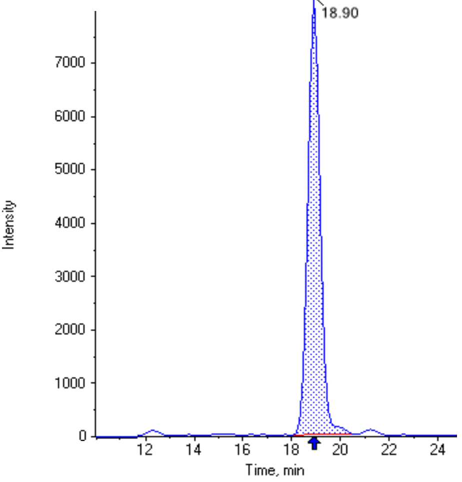

Figure D.29: DDAC-C-8 chromatogram for atlantis bluefin tuna.

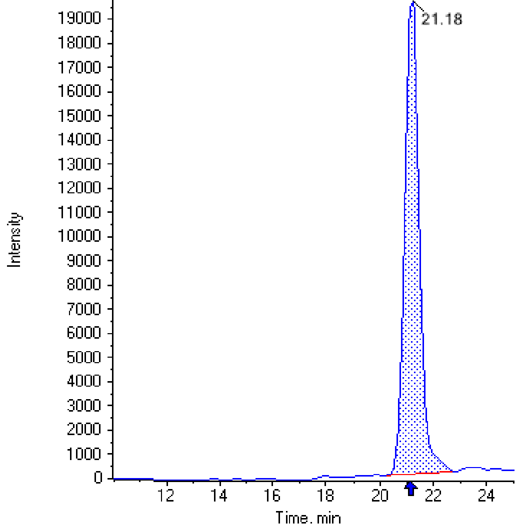

Figure D.30: DDAC-C-10 chromatogram for atlantis bluefin tuna.

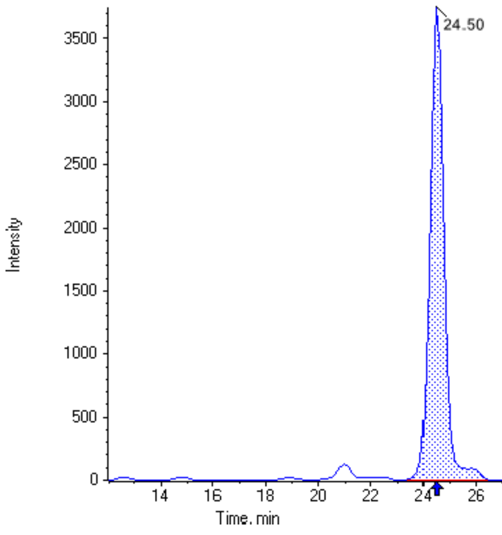

Figure D.31: DDAC-C-12 chromatogram for atlantis bluefin tuna.

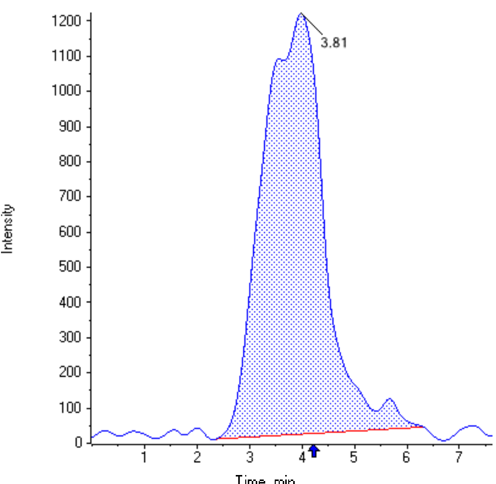

Figure D.32: Chlorate chromatogram for atlantis bluefin tuna.

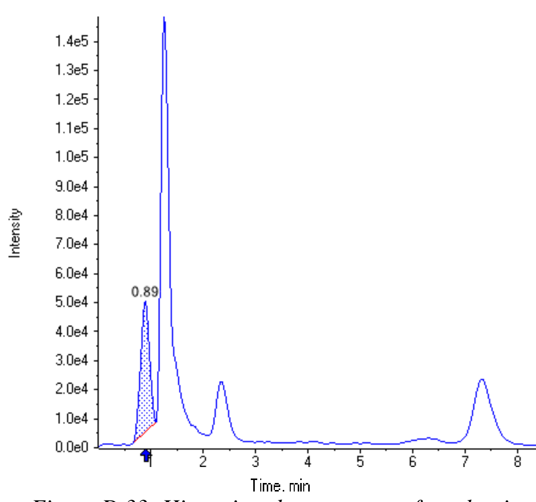

Figure D.33: Histamine chromatogram for atlantis bluefin tuna.

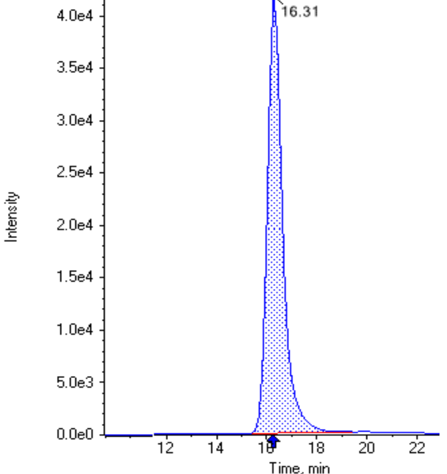

Figure D.34: BAC8 chromatogram for european parrotfish.

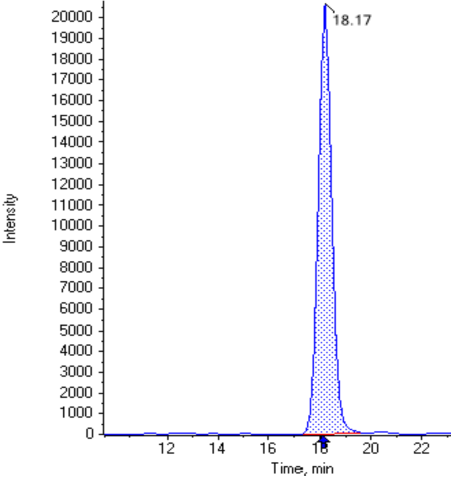

Figure D.35: BAC10 chromatogram for european parrotfish.

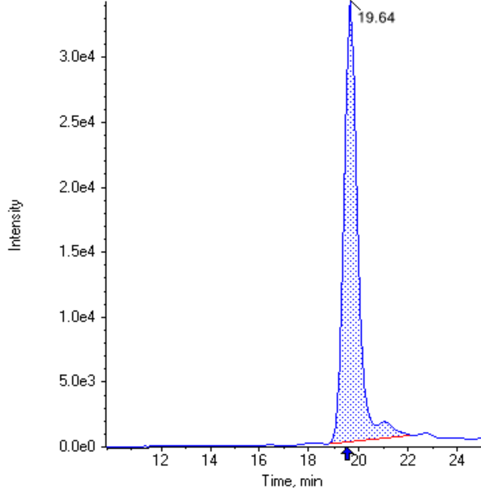

Figure D.36: BAC12 chromatogram for european parrotfish.

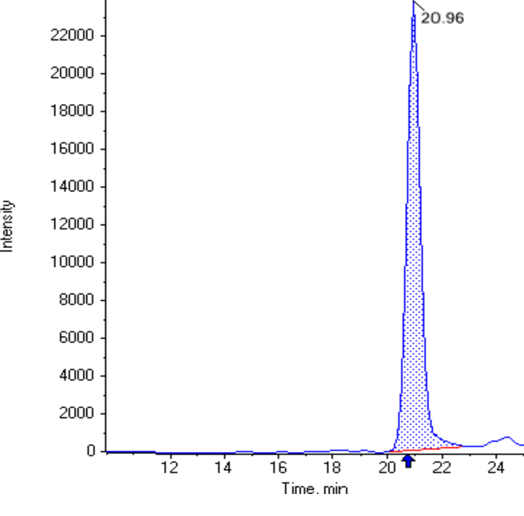

Figure D.37: BAC14 chromatogram for european parrotfish.

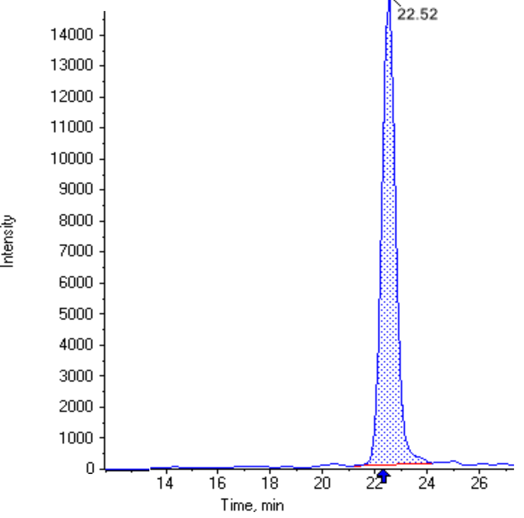

Figure D.38: BAC16 chromatogram for european parrotfish.

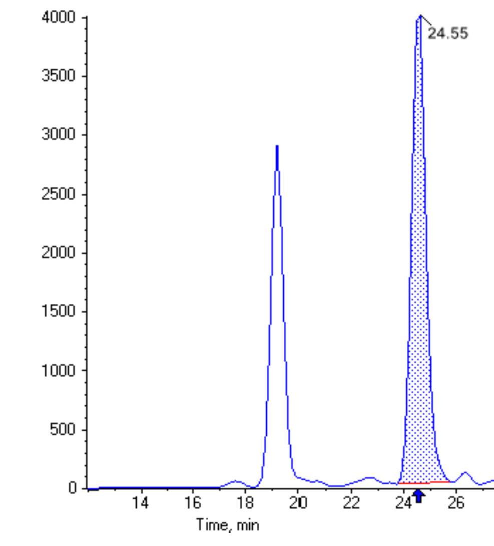

Figure D.39: BAC18 chromatogram for european parrotfish.

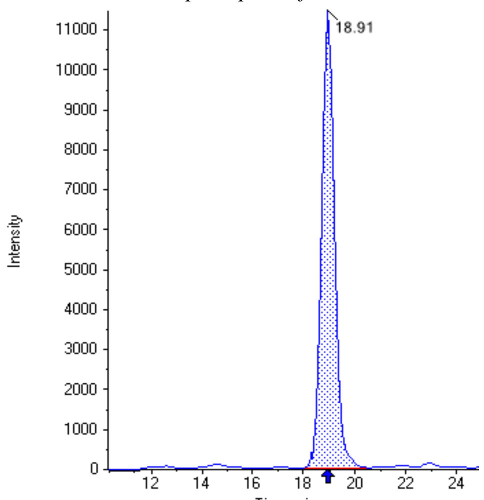

Figure D.40: DDAC-C-8 chromatogram for europeanparrotfish.

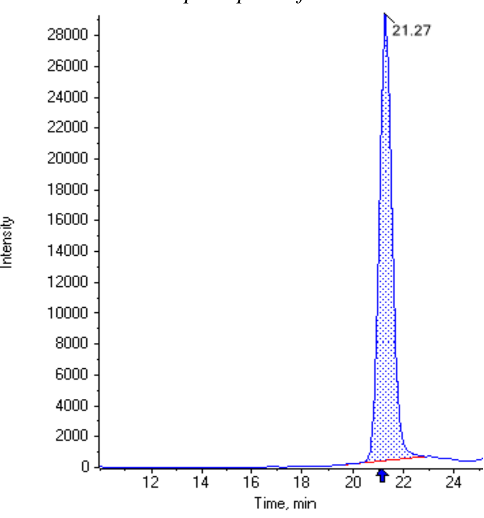

Figure D.41: DDAC-C-10 chromatogram for european parrotfish.

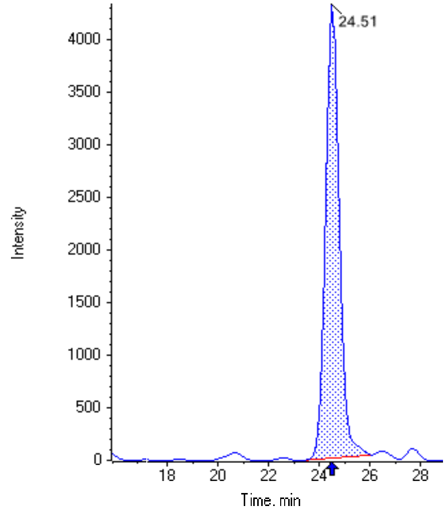

Figure D.42: DDAC-C-12 chromatogram for european parrotfish.

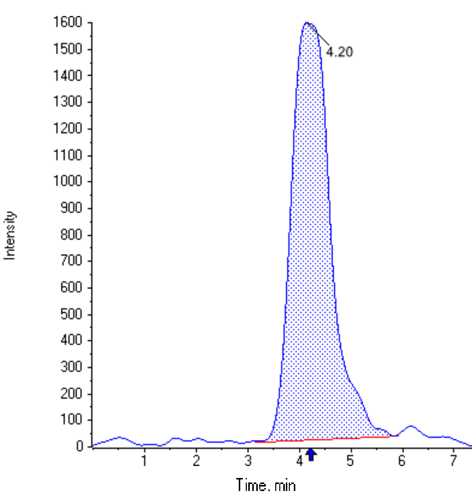

Figure D.43: Chlorate chromatogram for european parrotfish.

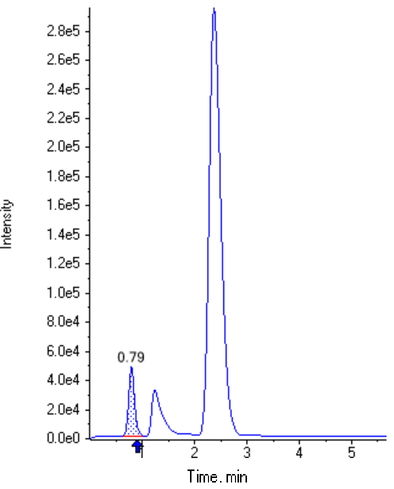

Figure D.44: Histamine chromatogram for european parrotfish.

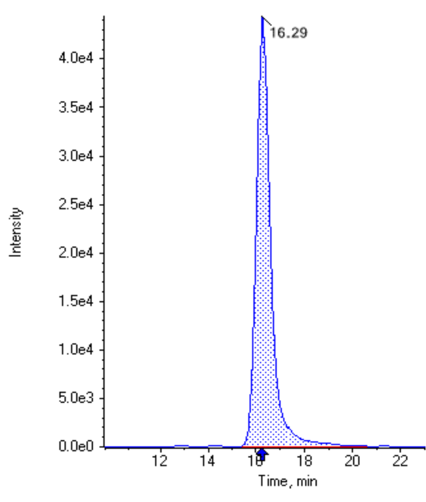

Figure D.45: BAC8 chromatogram for black scabbardfish.

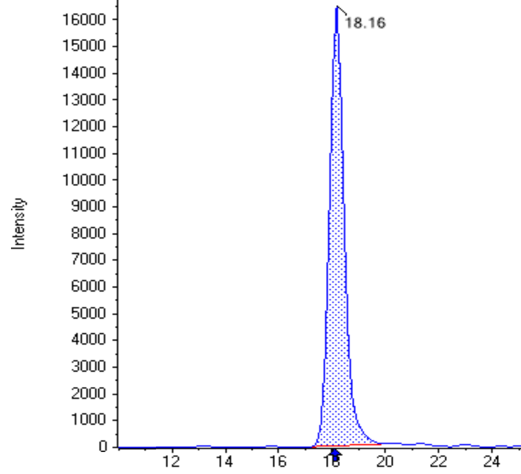

Figure D.46: BAC10 chromatogram for black scabbardfish.

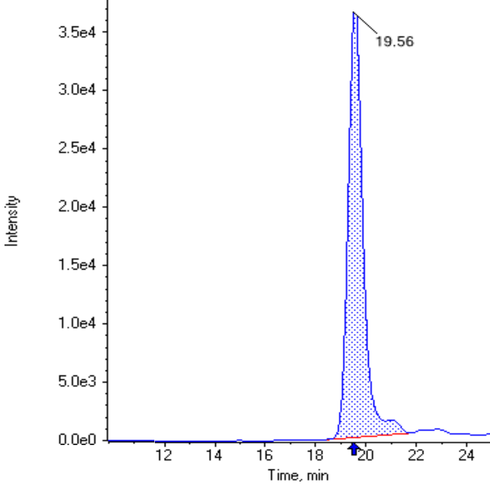

Figure D.47: BAC12 chromatogram for black scabbardfish.

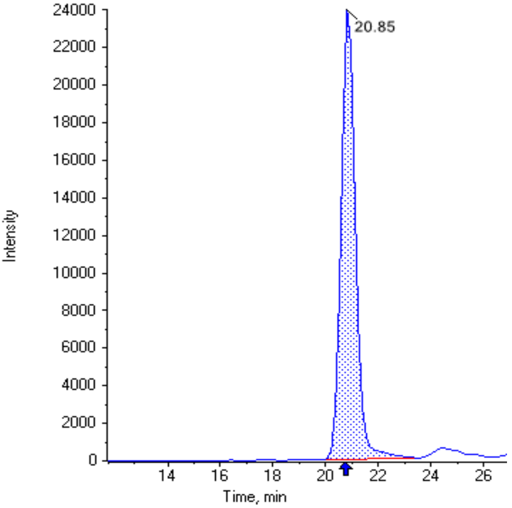

Figure D.48: BAC14 chromatogram for black scabbardfish.

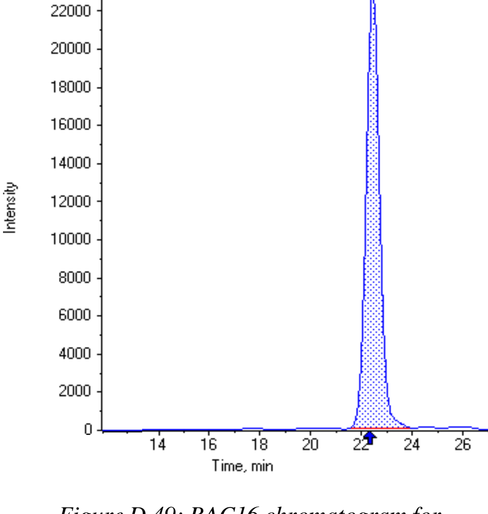

Figure D.49: BAC16 chromatogram for black scabbardfish.

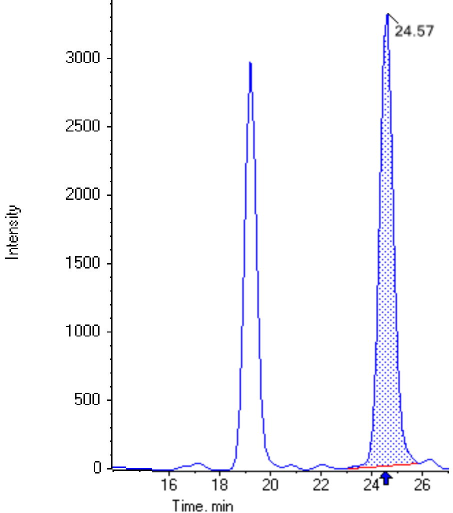

Figure D.50: BAC18 chromatogram for black scabbardfish.

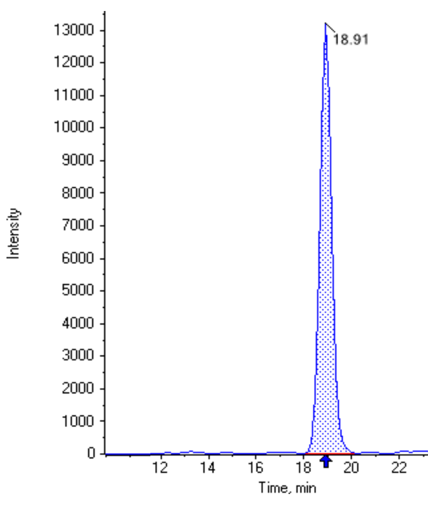

Figure D.51: DDAC-C-8 chromatogram for black scabbardfish.

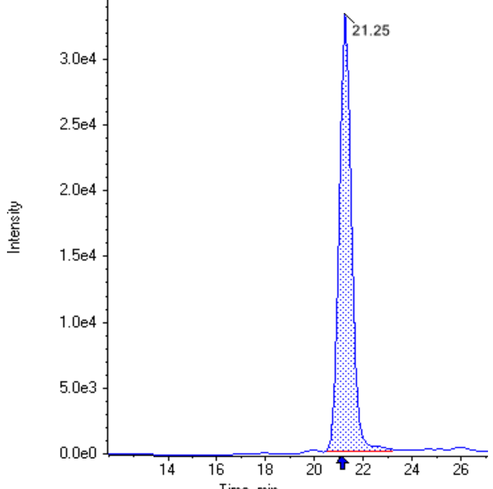

Figure D.52: DDAC-C-10 chromatogram for black scabbardfish.

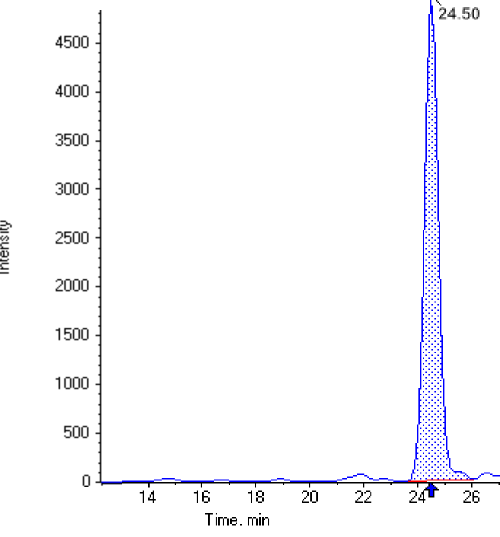

Figure D.53: DDAC-C-12 chromatogram for black scabbardfish.

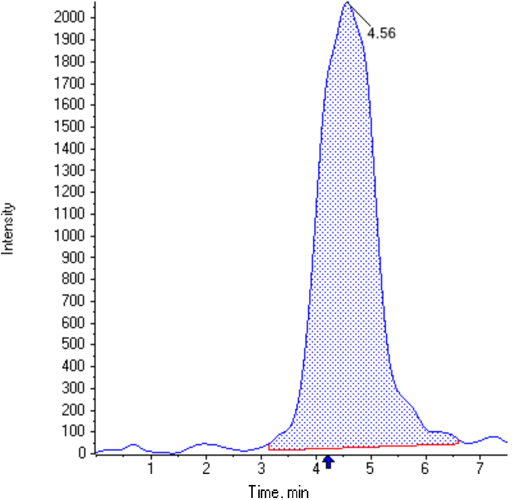

Figure D.54: Chlorate chromatogram for black scabbardfish.

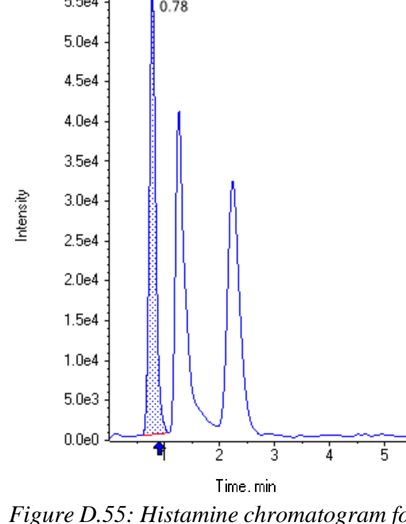

Figure D.55: Histamine chromatogram for black scabbardfish.

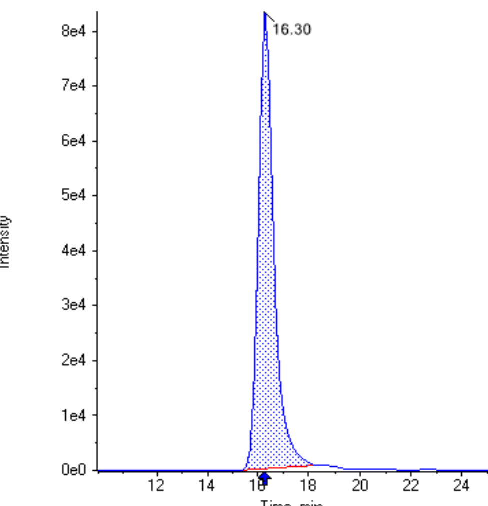

Figure D.56: BAC8 chromatogram for european seabass.

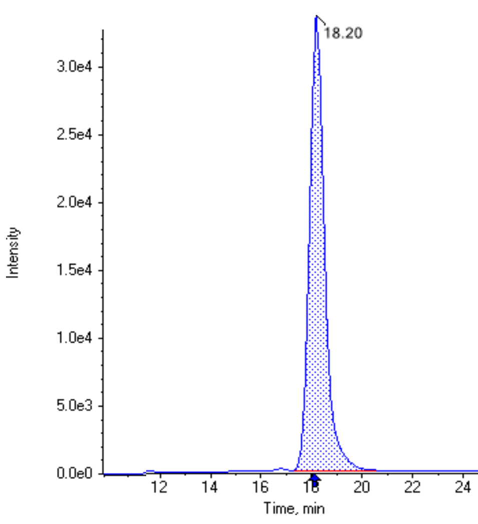

Figure D.57: BAC10 chromatogram for european seabass.

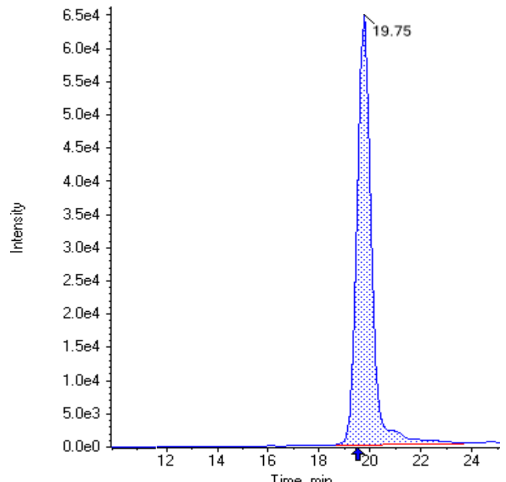

Figure D.58: BAC12 chromatogram for european seabass.

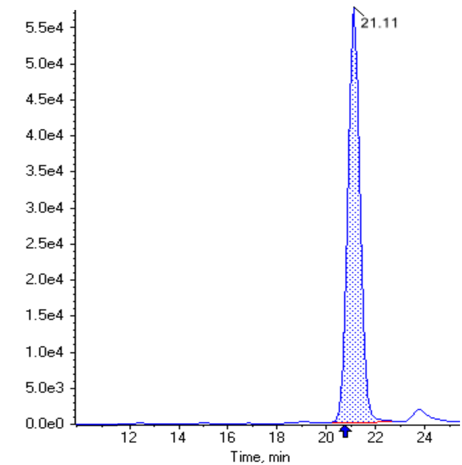

Figure D.59: BAC14 chromatogram for european seabass.

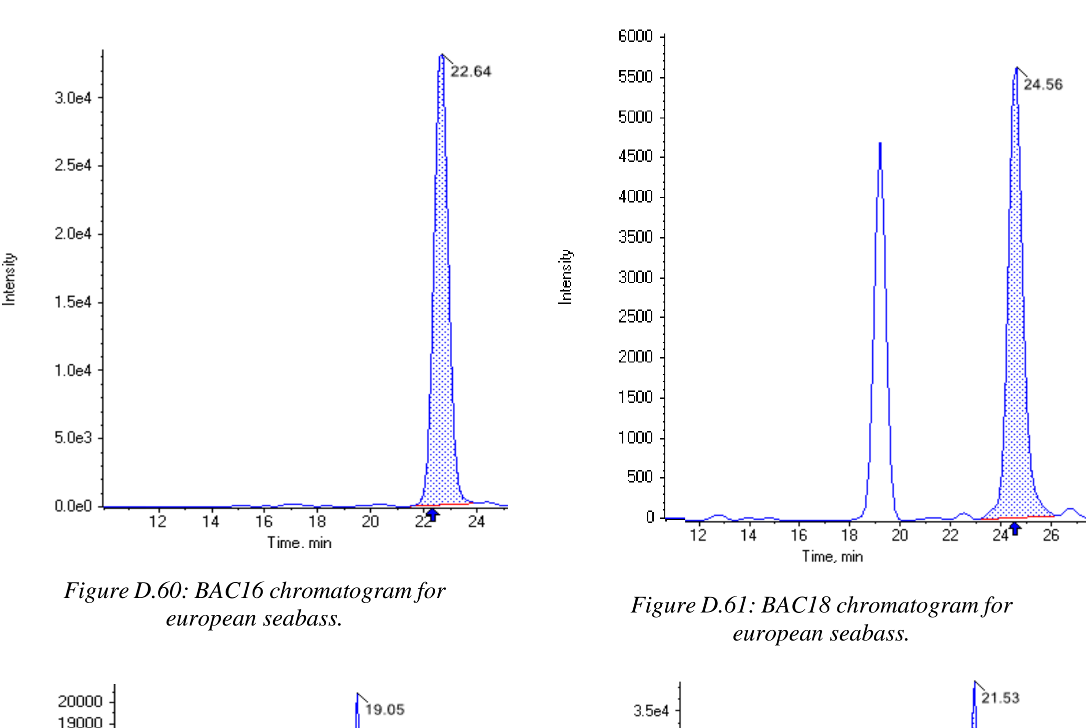

Figure D.60: BAC16 chromatogram for european seabass.

Figure D.61: BAC18 chromatogram for european seabass.

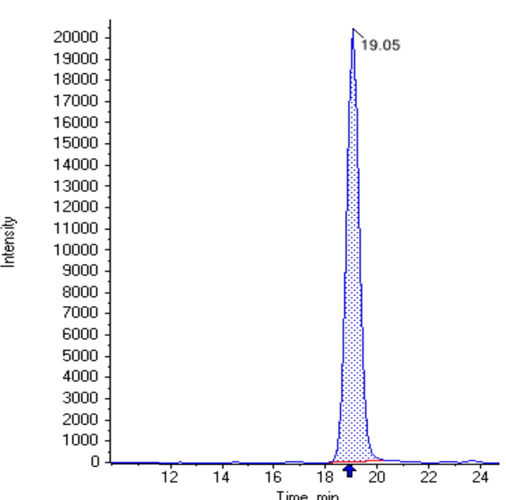

Figure D.62: DDAC-C-8 chromatogram for european seabass.

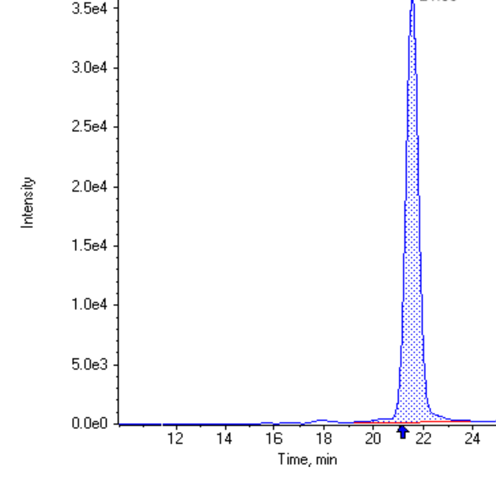

Figure D.63: DDAC-C-10 chromatogram for european seabass.

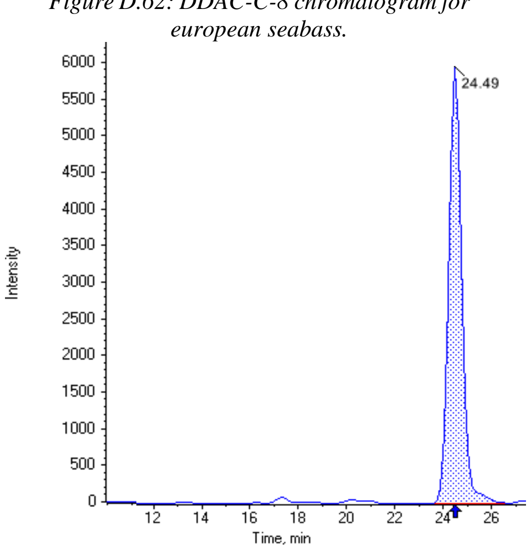

Figure D.64: DDAC-C-12 chromatogram for european seabass.

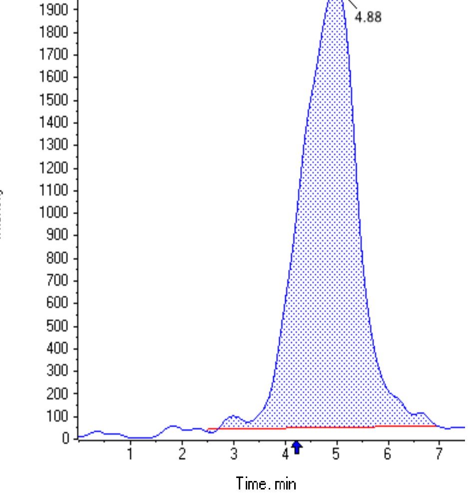

Figure D.65: Chlorate chromatogram for european seabass.

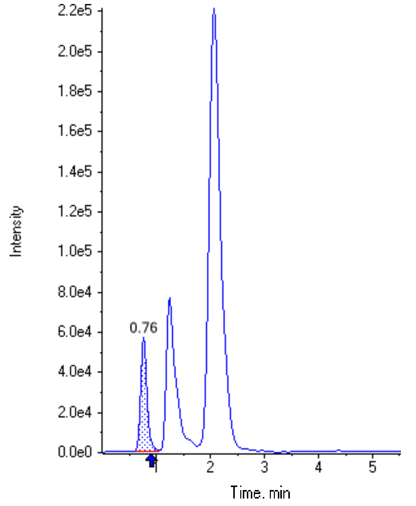

Figure D.66: Histamine chromatogram for european seabass.

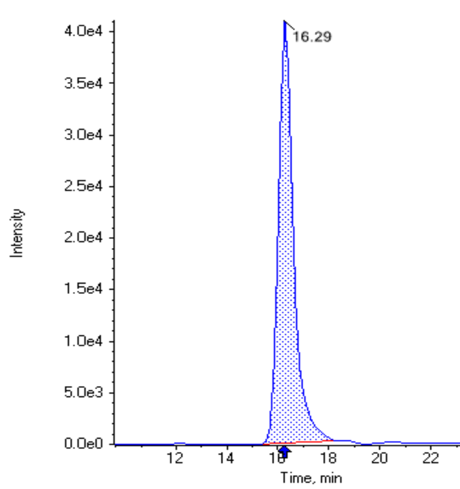

Figure D.67: BAC8 chromatogram for pink dentex.

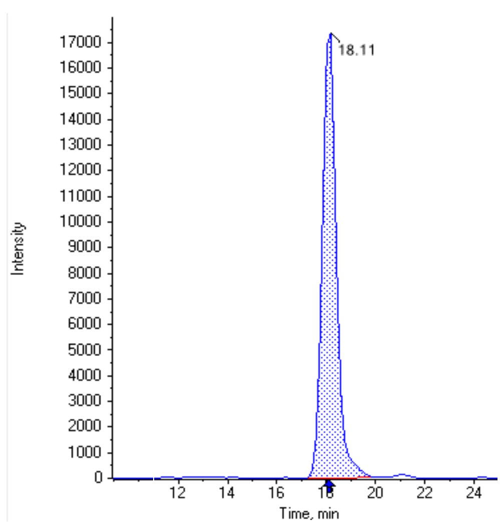

Figure D.68: BAC10 chromatogram for pink dentex.

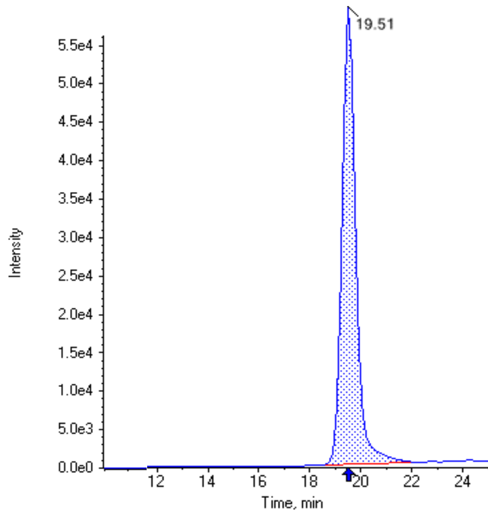

Figure D.69: BAC12 chromatogram for pink dentex.

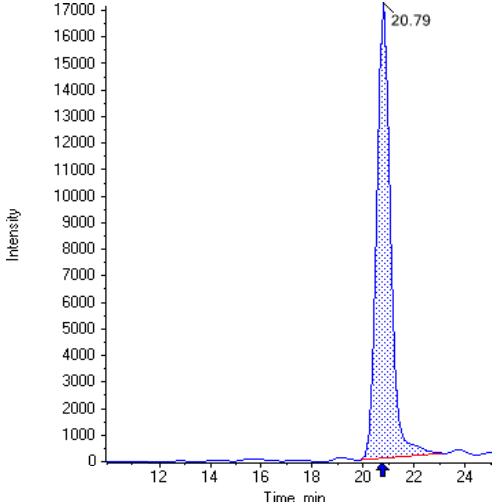

Figure D.70: BAC14 chromatogram for pink dentex.

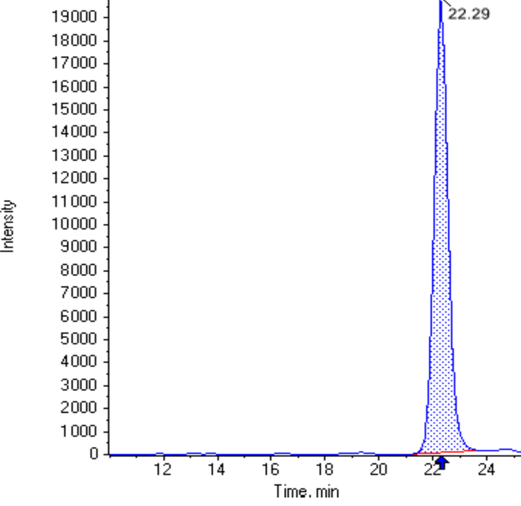

Figure D.71: BAC16 chromatogram for pink dentex.

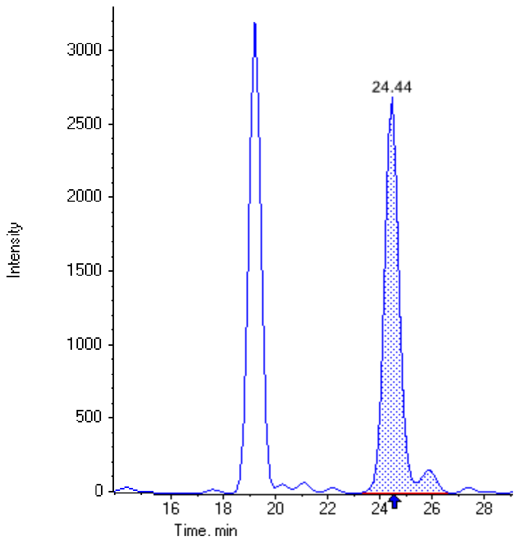

Figure D.72: BAC18 chromatogram for pink dentex.

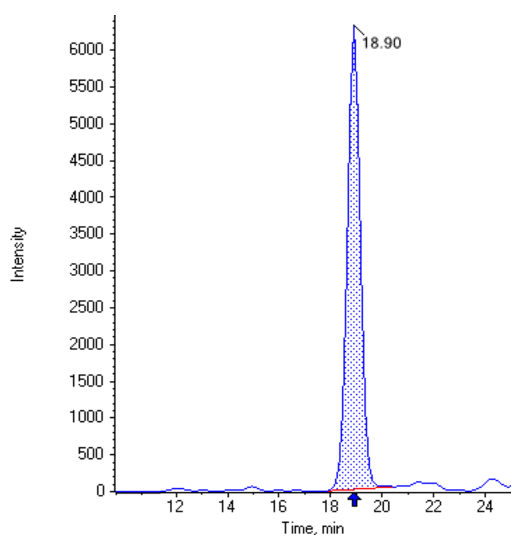

Figure D.73: DDAC-C-8 chromatogram for pinkdentex.

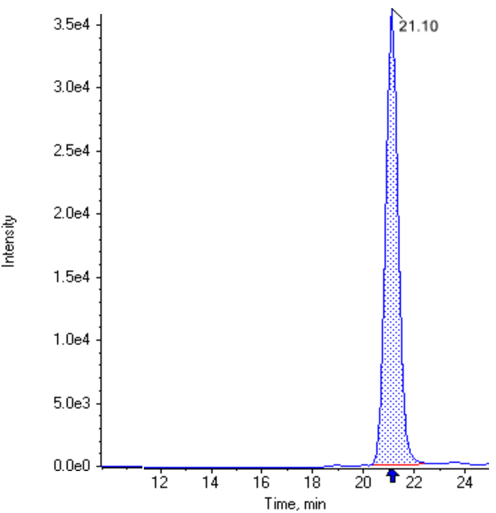

Figure D.74: DDAC-C-10 chromatogram for pinkdentex.

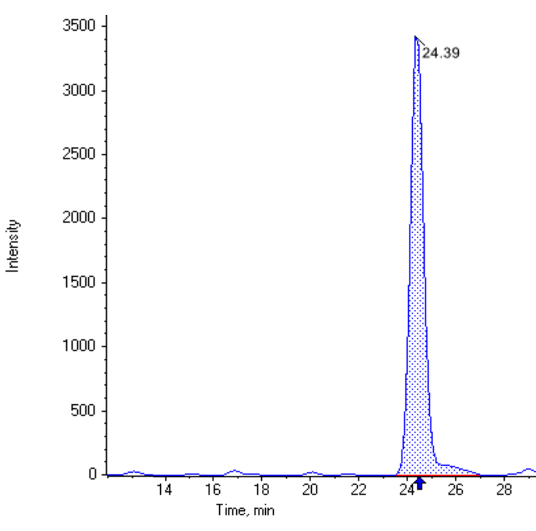

Figure D.75: DDAC-C-12 chromatogram for pinkdentex.

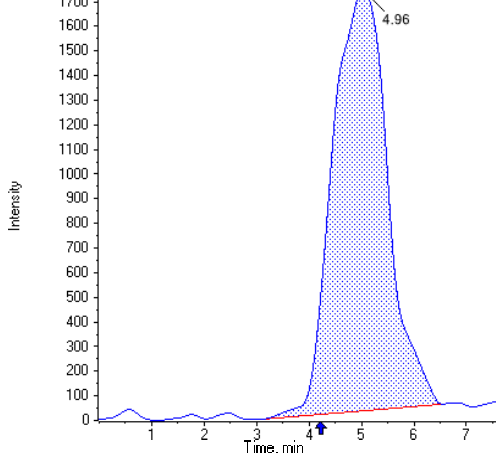

Figure D.76: Chlorate chromatogram for pink dentex.

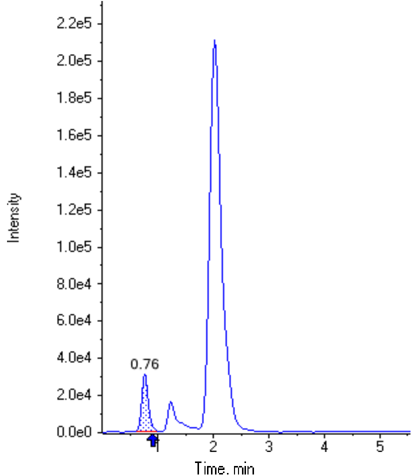

Figure D.77: Histamine chromatogram for pink dentex.

E. ANOVA tables performed in the precision test

Table E.1 to Table E.11 display the ANOVA analyses carried out on the precision test.

Table E.1: ANOVA table for BAC8.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	410.18	1	410.18	16.34	0.003	5.32
Within Groups	200.87	8	25.11			
Total	611.04	9				

Table E.2: ANOVA table for BAC10.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	167.04	1	167.04	6.05	0.04	5.32
Within Groups	220.71	8	27.59			
Total	387.75	9				

Table E.3: ANOVA table for BAC12.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	128.78	1	128.78	13.01	0.007	5.32
Within Groups	79.19	8	9.90			
Total	207.97	9				

Table E.4: ANOVA table for BAC14.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	256.34	1	256.34	26.64	0.001	5.32
Within Groups	76.99	8	9.62			
Total	333.33	9				

Table E.5: ANOVA table for BAC16.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	328.79	1	328.79	16.28	0.004	5.32
Within Groups	161.54	8	20.19			
Total	490.33	9				

Table E.6: ANOVA table for BAC18.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	690.10	1	690.10	4.72	0.06	5.32
Within Groups	1168.55	8	146.07			
Total	1858.64	9				

Table E.7: ANOVA table for DDAC-C-8.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	887.72	1	887.72	17.52	0.003	5.32
Within Groups	405.35	8	50.67			
Total	1293.07	9				

Table E.8: ANOVA table for DDAC-C-10.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1756.66	1	1756.66	11.18	0.01	5.32
Within Groups	1257.19	8	157.15			
Total	3013.85	9				

Table E.9: ANOVA table for DDAC-C-12.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	372.87	1	372.87	1.17	0.31	5.32
Within Groups	2550.91	8	318.86			
Total	2923.78	9				

Table E.10: ANOVA table for chlorate.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.70	1	14.70	0.05	0.83	5.32
Within Groups	2469.96	8	308.74			
Total	2484.65	9				

Table E.11: ANOVA table for histamine.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	810.20	1	810.20	2.99	0.12	5.32
Within Groups	2171.37	8	271.42			
Total	2981.57	9				

F. Matrix Effect

The values obtained for the matrix effect, in particular for each matrix analysed, can be found in Table F.1.

Table F.1: Matrix effect for the samples analysed.

Pesticide	Bigeye tuna (1)	Blue jack mackerel (2)	Black cardinal fish (3)	Phycis phycis (4)	Bigeye tuna (5)	Atlantis bluefin tuna (6)	European parrotfish (7)	Black scabbardfish (8)	European seabass (9)	Pink dentex (10)
BAC8	100.250	218.477	230.185	209.851	220.854	235.426	231.044	240.030	468.291	239.127
BAC10	92.882	42.294	48.573	42.375	44.902	44.624	53.716	46.994	96.447	48.724
BAC12	89.352	53.324	58.476	83.786	91.396	83.548	57.696	68.604	100.130	82.984
BAC14	82.014	42.286	44.844	42.868	43.540	39.484	43.671	52.164	92.662	31.658
BAC16	70.087	33.798	64.785	45.472	44.382	43.201	38.646	48.421	79.074	46.398
BAC18	92.413	52.382	51.007	49.452	52.085	54.953	55.809	53.939	89.995	43.806
DDAC-C-8	83.975	40.418	36.559	26.910	28.968	26.588	44.491	38.561	72.565	21.860
DDAC-C-10	73.300	100.643	39.785	56.676	52.808	61.550	66.529	55.545	79.372	66.921
DDAC-C-12	96.031	54.841	52.967	52.431	52.282	60.293	61.584	60.507	98.247	50.923
Chlorate	162.476	114.073	120.136	226.444	283.690	219.327	202.645	335.450	343.902	277.785
Histamine	28.796	38.359	2.918	5.113	37.260	38.147	29.943	34.495	34.191	19.703

Attachments

I. Statistical tests

The Table I.1, Table I.2, and Table I.3 show the critical values used in chapter for the Fisher, Conchran and t-student tests.

Table I.1: Fisher's critical values at 95% confidence.

Graus de liberdade do denominador	Graus de liberdade do numerador												
	1	2	3	4	5	6	7	8	9	10	12	15	20
1	161,448	199,500	215,707	224,583	230,162	233,986	236,768	238,883	240,543	241,882	243,906	245,950	248,013
2	18,513	19,000	19,164	19,247	19,296	19,330	19,353	19,371	19,385	19,396	19,413	19,429	19,446
3	10,128	9,552	9,277	9,117	9,013	8,941	8,887	8,845	8,812	8,786	8,745	8,703	8,660
4	7,709	6,944	6,591	6,388	6,256	6,163	6,094	6,041	5,999	5,964	5,912	5,858	5,803
5	6,608	5,786	5,409	5,192	5,050	4,950	4,876	4,818	4,772	4,735	4,678	4,619	4,558
6	5,987	5,143	4,757	4,534	4,387	4,284	4,207	4,147	4,099	4,060	4,000	3,938	3,874
7	5,591	4,737	4,347	4,120	3,972	3,866	3,787	3,726	3,677	3,637	3,575	3,511	3,445
8	5,318	4,459	4,066	3,838	3,687	3,581	3,500	3,438	3,388	3,347	3,284	3,218	3,150
9	5,117	4,256	3,863	3,633	3,482	3,374	3,293	3,230	3,179	3,137	3,073	3,006	2,936
10	4,965	4,103	3,708	3,478	3,326	3,217	3,135	3,072	3,020	2,978	2,913	2,845	2,774
11	4,844	3,982	3,587	3,357	3,204	3,095	3,012	2,948	2,896	2,854	2,788	2,719	2,646
12	4,747	3,885	3,490	3,259	3,106	2,996	2,913	2,849	2,796	2,753	2,687	2,617	2,544
13	4,667	3,806	3,411	3,179	3,025	2,915	2,832	2,767	2,714	2,671	2,604	2,533	2,459
14	4,600	3,739	3,344	3,112	2,958	2,848	2,764	2,699	2,646	2,602	2,534	2,463	2,388
15	4,543	3,682	3,287	3,056	2,901	2,790	2,707	2,641	2,588	2,544	2,475	2,403	2,328
16	4,494	3,634	3,239	3,007	2,852	2,741	2,657	2,591	2,538	2,494	2,425	2,352	2,276
17	4,451	3,592	3,197	2,965	2,810	2,699	2,614	2,548	2,494	2,450	2,381	2,308	2,230
18	4,414	3,555	3,160	2,928	2,773	2,661	2,577	2,510	2,456	2,412	2,342	2,269	2,191
19	4,381	3,522	3,127	2,895	2,740	2,628	2,544	2,477	2,423	2,378	2,308	2,234	2,155
20	4,351	3,493	3,098	2,866	2,711	2,599	2,514	2,447	2,393	2,348	2,278	2,203	2,124

Table I.2: Cochran's critical values at 95% confidence.

k \ n	2	3	4	5	6
2	0,9985	0,9750	0,9392	0,9057	0,8772
3	0,9669	0,8709	0,7977	0,7457	0,7071
4	0,9065	0,7679	0,6841	0,6287	0,5895
5	0,8413	0,6838	0,5931	0,5441	0,5065
6	0,7808	0,6161	0,5321	0,4803	0,4447
7	0,7271	0,5612	0,4800	0,4307	0,3974

Table I.3: Critical t-student values.

n \ p	0.75	0.8	0.85	0.9	0.925	0.95	0.975	0.99	0.995	0.999	0.9995
1	1.000	1.376	1.963	3.078	4.165	6.314	12.706	31.821	63.657	318.31	636.62
2	0.816	1.061	1.386	1.886	2.282	2.920	4.303	6.965	9.925	22.327	31.599
3	0.765	0.978	1.250	1.638	1.924	2.353	3.182	4.541	5.841	10.215	12.924
4	0.741	0.941	1.190	1.533	1.778	2.132	2.776	3.747	4.604	7.173	8.610
5	0.727	0.920	1.156	1.476	1.699	2.015	2.571	3.365	4.032	5.893	6.869
6	0.718	0.906	1.134	1.440	1.650	1.943	2.447	3.143	3.707	5.208	5.959
7	0.711	0.896	1.119	1.415	1.617	1.895	2.365	2.998	3.499	4.785	5.408
8	0.706	0.889	1.108	1.397	1.592	1.860	2.306	2.896	3.355	4.501	5.041
9	0.703	0.883	1.100	1.383	1.574	1.833	2.262	2.821	3.250	4.297	4.781
10	0.700	0.879	1.093	1.372	1.559	1.812	2.228	2.764	3.169	4.144	4.587
11	0.697	0.876	1.088	1.363	1.548	1.796	2.201	2.718	3.106	4.025	4.437
12	0.695	0.873	1.083	1.356	1.538	1.782	2.179	2.681	3.055	3.930	4.318

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