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MASTER THESIS

Development of a multi-residue methodology for the determination of thyreostats in bovine muscle tissue by Hydrophilic Interaction Liquid Chromatography-tandem Mass Spectrometry (HILIC-MS/MS)

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Development of a multi-residue methodology for the determination of thyreostats in food of bovine muscle tissue by Hydrophilic Interaction Liquid Chromatography-tandem Mass Spectrometry (HILIC-MS/MS)

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Ανάπτυξη πολύ-υπολειμματικής μεθοδολογίας για το προσδιορισμό θυρεοστατικών φαρμάκων σε μυϊκό ιστό βοοειδούς με Υγροχρωματογραφία Υδρόφιλων Αλληλεπιδράσεων συζευγμένη με διαδοχική Φασματομετρία Μαζών (HILIC-MS/MS)

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ABSTRACT

During the last decades, a large number of veterinary drugs have been used in order to improve animal health but also as growth promoters for intensive animal production. Antibiotics, anthelmintics and thyreostats are the most commonly used classes of drugs along with coccidiostats. Their improper use, non-respect of withdrawal periods, and cross-contamination can lead to the presence of veterinary drug residues in food of animal origin. The possible adverse effects on public health include allergic reactions in hypersensitive or sensitized individuals, and the development of resistant strains of bacteria following the ingestion of sub-therapeutic doses of antimicrobials.The possible presence of residues of veterinary drugs and other contaminants in edible tissues and food products is one of the main food safety issues of major concern to the public.

The aim of this study was the development of a sensitive, selective, resistant and effective analytical method for the determination of thyreostatic drugs 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil, methimazole (TAP), and 2-mercaptobenzimidazole (MBI) in food of animal origin using Hydrophilic Interaction Liquid Chromatography tandem Mass Spectrometry (HILIC-MS / MS).

Initially, an extended review of the veterinary drug classes and the existing methodology for the determination of thyreostatic drugs is presented. The experimental section of the thesis is constituted of two main parts: (1) Development and optimization of an analytical method for the determination of 6 thyreostatic drugs and (2) Method validation.

SUBJECT AREA: Analytical Chemistry

Keywords: LC-MS/MS, HILIC, thyreostats, residue analysis, food of animal origin

ΠΕΡΙΛΗΨΗ

Τις τελευταίες δεκαετίες, ένας μεγάλος αριθμός κτηνιατρικών φαρμάκων έχει χρησιμοποιηθεί για τη βελτίωση της υγείας των ζώων αλλά και χρησιμοποιούνται ωστόσο και ως αυξητικοί παράγοντες για την εντατική ζωική παραγωγή. Η ακατάλληλη χρήση τους, η μη τήρηση των περιόδων αναμονής για την απομάκρυνσή τους, αλλά και η διασταυρούμενη επιμόλυνση μπορούν να οδηγήσουν στην παρουσία καταλοίπων κτηνιατρικών φαρμάκων σε τρόφιμα ζωικής προέλευσης. Τα κατάλοιπα αυτά εμφανίζουν δυσμενείς επιπτώσεις στη δημόσια υγεία περιλαμβάνοντας αλλεργικές αντιδράσεις, ανάπτυξη ανθεκτικών στελεχών βακτηρίων και άλλες επιδράσεις.

Η πιθανή παρουσία υπολειμμάτων κτηνιατρικών φαρμάκων και άλλων ρυπαντών σε βρώσιμους ιστούς και στα προϊόντα διατροφής είναι ένα από τα βασικά θέματα για την ασφάλεια των τροφίμων που προκαλεί μεγάλη ανησυχία στην κοινή γνώμη.

Ο κύριος στόχος της παρούσας ερευνητικής εργασίας είναι η ανάπτυξη ευαίσθητης, εκλεκτικής, ανθεκτικής και αποτελεσματικής αναλυτικής μεθόδου για τον προσδιορισμό των θυρεοστατικών φαρμάκων 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil, methimazole (TAP) και 2-mercaptobenzimidazole (MBI) σε τρόφιμα ζωικής προέλευσης με τη χρήση Υγροχρωματογραφίας Υδρόφιλων Αλληλεπιδράσεων συζευγμένης με διαδοχική Φασματομετρία Μαζών (HILIC-MS/MS).

Αρχικά, παρουσιάζεται μια εκτενής ανασκόπηση των κατηγοριών των κτηνιατρικών φαρμάκων καθώς και των υπαρχόντων μεθόδων για τον προσδιορισμό των θυρεοστατικών φαρμάκων. Το πειραματικό μέρος αποτελείται από δύο μέρη: (1) Ανάπτυξη και βελτιστοποίηση αναλυτικής μεθόδου για τον προσδιορισμό των 6 θυρεοστατικών φαρμάκων (2) Επικύρωση της μεθόδου.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αναλυτική Χημεία

Λέξεις-κλειδιά: LC-MS/MS, HILIC, θυρεοστατικά, τρόφιμα ζωικής προέλευσης, υπολειμματική ανάλυση

CONTENTS

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INDEX OF FIGURES

INDEX OF TABLES

PREFACE

This work was conceived and performed at the Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece under the supervision of Professor Nikolaos S. Thomaidis.

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CHAPTER 1

Veterinary drug residues and the role of analytical chemistry

1.1 Introduction

Since always, animal breeding and agriculture have been major human activities, but nowadays they have been evolved into an important economic activity and they have a clear impact on food safety. Over the years, increasing interest has been directed toward maximizing the quantity of food product and at the same time reducing the cost. It is of vital importance to cover the needs for food supplies of an increasing world population, and also comply with legal limits regarding contaminants and veterinary drugs used. Thus, new practices in animal breeding have been designed by controlling various factors such as genetics, nutrition, health, management and the environmental conditions.

During the last decades, a large number of veterinary drugs has been used at therapeutic levels in the systems of livestock breeding in order to improve animal health and prevent stress-induced animal death but also as growth promoters for intensive animal production [1]. Antibacterials (including sulfonamides, tetracyclines, beta-lactams, macrolides etc) are widely used by farmers to fight against bacterial infections [2, 3]. Furthermore, other families of veterinary drugs, such as anthelmintics and coccidiostats, are used for the treatment of parasitic diseases and coccidiosis (an infectious disease caused by a microscopic protozoan parasite), respectively [4, 5].

Their improper use, non-respect of withdrawal periods, and cross-contamination can lead to the presence of residues of veterinary drugs, mainly antimicrobial agents, in food of animal origin. These residues may include the non-altered parent compound as well as metabolites and/or conjugates, and may have direct toxic effects on consumers, e.g. allergic reactions in hypersensitive individuals. Moreover, indirect problems in clinical treatment maybe caused through induction of resistant strains of bacteria (development of bacterial resistance) [6-8].

As a result increasing concern has been expressed for the safeguarding of the public health. In that direction, several associations and international systems of legal control are working on the quality assurance and control of the animal products entering the food supply.

1.2 Classification of veterinary drugs

1.2.1 Antibacterials

Antibacterial agents can be classified based on their mechanism of action, chemical structure, spectrum of activity or source. Most commonly, the classification is based on the chemical structures, which can provide information on chemical, physical and biological properties. The classes are: aminoglycosides, amphenicols, β-lactams, lincosamides, macrolides, nitrofurans, quinolones, sulfonamides, tetracyclines and miscellaneous.

1.2.1.1 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics isolated from *Streptomyces* and *Micromonospora* bacteria that exert their antibacterial effect by targeting the bacterial ribosome, thus inhibiting protein synthesis [9]. Their structure contains two or more aminosugars linked by glycosidic bond to an aminocyclitol group, which is 2-deoxystreptamine in most aminoglycosides or streptidine in streptomycin and dehydrostreptomycin. Most aminoglycosides are mixtures of several very similar components differing only in degree of methylation or stereochemistry of the sugar units. Closely related aminocyclitols, such as spectinomycin or apramycin, that also contain an aminocyclitol group but slightly differ in structure, are generally considered part of the aminoglycoside class of antibiotics. They are administered both therapeutically and prophylatically to treat cattle, swine and poultry [10]. Aminoglycosides are not absorbed orally and so are usually administered via intramuscular injection. Residues of these drugs tend to concentrate in the kidney as they are generally excreted through the urinary tract [11].

1.2.1.2 Amphenicols

Amphenicols (chloramphenicol, florfenicol, and thiamphenicol) are broad-spectrum antibiotics with a phenylpropanoid structure, active against a variety of pathogens. They function by blocking the enzyme peptidyl transferase on a ribosome subunit of bacteria [9]. Chloramphenicol was first isolated from cultures of *Streptomyces venezuelae* but is now produced synthetically. It readily forms conjugates with glucuronic acid in the liver of treated animals and therefore appears in kidney mainly as the corresponding glucuronide [12]. However due to the reports of serious side effects (mainly aplastic anemia) in humans, chloramphenicol was banned in the EU, the USA and Canada in the 1990s. Structurally similar thiamphenicol and florfenicol, in which the nitro group of chloramphenicol is replaced by a methyl sulphonyl group (in florfenicol, a hydroxyl group is also replaced by a fluorine), have been permitted as chloramphenicol substitutes.

1.2.1.3 β-Lactams

β-Lactam antibiotics are probably the most widely applied antimicrobial drugs in current veterinary practice. They are divided into two subcategories: penicillins and cephalosporins. These antibacterials have as their basic structure a thiazolidine ring, a β-lactam ring and variable side chains that account for the major differences in their chemical and pharmacological properties [13]. In penicillins, the ring is fused to a five-member thiazolidine ring, while for cephalosporins the ring is fused to a six-member ring. The *β*-lactam ring is responsible for the antimicrobial activity and also for a reduced stability of *β*-lactams. They are thermolabile, unstable in alcohols and acidic conditions [14].

Their mode of action is based on inhibiting bacterial cell wall biosynthesis, which has lethal effect on bacteria. However, bacteria have shown resistant against β-lactam antibiotics [15]. Penicillins are derived from *Penicillium fungi* and are historically significant because they are the first drugs that were effective against many previously serious diseases. They are used in the treatment of bacterial infections caused by susceptible, usually Gram-positive,

organisms [16]. Cephalosporins are originally derived from the fungus *Acremonium*, previously known as *Cephalosporium*. First-generation cephalosporins were active predominantly against Gram-positive bacteria but successive generations have increased activity against Gram-negative bacteria, as well.

1.2.1.4 Macrolides and Lincosamides

Macrolides are basic macrocyclic antibiotics that have a common 14-, 16-, or 17-membered ring in their structure, which is linked by glycoside bonding to one or more molecules of deoxy sugars, usually cladinose and desosamine. They are widely used in veterinary practice to treat respiratory diseases and to promote growth and are usually used against Gram-positive organisms that are resistant to penicillin treatment. Erythromycin and tylosin are the drugs most commonly given to food-producing animals. Macrolide antibiotics are weak bases readily soluble in common organic solvents [17]. Lincosamides (lincomycin, clindamycin, and pirlimycin) are monoglycosides with an amino acid side chain. The first lincosamide to be discovered was lincomycin, isolated from *Streptomyces lincolnensis*. They are highly effective against a broad spectrum of gram-positive and anaerobic bacteria. Both macrolides and lincosamides target the bacterial ribosome and inhibit protein synthesis [13, 14, 17].

1.2.1.5 Nitrofurans

Nitrofurans are synthetic antibacterial compounds, which contain a characteristic 5-membered nitrofuran ring in their structure. They are used to treat infections caused by protozoa or by certain Gram-positive or Gram-negative bacteria and do not contribute to the development of antimicrobial resistance [9, 13]. The precise mechanism by which nitrofurans exert their antimicrobial effects is not completely clarified, but it is based on inhibition of enzyme systems [18]. They are used in the poultry industry as well as for the treatment of cattle and pigs and residues of them have also been found in farm-raised shrimp and honey [11]. However due to their toxicological effects (carcinogenity and mutagenicity), nitrofurans (nitrofurazone, nitrofurantoin,

furaltadone, furazolidone and later also nifursol) were banned in many countries, including the US, the EU, Japan and Australia, starting in mid-1990s to early 2000s.

1.2.1.6 Quinolones

Quinolones are broad spectrum synthetic antibiotics (derived from 3-quinolenecarboxylic acid) that are widely used in aquaculture and poultry farming. They prevent bacterial DNA from unwinding and duplicating.

The first generation of quinolones includes mainly oxolinic acid and nalidixic acid that are effective only against Gram-negative bacteria, while the second-generation quinolones are fluoroquinolones, such as enrofloxacin, danofloxacin and ciprofloxacin. Fluoroquinolones contain a fluorine atom at the C-3 position and a piperazinyl group at the C-7 position, which increases the activity against Gram-positive and Gram-negative bacteria, respectively, and the majority of quinolones in clinical use belong to this subclass [19]. Quinolones are also highly important human drugs, and their widespread use in food-producing animals is of high concern due to the recent evidence of development of bacterial resistance to these antibiotics.

1.2.1.7 Sulfonamides

Sulfonamides are synthetic antibiotics that are used for prophylactic and therapeutic treatment of bacterial and protozoal infections. They share a common chemical nucleus that comes from sulfanilamide and is responsible for the exhibited antimicrobial activity [14]. They have been used clinically for more than 50 years, and during this time over 5000 derivatives have been tested. Sulfonamides show large variations in polarity and exhibit amphoteric properties. In bacteria, antibacterial sulfonamides act as competitive inhibitors of the enzyme dihydropteroate synthetase (DHPS), an enzyme involved in folate synthesis (vitamin B9). As such, the microorganism will be "starved" of folate and die. On the contrary, humans, acquire folate through the diet [20]. Sulfonamides are often administered together with synthetic diaminopyrimidines, such as baquiloprim, ormetoprim or trimethoprim, which act as potentiators of sulfonamides.

1.2.1.8 Tetracyclines

Tetracyclines are broad-spectrum antibiotics that consist of a substituted 2-napthacenecarboxamide molecule. They are widely used in veterinary medicine for cost-effective prophylactic and therapeutic treatment and also as growth-promoting substances in cattle and poultry but their usefulness has been reduced with the onset of bacterial resistance. Tetracycline antibiotics are protein synthesis inhibitors, inhibiting the binding of aminoacyl-tRNA to the mRNA-ribosome complex [21].

1.2.1.9 Other antibacterials

Unlike the compounds in the preceding groups, several individual antibacterials have heterogenous nature. A tabulated survey of their properties is not possible. However, there are a number of subgroups including diaminopyrimidines quinoxalines, pleuromutilins, peptides or novobiocin and dapsone that merit discussion.

Diaminopyrimidines are a class of organic chemical compounds that include two amine groups on a ring. They include many dihydrofolate reductase inhibitor drugs and the antibiotics iclaprim and trimethoprim. Trimethoprim blocks folic acid synthesis in bacteria at a step later than the sulfonamides [22].

Carbadox and olaquindox are both quinoxaline-1, 4- dioxide antibacterials that are synthetically produced. They are light-sensitive compounds and require special handling precautions during analysis to prevent their decomposition. Metabolism studies have shown that carbadox is rapidly converted into its mono-oxy and desoxy metabolites whereas quinoxaline-2-carbonic acid is considered to be the last remaining major metabolite and may serve as a marker residue. Both carbadox and its desoxy metabolite are carcinogenic compounds [23].

Pleuromutilin and its derivatives are antibacterial drugs that inhibit protein synthesis in bacteria by binding to the peptidyl transferase component of the 50S subunit of ribosomes. This class of antibiotics includes retapamulin,

valnemulin and tiamulin [24]. Among the peptides, the main antibacterials are avoparcin, bacitracin, efrotomycin, polymyxin and virginiamycin. Most are complex multicomponent compounds that possess large peptide molecules that often contain D-amino acids in contrast to naturally occurring proteins, which are composed of L-amino acids.These peptides disrupt both Gram positive and Gram negative bacteria by interfering with cell wall and peptidoglycan synthesis [13].

Novobiocin, also known as albamycin or cathomycin, is an aminocoumarin antibiotic that is produced by the actinomycete Streptomyces niveus. Aminocoumarins are very potent inhibitors of bacterial DNA gyrase, with higher potency than fluoroquinolones, but at a different site on the enzyme. Finally, dapsone (diamino-diphenyl sulfone), according to its chemical structure, is not comprehended in any antibacterial class but according to its mechanism of action, it falls onto the sulfonamide group. As an antibacterial, dapsone inhibits bacterial synthesis of dihydrofolic acid, via competition with para-aminobenzoate for the active site of dihydropteroate synthetase. It is used for the treatment of Mycobacterium leprae infections (leprosy) and for a second-line treatment against Pneumocystis jirovecii [13].

1.2.2 Anthelmintics

Anthelmintics (also called parasiticides, endectocides and nematocides) are drugs used to treat parasitic warm infections, including flatworms (tapeworms and flukes) and roundworms (nematodes), which usually infect human, livestock and crops, affecting food production.

They are usually classified into several types on the basis of similar chemical structure and mode of action. Basically, three main families can be distinguished: benzimidazoles, nicotinic receptor agonists and macrocyclic lactones (avermectines and milbemycins) [25]. The benzimidazoles consist of a ring system composed of a benzene ring fused with an imidazole ring. They exert their effect by binding selectively and with high affinity to the beta-subunit of helminth microtubule protein. The target site of the nicotinic agonists (e.g. levamisole, tetrahydropyrimidines) is a pharmacologically

distinct nicotinic acetylcholine receptor channel in nematodes. The macrocyclic lactones (e.g. ivermectin, moxidectin) are a group of complex compounds isolated from Streptomyces avermitilis. They act as agonists of a family of invertebrate-specific inhibitory chloride channels that are activated by glutamic acid [26].

The most frequently used anthelmintic compounds are levamisole, several compounds from the benzimidazole group (albendazole, cambendazole, fenbendazole, oxfendazole and thiabendazole) and ivermectin [1]. Other important anthelmintics are dichlorvos and haloxon (organophosphorus cholinesterase antagonists) and piperazine (gamma-amino-butyric acid agonist at receptors on nematode muscles causing flaccid paralysis). Praziquantel has a selective effect on the tegument of trematodes and increases permeability of calcium while salicylanilides: rafoxanide, oxyclozanide, brotianide and closantel and the substituted phenol, nitroxynil, are proton ionophores [27].

Anthelmintic resistance is wide-spread and a serious threat to effective control of helminth infections and, therefore, new classes of anthelmintics with new new modes of action are being proposed. Thus, a new anthelmintic class named aminoacetonitrile derivative (AAD) has been developed, which is well tolerated and has low toxicity to mammals. The AAD monepantel is effective against some nematodes resistant to other drugs because its mode of action, which is based on a nematode-specific clade of acetylcholine receptor subunits, is different [24].

1.2.3 Beta-agonists

B-agonists are synthetic phenethanolamine compounds and were originally used as therapeutic treatments for asthma and preterm labour in humans [28]. However, these compounds have also been misused as nutrient repartitioning agents in livestock, where they served to divert nutrients from fat deposition in animals to the production of muscle tissues [29]. B-agonists have been banned as growth promoters in many countries including European Union countries and China because of their well-

documented adverse effects on human health. Because of diversified analogues and rapid metabolism, highly sensitive analytical methods for quantification and confirmation of trace residues in cattle tissues are necessary for surveillance of feeding processes and food animal origin [30].

1.2.4 Coccidiostats

Coccidiostats are antiprotozoal agents that act upon Coccidia parasites by inhibiting reproduction and retarding the development of the parasite in a host cell [11, 31]. Even minor lesions of the intestinal wall due to coccidiosis can lead to poorer growth of the animal and lower feed conversion, reducing economic viability. They are most commonly used in poultry populations by addition in the feed at the authorized levels and observing the prescribed hygiene requirements. The disease can also occur in other food producing animals including pigs, calves, and lambs [5].

Coccidiostats can be grouped in two major classes: the polyether ionophore antibiotics (monensin, lasalocid, maduramycin, narasin, salinomycin and semduramycin) and the nonpolyether ionophores (often reffered as synthetic compounds or chemicals). Polyether ionophore antibiotics are produced by fermentation with several strains of *Streptomyces* spp. and *Actinomadura* spp. They have both anticoccidial and antibacterial activity and they are also used as growth-promoting agents and as an active compound against clostridiosis [31].

1.2.5 Hormones

1.2.5.1 Anabolic steroids

Anabolic steroids (ASs) have been extensively used in husbandry practice with beneficial effects such as animal growth promotion and feed efficiency. The use of anabolic steroids for growth promotion purposes in meat producing animals results in an improvement in muscle growth and more lean meat. However, toxicological/epidemiological studies show that there are harmful effects to consumers; as a result the public health is placed in risk. As a

consequence, the use of anabolic steroids for fattening purposes has been banned in the European Union since 1986 [32].

1.2.5.2 Corticosteroids

Endogenous corticosteroids are produced by the adrenal cortex (e.g. cortisol) and have important effects on a variety of metabolic events, including glucose and protein metabolism. The overall effect is to increase the blood glucose level by stimulating hepatic synthesis of glucose from amino acids [33]. Nowadays, several exogenous corticosteroids (prednisolone, dexamethasone, betamethasone, methylprednisolone) are authorized for therapy in both human and veterinary practices. They are widely used to combat inflammatory diseases in food-producing animals, but they are also frequently employed as growth promoters. The European Union banned their administration for fattening purposes in 1996 [34].

1.2.5.3 Thyreostats

Thyreostats are orally active drugs, which upon administration disturb the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine. This goitrogenic activity may be attributed to the presence of athiocarbamidegroup. In livestock,the administration of thyreostats results in a considerable live weight gain, mainly caused by increased water retention in edible tissue and augmented filling of the gastrointestinal tract [35]. Consequently, these growth promoting agents negatively affect the meat quality of treated animals. In addition, xenobiotic thyreostats are listed as compounds with teratogenic and carcinogenic properties and thus pose a possible human health risk. These arguments led in 1981 to a ban on their use for animal production in the European Union [36]. A thorough description of Thyreostats presented in Chapter 2.

1.2.6 Tranquilizers

Tranquilizers are administered to animals for sedation prior to anesthesia before transport to the market. Stress in animals is known to produce a deterioration of meat quality and pigs, in particular, easily become stressed

during transport [1]. Some tranquilizers have analgesic effects (α_2-agonists) but these are the exception since analgesia is not a hall mark of tranquilizers [37].

Tranquilizers are classified into two broad categories in veterinary medicine: major and minor tranquilizers. Major tranquilizers include phenothiazides (acepromazine, promazine, and chlorpromazine), butyrophenones (azaperone, droperidol) and α_2-agonists (xylazine, detomidine, medetomidine, dexmedetomidine etc.) while minor consist of benzodiazepines (diazepam, midazolam, zolazepam) [37]. Most tranquillizers are rapidly metabolized in the animal's body and any residues are concentrated in the liver and/or kidney. These organs should be discarded if tranquillizers have been administered shortly before slaughter [1].

1.3 Veterinary drug residues in food of animal origin

Organic contaminants that might be present in food, whether from natural or anthropogenic origin, can be divided into four main categories, namely pesticides, persistent environmental chemicals, naturally occurring toxins and veterinary drugs. In the field of food safety, scientists and regulatory agencies need to identify any potential risks to consumers related to the consumption of food [2].

Taking into consideration the inevitable use of veterinary drugs and the assurance of the public health, there are several measures required in order to eliminate the possibility of contamination; extensive analytical control of food, determination of the sources of contamination and strict legislation [38].

The veterinary drug residues in food are a crucial issue in food safety and thus in public health. The concept of zero tolerance, which refers to the total absence of residues, is unrealistic, since the power of analytical chemistry is not limitless. For quite some time, this concept seemed to guarantee the highest degree of food safety as residues could not be found in meat, milk and eggs, due to high detection limits. As the power of analytical chemistry increases, the types of chemicals that can be detected increase, and the limits of concentration at which they can be measured are continually reduced.

Analytical Chemistry is the mean to expand and refine our ever-changing perspective of food safety. Since it is impossible to entirely abandon the use of veterinary drugs, a complete risk assessment must be performed in order to evaluate the possible hazards against public health.

1.3.1 Risk evaluation

Although residues from veterinary drugs in food products of animal origin are generally considered safe and well tolerated, they have been associated with a wide range of adverse effects and can represent a risk for consumers. However, the adverse effects from consuming food of animal origin, like meat, milk and eggs, are not very probable since the residues are present at very low concentrations, and thus acute human toxicity is rather unlikely [13].

The main side-effect of the presence of antibacterial residues in food is the development of resistant bacterial strains. Such resistance could be transferred to other bacteria, pathogenic or not, and can be related to the appearance of antibacterial-resistant microorganisms [8]. Although increased bacterial resistance has several causes, two are the main key factors; the overuse and misuse of antibiotics. Such resistant bacteria may enter the human food supply and cause infectious diseases that can no longer be successfully treated by the antibacterial agent. Furthermore, some substances must receive particular attention due to allergic reactions [39].

Although prophylactic medication with coccidiostats in the feed remains the major way of preventing coccidiosis, the development of resistance by the coccidium to all medications available has been the greatest problem associated with this control [31]. Also, anthelmintic resistance has become entrenched as a perennial programme favourite at any gathering of veterinary parasitologists. Anthelmintic resistance is likely to develop wherever anthelmintics are frequently used and be detected if it is investigated. Worm count or egg count reduction after treatment is useful for the detection of all types of anthelmintic resistances. More economical, faster and more sensitive in vitro assays for the detection of anthelmintic resistance have been developed [40].

Finally, growth promoters (β-agonists, hormones) have been banned in many countries, including European Union countries, because of their well-documented adverse effects on human health, such as food poisoning and cardiovascular and central nervous diseases [16,41], as well as their teratogenic and carcinogenic properties [36].

Risk assessments of veterinary drugs residing in foods are performed by following the integrative steps of hazard identification, hazard characterization, exposure assessment, and risk characterization [42]. At the step of hazard identification, known or potential adverse health effects in humans are identified, which are induced by a veterinary drug or its metabolites that may be present in a particular food.

Toxicological evaluations, toxicokinetic assessments, and cancer/non-cancer evaluations are mainly performed for hazard identification. At the hazard characterization step, the characteristics of the adverse effects associated with a veterinary drug or its metabolites present in food are demonstrated. In addition, the levels that clearly do not cause any adverse effects on human health are evaluated according to dose-response relationships [43].

Maximum acceptable or tolerable levels for chemicals which are neither genotoxic nor carcinogenic, such as acceptable daily intake (ADI), reference dose (RfD), tolerable daily intake (TDI) and provisional tolerable weekly intake (PTWI) for contaminants which may accumulate in the body, are set. Dose–response information is essential for quantifying an adverse health effect. NOAEL (No Observed Adverse Effect Level) is the highest dose of a substance which causes no detectable adverse alteration in line with defined treatment conditions. ADI is generated using conservative statistical extrapolation to humans [44]. The ADI is an estimate of the residue, expressed in term of mg or mg per kg bodyweight, which can be ingested daily over a lifetime with a health risk to the consumer. In calculating an MRL, the ADI, the residue depletion patterns of a compound in the edible tissues of a particular food-producing animal and the theoretical food intakes are taken into account [45].

In case the chemical is evaluated as a complete carcinogen, which means a genotoxic carcinogen, it is recommended to operate a policy of prohibition and control levels “as low as reasonably practicable” [42].

Furthermore, toxicity assays involve the determination of acute toxicity, designated as LD_{50} (the dose that will kill 50% of the animals in a test series), subacute toxicity, determined by animal feeding tests lasting four weeks and chronic toxicity, assessed by animal feeding tests lasting 6 months to 2 years. In chronic toxicity tests attention is especially given to the occurrence of carcinogenic, mutagenic and teratogenic symptoms [38].

1.3.2 Legislative framework for veterinary drug residues

In order to ensure the safety of the consumers, many agencies worldwide regulate the use of antimicrobials, particularly in food-animal species. The US Department of Agriculture’s (USDA) Food Safety Inspection Service (FSIS) is responsible for the safety of meat, poultry, and egg products in the USA. The European Food Safety Authority (EFSA) is the keystone of the European Union’s (EU) risk assessment regarding food and animal feed safety. The Codex Alimentarius Commission (created by the FAO and WHO) develops food standards, guidelines and related texts such as codes of practice under the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Moreover, VICH, a trilateral (EU-Japan-USA) program aimed at harmonizing technical requirements for veterinary product registration was officially launched in April 1996.

The European Union (EU) has strictly regulated controls on the use of antibacterial agents, particularly in food–animal species, by publishing different Regulations and Directives. The use of veterinary drugs was regulated through EU Council Regulation 2377/90/EC [46], which has been repealed by Council Regulation 470/2009/EC [47] and describes the procedure for establishing Maximum Residue Limits (MRLs) for veterinary medicinal products in foodstuffs of animal origin.

In 1996, the prohibition of the use of growth promoters is laid down in Council Directives 96/22/EC and 96/23/EC, which also contain guidelines for

controlling veterinary drug residues in animals and their products with all the necessary information to set up national monitoring plans [48, 49]. In regulation (EC) No 1831/2003 the European Union (EU) has prohibited the use of antimicrobials as feed additives but allows the usage of anticoccidial drugs to allow for the prevention of coccidiosis, a disease that may cause serious economical consequences [50]. EU-wide surveillance conducted during 2009, reported that coccidiostats continue to be a problem with non-compliance rates of 2.05 and 1.19% in poultry meat and eggs, respectively [5]. In response, the European Food Safety Authority set maximum levels (MLs) for 11 coccidiostats in edible tissues (including eggs) [51].

Most recently, Regulation 37/2010/EC [52] lists pharmacologically active substances and their maximum residue level (MRL) in foodstuffs of animal origin, as well as compounds for which no MRL has been set because no hazard for public health has been observed. As regards the coccidiostat lasalocid, regulation 37/2010/EC was amended in 2012 [53].

The requirements for performance and validation of analytical methods employed in the official residues control for screening and confirmatory purposes are described in European Decision 2002/657/EC [54]. Validation shall demonstrate that the analytical method complies with the criteria applicable for the relevant performance characteristics. Different control purposes require different categories of methods. The following table determines which performance characteristic shall be verified for which type of method [54].

Table 1: European Decision's 2002/657/EC requirements

		Detection limit CCß Decision limit CCα		*Trueness/ Recovery*	*Precision*	*Selectivity/specificity*	*Applicability/ ruggedness/*
Qualitative methods	S	+	-	-	-	+	+
	C	+	+	-	-	+	+
Quantitative methods	S	+	-	-	+	+	+
	C	+	+	+	+	+	+
S = screening methods; C = confirmatory methods; + = determination is mandatory.							

Amending the Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin, a Commission Decision, 2003/181/EC, was regulated [55].

1.4 Determination of thyreostats in food of animal origin

1.4.1 Thyreostatic drugs

Nowadays the term ‘thyreostats’ or ‘thyreostatic drugs’ (TS) is used to refer to a complex group of substances that inhibit the thyroid function, resulting in a decreased production of thyroid hormones triiodothyronine (T3) and thyroxine (T4) [56]. In the past also ‘anti-hormones’ was used, although this nomenclature was not correct. Anti-hormones counteract the action of a hormone, not its production [57].

The use of this group of compounds for animal fattening purposes results in a weight gain caused by the increased filling of the gastro-intestinal tract as well as the retention of water in edible tissues, by inhibiting the thyroid hormone production [58].

Thyreostats can be divided into two main groups, respectively the xenobiotic and the natural occurring sulfur compounds [57]. These are polar and amphoteretic thioamides, characterized with a low molecular weight. Within their formula a common element is displayed, the nitrogen–carbon–sulfur sequence (thioamide), presumed responsible for the thyroid-inhibiting activity.

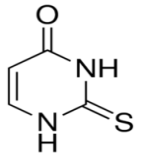
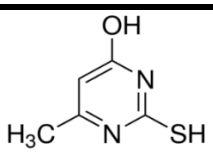
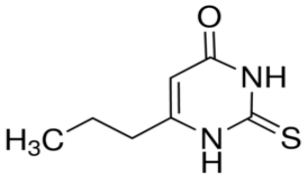
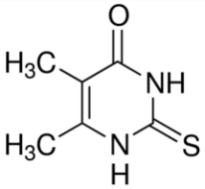
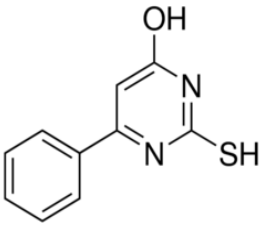
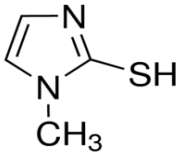
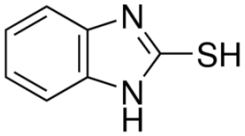
Additionally, a large number of other molecules like inorganic ions such as lithium (Li^{+}), perchlorate ($ClO4^{-}$) and thiocyanate (SCN), but also veterinary drugs like sulfonamides may have a thyreostatic action.

The group of natural occurring thyreostats comprises thiocyanates and oxazolidine-2-thiones (OZT’s). Thiocyanates may be classified within the group of inorganic ions because besides their natural origin, they can be chemically synthesized. The group of natural TS originates from precursors

(glucosinolates), present in plants of Brassicaceae (syn. Cruciferae) and related families [59].

Within the framework of residue control of xenobiotic TS, 4(6)-R-2-thiouracil (R=hydrogen, methyl, propyl, phenyl), tapazole (TAP) and 2-mercaptobenzimidazole (MBI) are of most interest [56]. These synthetic drugs are the most powerful TS agents. Above this, they were cheap and readily available on the black market. The most basic xenobiotic compounds that show thyreostatic action are presented in the table below (Table 1.2).

Table 2: Abbreviations, structures, characteristics and physico-chemical parameters of thyreostats [].

THYREOSTATS(TS)	***SYNONYMS***	***STRUCTURE***	***CHARACHTERISTICS***
2-thiouracil (TU)	4-Hydroxy-2-mercaptopyrimidine		Chem. formula: $C_4H_4N_2OS$ Exact mass: 128.0044u
6-methyl-2-thiouracil (MTU)	4-hydroxy-2-mercapto 6-methylpyrimidine		Chem. formula: $C_5H_6N_2OS$ Exact mass: 142.0201u
6-propyl-2-thiouracil (PTU)	2,3-dihydro-6-propyl-2-thioxo-4(1*H*)-pyrimidinone 4-hydroxy-2-mercapto-6-propylpyrimidine		Chem. formula: $C_7H_{10}N_2OS$ Exact mass: 170.0514u
5,6-dimethyl-2-thiouracil (DMTU)	-		Chem. formula: $C_6H_8N_2OS$ Exact mass: 150.0252u
6-phenyl-2-thiouracil (PhTU)	-		Chem. formula: $C_{10}H_8N_2OS$ Exact mass: 204.0357u
tapazole (TAP)	methimazole, 1-methyl-2-imidazolethiol, 2-mercapto-1-methylimidazole		Chem. formula: $C_4H_6N_2S$ Exact mass: 114.0252u
2-mercaptobenzimidazole (MBI)	1,3-dihydro-2*H*-benzimidazole-2-thione, 2-benzimidazolethiol		Chem. formula: $C_7H_6N_2S$ Exact mass: 150.0252u

1.4.2 Legislative framework

When thyreostatic drugs are administrated in livestock breeding, residues may occur in edible matrices derived from these treated animals. Due to the potential human health risk, the European Union (EU) issued certain regulations concerning the use of substances with thyreostatic action. Subsequently, guidelines and criteria were set for the detection of TS abuse [59].

The European Community has always been sensitive to the health and safety of its citizens, especially of the most vulnerable ones. That is why several laws regulating the presence of xenobiotics in foods were promulgated. Unexpectedly, there is a lack of legislation regarding residues in foods intended for children, with the exception of the Commission Directive 2006/ 125/EC, which establishes specific maximum residue levels of pesticides or their metabolites in processed cereal-based baby foods. In the legislative void of such foodstuffs, the maximum precaution principle and zero tolerance policy are usually applied so that drug residues in baby foods are consider illegals at any levels [60].

Unlike the European Union, the livestock industry of some countries, such as the United States, Canada and Australia, permits the administration of growth promoters to increase the weight and muscle mass of animals and to produce leaner and affordable meat. Different classes of pharmacologically active compounds exhibit anabolic effects: hormones, microbial agents and drugs that interfere with the endocrine functions. Thyreostats belong to this last group.

In Belgium, substances with thyreostatic action were prohibited since 1974 (KB 12.04.1974). The European Community has prohibited the use of growth promoters in stock farming since 1981 (Council Directive 81/602/EEC) and even meat importation from third countries which allow their employment. Later on, Council Directive 85/358/EEC amended Council Directive 81/602/EEC, to guarantee a uniform application for detection and monitoring of TS in all Member States. Council Directive 96/22/EC, the revision of

Council Directive 81/602/EEC described the prohibition on the use of certain substances with hormonal or thyreostatic action in stock farming [59].

Directive 81/602/EEC described the prohibition on the use of certain substances with hormonal or thyreostatic action in stock farming. Additionally, it promulgates that Member States have to prohibit the import of meat from treated animals, from third countries. The measures to monitor the residue control of certain substances (listed in Annex I), e.g. thyreostats in live animals and animal products are described by the Council Directive 96/23/EC. Two groups of substances are included in Directive 96/23/EC listed in Annex I, based on Commission Regulation No. 2377/90. Group A comprises substances having hormonal or thyreostatic action, β-agonists (Directive 96/22/EC) and veterinary drugs that now have been banned (included in Annex IV of Council Regulation (EEC) No 2377/90). Group B comprises other veterinary drugs and contaminants [59].

For good implementation of directive 96/23/EC, it is necessary to determine common criteria for the interpretation of test results of official control laboratories. Also important, in particular for substances not authorized or prohibited by the EU, is the progressive establishment of a minimum required performance limit (MRPL) of analytical methods. For thyreostats a suggested MRPL is fixed at 100μgL^{-1} or μgkg^{-1}.

In recent years, active researchers in the food safety area have supposed a natural origin for the low levels of TU (1–10 μg L^{-1}) frequently found in the urine of non-treated animals [61]. For these reasons, the European Community Reference Laboratories' guidance paper of 2007 has stated that concentrations below 10 μg L^{-1} might derive from the consumption of glucosinolate rich Brassicaceae plants [62]. Therefore, this value has been set as the recommended concentration (RC) for urine and the thyroid gland, even if many researchers also use it for other matrices, such as muscle tissue. After slaughter, the possible administration of thyreostats can be deduced by veterinarians who inspect the thyroid of animals and, if dimensions and morphological appearance seem altered, they can require a histological or chemical investigation. On the other hand, the chemical assay is the only

solution for verifying the compliance of meat-based processed products such as, for example, baby foods.

Commission Decision 2002/657/EC lays down the technical guidelines and performance criteria for residue control. Within this Commission decision (2002/657/EC) [70], a system of identification points (IPs) is introduced in order to interpret the obtained data (chromatograms, spectra) when detection methods are used other than full-scan techniques. This system is based on the number and the ratio of the ions in the obtained MS spectrum. For the confirmation of the banned substances, listed as group A (e.g. thyreostats), a minimum of four IPs is required. Since the implementation of the 2002/657/EC criteria [70], few studies describe the applicability of these guidelines for determination of thyreostats in urine and thyroid [68]. Parameters that need to be evaluated during the validation procedure are selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, stability, repeatability, reproducibility and decision (CCα) and detection limits (CCβ).

1.4.3 Effects in human health

Unlike other anabolic agents such as the natural hormones [71], the agreement on the ban of these drugs is international due to the inferior quality of meat that contains high water concentration and residues dangerous to human health. As a matter of fact, the toxicity of thyreostats ranges from minor effects (urticaria, vomiting, arthralgia and lethargy) to severe effects (liver damage, agranulocytosis [72], thrombocytopenia, leukopenia, eosinophilia, lymphocytosis, and autoimmune events [73]. TU, MTU and PTU are listed in Group 2B (possibly carcinogenic to humans) by the International Agency for Research on Cancer (2001). Their negative effects have a higher impact on infants, who represent the most vulnerable group of the population. Moreover, in comparison with adults, they have a high food consumption rate per weight unit, which makes them even more exposed to xenobiotics, especially when consuming baby food products [60]. Besides that, it has been proposed that residues of TS may be teratogenic and carcinogenic.

1.4.4 Used analytical methodologies

1.4.4.1 Extraction

It must be pointed out that sample pretreatment prior to the analysis is an absolute requirement for eliminating interfering substances. To extract thyreostats from matrices (e.g. animal tissue, excreta, plasma and organs) most often methanol is used as a solvent, but also acetonitrile and ethyl acetate have been reported. In the beginning, a mercurated affinity column was used for the clean-up of extracts nowadays solid phase extraction (SPE) gained in interest. In most cased. Silica SPE cartridges are mentioned, but anion exchange, amino-propyl and alumina cartridges have also been reported. Some even resort to the technology of matrix solid-phase dispersion (MSPD), because homogenization, disruption, extraction and clean-up are combined in one procedure [74].

1.4.4.2 Detection

Since the 1960s, separation methods based on gas- and liquid chromatography gained in popularity, due to the achievement of higher specificity and selectivity. With the introduction of gas chromatography (GC) in TS residue analysis, it became clear that coupling of GC with mass spectrometry (MS) was a requirement to achieve quality control criteria. Using derivatization agents like benzylchloride, pentafluorobenzylbromide or methylation prior to GC–MS analysis lower limits of detection (LOD) could be reached. Unfortunately, the recovery of certain TS compounds, like tapazole remained low. Therefore, De Brabander et al. proposed in 1992 an efficient method combining HPTLC and GC–MS analysis. In this case, a suspected spot originated on the TLC plate, it was scraped off. This was then eluted (diethyl ether), evaporated and derivatized with MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) to form silyl-derivatives that was subsequently analyzed by GC–MS. More recently, the derivatization (MSTFA) prior to the GC–MS analysis is combined with a second derivatization, executed before the purification of the sample. This supplementary derivatization solved problems concerning, recovery, repeatability and active

site adsorptions, for this NBD-Cl, PFBBr or 3-BrBBr [have been described. These methods were found suitable for confirmation of TS abuse in biological matrices (e.g. animal tissue, milk, thyroid and urine) [59].

Additionally, high-performance liquid chromatography (HPLC) methods were described for matrices such as urine, meat, serum, plasma and thyroid, all in the range of mg kg^{-1} levels. Only Buick et al. (1998) was able to reach a lower LOD, in the range of 10 $\mu g kg^{-1}$. As for the detection UV (ultraviolet), electrochemical, chemiluminescence and diode array detection (DAD) were reported. With the introduction of atmospheric pressure interfaces like ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization), liquid chromatography coupled to mass spectrometry (LC–MS) became the method of choice for (thyreostatic) residue control . The first LC–MS method was introduced by Blanchflower et al. (1997).Without derivatization 5 TS (TU, MTU, PTU, PhTU and TAP) were detected in urine and thyroid tissue at concentration levels of 25 $\mu g kg^{-1}$, based on APCI ionization and SIM-MS (single ion monitoring). Later on, LC–MS^n methods in combination with ESI were developed and applied on matrices such as urine, faeces, muscle, liver, animal feed and hair, which obtained higher sensitivity and specificity by derivatization with NBD-Cl or 3-iodobenzylbromide (3-IBBr). Within the advantages of ion trap mass spectrometry, multiple stage mass spectrometry (MS^n) originated. De Wasch et al. (1998) combined HPTLC with an ion trap mass spectrometer in MS^n mode for confirmatory purposes. Thus, in case of HPTLC suspected samples, the remainder of the extract was subjected directly into the ion trap mass spectrometer (Finnigan MAT LCQ), operating in MS^n. Ionization was performed by ESI and fragment ions were acquired up to MS^3 [75]. It must be pointed out that nowadays nearly all detection methods of TS use GC or LC coupled to multiple or tandem MS, in the attempt of improving the analytical accuracy as well as the sample throughput.

1.4.5 A brief literature review

In the following pages, a brief literature review for the determination of thyreostatic drugs is presented, containing the different matrices that

thyreostats were determined, the procedure of the sample preparation, the used analytical techniques and the results that were obtained.

Nevertheless, thyreostats are compounds hard to analyse -determine because of the manifold difficulties connected to their extraction and LC-MS determination: low molecular weight, amphoteric nature, high polarity, occurrence of tautomeric forms and oxidation. It is probably for these reasons that the literature has few analytical methods that address the confirmation - determination of these drugs in meat or meat-derived products [63, 64]. No publication is specifically devoted to thyreostatic residues in baby food from animal origin, except for PTU by Zhan et al. [65]. Among the several LC chromatographic techniques, LC-MS has long been established as the preferred technique for thyreostats' residues detection, whereas the developed extraction protocols differ according to the selected matrix [66]. For instance, Lega et al. [67] adopted QuEChERS for thyreostats' extraction from muscle and thyroid tissues, while Abuín et al [68] compared two clean-up strategies, one based on silica cartridges and the other on gel permeation chromatography, after extraction from thyroid gland with ethyl acetate. Recently, Chiesa et al. [69] applied a salting-out assisted liquid–liquid extraction to isolate thyreostats from bovine urine and the thyroid gland. The aim of this work was to develop a LC – MS/MS multi-residue method for the determination of thyreostatic agents in meat-based baby foods. The performance of a simple and quick extraction procedure based on MSPD was tested in terms of recovery yields. The advantages of the separation of the six thyreostats on a PFP column, packed with 2.6 μm core-shell particles, are reported. The whole method was validated according to the guidelines of the Commission Decision 2002/657/EC and applied to the analysis of some real samples.

Table 3: Quantitative applications in multi-residue analysis of thyreostats in different matrices

TS	*MATRIX*	*SAMPLE PREPARATION*	*ANALYTICAL TECHNIQUE*	*RESULTS*	*REFERENCE*
TU, MTU, PTU, PhTU, TAP	Bovine Urine and Thyroid Glands	Extraction by MeOH Clean-up with *Tert*-butyl methyl ether	**HPLC-ESI(+)-MS/MS** Reverse-phase HPLC Column: C18 (150x2.0 mm, i.d. 4µm). (Phenomenex) Mobile phase: A: 0.1% HCOOH B: MeOH Gradient program. Total run time: 30 min.	**(ngg^{-1}) CC_α CC_β** TAP 7.3 9.7 TU 7.4 9.7 MTU 7.0 8.7 PTU 7.4 9.6 PhTU 6.6 8.0 **Recovery (%):** TAP 96-104% TU 99-101% MTU 99-103% PTU 98-102% PhTU 100%	[69] L. M. Chiesa et al. (2016)
TU, MTU, PTU, PhTU, MBI,TAP	meat-based baby food	Extraction by MSPD	**LC-ESI(±)-MS/MS** Column: ph-core shell. Kinetex PFP (100x2.1 mm2.6 µm, 92 Å) with adherent (Phenomenex). Mobile phase: A: H2 O: ACN (50:50, v/v) B: 5mM HCOOH Gradient elution: 0%B/ 1min, 0%B-100%B/ 4min, 100%B/ 2min.	**Recovery (%) & CV %** TU 97 (14) MTU 100 (1) TAP 94 (5) PTU 93 (6) MBI 82 (8) PhTU 91 (5) **(ngg^{-1}) CC_α CC_β** TU 7.0 9.8 MTU 3.7 6.2 TAP 5.3 9.0 PTU 1.2 2.0 MBI 0.7 1.2 PhTU 2.8 4.8	[76] A. Gentili et al. (2016)
TU, MTU, PTU, TAP	cow's, lamb's, and goat's milk	Extraction with ACN and phosphate buffer 0.2 M pH = 8 Derivatization (1h, 40 ° C). Clean up with ethyl acetate,	**LC-ESI (+) - MS / MS** Column: Kinetex C18 100Å, 2.1x100 mm, 2.6 µm (Phenomenex). Mobile phase : Solvent A: CH3COOH 0.1%, Solvent B: ACN Gradient elution: 30% B (1 min), 85% B (9 min).	**(ngg^{-1}) CC_α CC_β** TAP 0.51 0.86 TU 0.87 1.49 MTU 0.73 1.24 PTU 0.28 0.48	[77] A. Cirkva et al. (2013)
TU, MTU, PTU, PhTU, MBI, TAP, DMTU	muscle and thyroid tissues	Extraction by QuEChERS (with ethyl acetate) without further derivatization.	**UPLC-ESI–MS/MS** Column: Acquity UPLC BEH C18 column (1.7µm, 100 x 2.1 mm), (Waters) Mobile phase: A: 0.1% (v/v) HCOOH B: 0.1% (v/v) HCOOH in ACN Isocratic conditions: 100% A (2min) Linear gradient: 80% A (3min). Total time of analysis: 9min	**Recoveries:** TU >60% MTU 55% PTU 45% TAP 40% MBI 40% PhTU 40%	[67] F. Lega et al. (2013)

TS	MATRIX	SAMPLE PREPARATION	ANALYTICAL TECHNIQUE	RESULTS	REFERENCE
TU, MTU, PTU, PhTU, TAP	bovine and porcine urine and muscle tissues	**Urine sample:** Extraction by Britton-Robinson buffer, pH 8.0 in MeOH Derivatization by 3-IBBr (dark, 40°C, 1h). Clean-up with diethyl ether. **Muscle tissue sample:** Extraction by MeOH and Britton-Robinson buffer, pH 8.0 (30:20 (v/v)). Defatting with 4 mL of petroleum ether.	**LC-MS^2 (IT)** Column: octadecyl grafted with SiO2 Nucleosil® 100-5 C18 AB (125 mm x 2 mm, 5 µm with CC 8/3 column). Mobile phase: A: ACN, B: CH_3COOH 0.1% Gradient elution (A: B (v / v)): 15:85 (0-5min) , 70: 30 20min, 15:85 (35-45min) **LC-ESI(+)-MS/MS** Same column and solvents Mobile Phase (A: B (v /v)): 30:70 (0 min), 70:30 (20min), 30:70 (25-30min)	**Recoveries:** urine: 82% -117% muscle tissue: 84%-102% **CC_α and CC_β:** <10 µg L^{-1}(kg^{-1}).	[78] J. Vanden Brussche et al. (2011)
TU, MTU, PTU, PhTU, TAP	cow's milk	Extraction by MeOH and H_2O Clean-up with petroleum ether.	**LC-MS/MS** Column: Poroshell 120-EC C18 column (150 mm × 2.1 mm, 2.7 µm) with octadecyl guard cartridge (4 mm × 2 mm) (Phenomenex). Total run time: 25 min. Mobile phase: A: 0.1% CH_3COOH, B: ACN Gradient elution: 25%B (0min), 65%B (4min), 70%B (15min), 25%B (18min).	**CC_α and CC_β:** <2.2 µg L^{-1} and <3.8 µg L^{-1}.	[79] B. Wosniak et al. (2014)
TAP, TU, MTU, PTU, PhTU	animal feeding stuffs	2 extractions: Liquid-liquid extraction with petroleum ether and diethyl ether. Derivatization with 3-IBBr(dark, 40 °C,1 h).	**LC-ESI(+)- MS/MS** Column: Poroshell 120-EC C18 column 150 mm × 2.1 mm, 2.7 µm) with octadecyl guard cartridge(4 mm × 2 mm) (Phenomenex) Mobile phase: A: 0.1% CH_3COOH, B: ACN Gradient elution: 25%B (0min), 65%B (4min), 70%B (15min), then returned 25%B (18min). Total run time: 25 min.	**CC_α (µgkg^{-1}) :** 1.63- 3.9 **CC_β (µgkg^{-1}) :** 2.74-6.73 **Recovery (%):** 82%-97.5% for all examined compounds	[80] B. Wosniak et al. (2014)

TS	MATRIX	SAMPLE PREPARATION	ANALYTICAL TECHNIQUE	RESULTS	REFERENCE
TU	feedstuffs used in animal husbandry	Liquid/liquid extraction with diethyl ether Clean-up with SPE.	**LC-HESI^{+}-MS^{2}** Column: Symmetry C18 column (5 µm×150 mm×2.1 mm), (Waters) Mobile phase 50/50 (v/v): A: 0.5% CH_3COOH, B: MeOH Linear gradient elution: A/B 50/50 (3 min) , 0/100 (20 min), 50/50 Total run time: 35 min.	**LOQ:** 0.5 ng g−1. **Recovery (%):** 90.9- 99.7 %. **Repeatability:** ≤6.0 **Intra-laboratory reproducibility (RSD%):** ≤5.2 %	[66] J. A. L. Kiebooms et al. (2014)
TU, MTU, PTU, PhTU, TAP, MBI	thyroid samples	Extraction with MeOH Clean-up with SPE	**LC-ESI(+)–MS/MS** Column: C18 Acquity UPLC BEH, Waters (100mm×2.1mm; 1.7 µm) Mobile phase A: 0.1% HCOOH B: ACN- 0.1% HCOOH Gradient elution: (time (min), % A): (0, 100), (2, 100), (8, 50), (9, 50), (9.1, 100).	**CC_{α}:** 4.3-16.1µgkg^{-1} **CC_{β}:** 8.7–20.7µgkg^{-1} **Reproducibility (RSD %):** 5.6-10.3%.	[82] S. Abuin (2008)
TU, MTU, PTU, PhTU, TAP, MBI	urine and thyroid gland	Extraction by MeOH Clean up with diethyl ether.	**UPLC-ESI(±)–MS/MS** Column: Acquity UPLCTM BEH C18 (1.7µm, 2.1mm×100 mm). A: H_2O: ACN: CH3COOH (80:20:0.1) B: H_2O: ACN: CH3COOH (10:90:0). Gradient separation: 100%A (0–1 min) / 100%B (1-8 min) Isocratic composition: 100% A (2min./10-11min)	**CC_{α} and CC_{β}:** below 10 µgL^{-1} (kg^{-1}).	[83] M. Luhmus (2009)
TU, MTU, DMTU, PTU, PhTU, TAP,	bovine feces	Extraction with mixture of methanol and buffer (pH = 8). Derivatization with 3-IBBr	**UPLC-ESI(+)–MS/MS** Column: B-C18 (50 × 2.1 mm; 1.8 µm, Agilent) Gradient elution: ACN/0.1% CH_3COOH within 7.5 min	**Recovery (%)** TAP 97.5 TU 109.7 MTU 110.5 PTU 101.6 PhTU 109.0 DMTU 98.7	[124] S. Witek (2017)

CHAPTER 2

Used Analytical Methodologies and Techniques

2.1 Analytical methodologies

For all the reasons mentioned above, the widespread use of veterinary drugs created the necessity to develop rapid, sensitive and reliable analytical methods for the determination of veterinary drug and pharmaceutical residues in food of animal origin. An overview of the analytical methodologies developed so far for the multi-residue analysis of thyreostats in food matrices using liquid chromatography and mass spectrometric techniques is presented in **Table 1.2**.

2.1.1 Sample preparation

Sample preparation is the process which includes the isolation and/or preconcentration of compounds of interest from various matrices, the removal of any matrix interferences that may affect the detection system as well as making the analytes more suitable for separation and detection. Even with the advances in the development of highly efficient analytical instrumentation for their final determination, sample preparation is a vital part of the analytical procedure and effective sample preparation is essential for obtaining accurate quantitative results and maintaining instrument performance.

A typical sample preparation technique consists of an extraction step of the analytes from the matrix and a subsequent purification step of the extract.

2.1.1.1 Sample extraction techniques

2.1.1.1.1 Liquid extraction (LE)

Liquid extraction is a very popular sample treatment technique. LE entails conventional liquid–liquid extraction (LLE) of target compounds from liquid matrices, such as milk, and the liquid extraction of homogenized tissues such as liver, kidney, and meat, referred to as solvent extraction (SE). To obtain

optimal results, the extraction solvent has to be selected in such way that efficient extraction of the target compounds is obtained, whereas the extraction of matrix constituents remains limited in order to prevent excessive matrix effects (ME). The selection of the solvent therefore depends not only on the target compounds, but also on the matrix.

Simple extraction with aqueous buffers (e.g. McIlvaine buffer or succinate buffer) is advantageous for highly polar residues because they reduce non-polar matrix components (e.g. lipids) and extracts can be enriched on reversed phase SPE [84-88]. A disadvantage is that strongly protein-bound residues are not fully extracted and polar matrix components are co-extracted. Complexing agents are reported to be essential for the extraction of tetracyclines, quinolones and some macrolides, because these compounds have a strong tendency to form chelates with divalent metallic cations present in food samples [84,89].

In general, the majority of methods employ more efficient organic solvents as extracting agents. Methanol (MeOH) and acetonitrile (ACN) are more adequate as extraction solvents as they can simultaneously precipitate the proteins and extract the target analytes. Many authors, prefer ACN over MeOH or ethyl acetate as extraction solvent, because MeOH and ethyl acetate extract too many matrix compounds, complicating the following clean-up steps. However, ACN does not sufficiently extract polar analytes.

A great number of multi-residue analytical methods developed use a combination of water or aqueous buffer and organic solvent as the extraction mixture of the target compounds from the matrix . Kaufmann et al. proposed a bipolar extraction, combining an extraction with ACN and one using a McIlvain buffer-containing complexing agent [90]. With one of the greater challenges in sample preparation being the development of a generic extraction method which should not only cover a vast number of target analytes, but should also be applicable to different types of food and feed matrixes, Mol et al. reported a thorough research comparing the use of ACN, MeOH and acetone (ACE) for the extraction of veterinary drugs, pesticides and toxins from honey, milk, eggs and muscle [90]. However, in the area of

multi-residue analysis there is always a compromise between recovery and purity of sample extracts.

Liquid–liquid extraction (LLE) is a widely applied extraction procedure in residue analysis due to its high selectivity compared to simple solvent extraction (SE). LLE applications can also include polar ionisable compounds, which can be extracted by non-polar organic solvents using the ion-pair technique: transforming positively charged substances into non-polar neutral compounds in the presence of organic anions, or vice versa [91,92].

Anastassiades et al. developed a variation of LLE, called QuEChERS sample preparation procedure (standing for Quick, Easy, Cheap, Effective, Rugged and Safe), which has been successfully applied to the analysis of hundreds of pesticide residues [93]. In QuEChERS approach, the high-moisture sample (H_2O is added to dry foods) is extracted with an organic solvent (mainly ACN, but also ethyl acetate or acetone) in the presence of salts (MgSO4, NaCl and/or buffering agents). The addition of salts induces phase separation of the solvent from the aqueous phase. The residues of interest and matrix co-extractives are separated into the relevant liquid phase based on their polarity with the residues partitioning into the organic phase and matrix co-extractives into the aqueous phase. The extract is subjected to further purification using dispersive-SPE (d-SPE), which entails mixing sorbents with the extract.

Although veterinary drugs present greater diversity in the chemical properties compared to pesticides, making their simultaneous extraction more difficult, many methods have been developed for antibacterial determination using this technique. The majority of methods based on the QuEChERS approach involve SE with acidic ACN in the presence or absence of EDTA followed by phase separation using anhydrous magnesium sulfate as drying agent. A few methods include a subsequent d-SPE procedure using C18, primary secondary amine (PSA) or a combination of both as sorbent. A thorough optimization of the QuEChERS procedure for the extraction of antibactrerials from animal tissues was performed from Stubbings & Bigwood [108]. QuEChERS flexibility, coupled to low cost and ease of use will undoubtedly result in an increase in its application to residue analysis.

2.1.1.1.2 Ultrasound-assisted extraction (USAE)

Ultrasound-assisted extraction (USAE) is an interesting process to obtain high valuable compounds and could contribute to the increase in the value of some food by-products when used as sources of natural compounds. The main benefits will be a more effective extraction, thus saving energy, and also the use of moderate temperatures, which is beneficial for heat-sensitive compounds. For a successful application of the USAE, it is necessary to consider the influence of several process variables, the main ones being the applied ultrasonic power, the frequency, the extraction temperature, the reactor characteristics, and the solvent–sample interaction. The highest extraction rate is usually achieved in the first few minutes, which is the most profitable period [162].

2.1.1.2 Sample clean-up/purification techniques

2.1.1.2.1 Solid-Phase Extraction (SPE)

SPE is the most important sample purification technique in residue analysis and has gradually replaced liquid-liquid extraction and liquid-liquid partitioning. A number of books and review papers have already been written on this topic and can be consulted for more detail [100-103].

A wide choice of sorbents is available which rely on different mechanisms for extraction/retention of analytes. Alumina, amino or strong cation exchangers (SCX) have been proposed for ionic antibacterials, while C18 or polymeric sorbents, especially Hydrophilic-Liphophilic Balance (HLB) polymeric reversed phases are used for neutral or ionisable compounds working at a pH lower than the pKa of the analytes. HLB sorbent consists of a copolymer of N-vinylpyrrolidone and divinylbenzenes. The hydrophilic N-vinyl pyrrolidone increases the water wettability of the polymer and the lipophilic divinylbenzene provides the reversed-phase retention necessary to retain analytes.

For compounds with varied chemical properties, mixed-mode sorbents are recommended (e.g., Bond Elut SCX cartridges for multiresidue of basic drugs

[104]. SPE can be directly used for the extraction of veterinary drugs from liquid food only (e.g., milk, or honey, which can be dissolved in aqueous media). Applications of SPE in multi-residue analysis of veterinary drugs in food matrices are presented in Table 1.2.

2.1.1.2.2 Dispersive SPE (d-SPE)

Dispersive-SPE (d-SPE) is a clean-up technique that involves mixing sorbent with a sample that has been pre-extracted with an appropriate solvent. It is typically part of the QuEChERS method where it follows the extraction step. The appropriate sorbent adsorbs matrix co-extractives onto its surface, leaving analytes of interest in the solvent. C18 sorbents remove highly lipophilic compounds and other sorbents, like amino- or carbon-based phases, are employed mainly for the removal of fatty acids and pigments, respectively. MgSO4 is added to provide additional clean-up by removing residual H2O and some other compounds via chelation. It is an extremely fast, simple and inexpensive process that provides high recovery and reproducibility for many LC- and GC-amenable analytes.

Several analytical methods have used d-SPE as a clean-up step in veterinary residue analysis, mainly using C18 as a sorbent [105-107]. PSA, amine (NH2) and silica have also been reported [108-112]. d-SPE does not provide the same degree of clean-up as SPE. However, it does provide good recovery and reproducibility, coupled with practical (speed) and cost advantages.

2.2 Instrumental analysis

2.2.1 Screening tests

Very popular and quite often used methods for residue screening are methods based on microbial or immunological assay or bioassays [113,114]. Screening methods usually can provide semi-quantitative or quantitative results, with low rate of false compliant samples. They can also assure high throughput, ease of use, short analysis time, good selectivity, and low cost. It is common practice for routine laboratories to apply screening methods,

covering families of antibiotics, and samples found to be non-compliant are then analyzed by confirmatory methods [122]. However, this approach would not be sufficient itself. Positive responses from the rapid tests would need to be correlated with an actual presence of residues in the samples. Thus, very often, screening tests are accompanied by confirmatory methods [123].

2.2.2 Confirmatory methods

Separation techniques, for example gas chromatography (GC), high-performance liquid chromatography (LC), and capillary electrophoresis, have been widely used for the analysis of veterinary drugs residues in food samples. Historically, the control of veterinary drug residues was based on chromatography coupled to non-specific technologies such as fluorimetric detector (FLD), ultra violet detector (UV) and electron capture detector (ECD). However, these techniques suffer some inherent drawbacks: each antibiotic class has to be tested separately, confirmation of the target analytes is based mainly on retention-time comparison to standards and some analytes have to be derivatized to obtain an appropriate limit of detection (LOD).

The first introduction of mass spectrometry (MS) in the 1980s was immediately considered as a revolution in the domain due to its outstanding specificity and sensitivity. Compared with older chromatographic methods based on the use of conventional detectors, the use of separation techniques coupled to very selective MS detector systems, besides supplying precious information about the identity of a specific compound, offers the additional advantage that older laborious and time-consuming sample treatment procedures can be greatly simplified, thereby resulting in faster and low-handling methodologies.

Public Health Agencies in many countries rely on detection by mass spectrometry, which, being a specific detector, affords unambiguous confirmation of contaminants in foodstuff. Commission Decision 2002/657/EC states that “Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods” [54].

2.2.2.1 Hydrophilic Interaction Liquid Chromatography (HILIC)

Liquid chromatography is a technique used to separate a sample into its individual parts. This separation occurs based on the interactions of the sample with the mobile and stationary phases. Because there are many stationary/mobile phase combinations that can be employed when separating a mixture, there are several different types of chromatography that are classified based on the physical states of those phases. Liquid-solid column chromatography, the most popular chromatography technique and the one discussed here, features a liquid mobile phase which slowly filters down through the solid stationary phase, bringing the separated components with it.

Hydrophilic Interaction Liquid Chromatography (HILIC) provides an alternative approach to effectively separate small polar compounds on polar stationary phases. The purpose of this work was to review the options for the characterization of HILIC stationary phases and their applications for separations of polar compounds in complex matrices. The characteristics of the hydrophilic stationary phase may affect and in some cases limit the choices of mobile phase composition, ion strength or buffer pH value available, since mechanisms other than hydrophilic partitioning could potentially occur. Enhancing our understanding of retention behavior in HILIC increases the scope of possible applications of liquid chromatography. One interesting option may also be to use HILIC in orthogonal and/or two-dimensional separations. Bioapplications of HILIC systems are also presented

Hydrophilic interaction liquid chromatography (HILIC) is an alternative high-performance liquid chromatography (HPLC) mode for separating polar compounds. For historical reasons, it has been reported that HILIC is a variant of normal phase liquid chromatography, but the separation mechanism used in HILIC is more complicated than that in NP-LC. While the acronym HILIC was first suggested by Alpert in 1990 [125], the number of publications on HILIC has increased substantially since 2003, as outlined in the well-constructed review by Hemström and Irgum [126].

2.2.2.1.1 Stationary Phases in HILIC

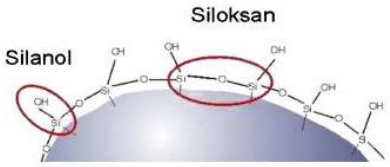
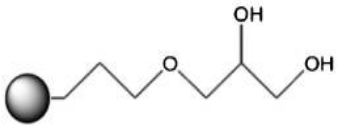
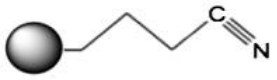
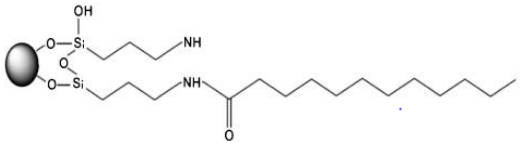
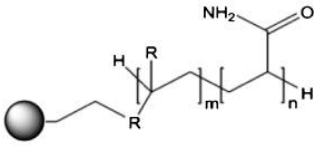
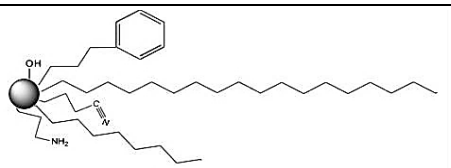
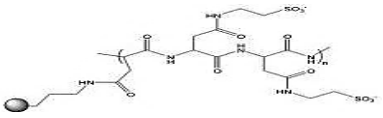
Any polar chromatographic surface can be used for HILIC separations. Typical HILIC stationary phases consist of classical bare silica or silica gels modified with many polar functional groups. Polymer-based stationary phases can also be used.

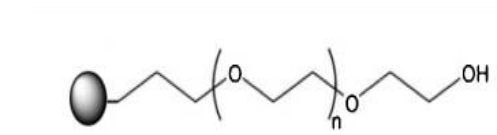
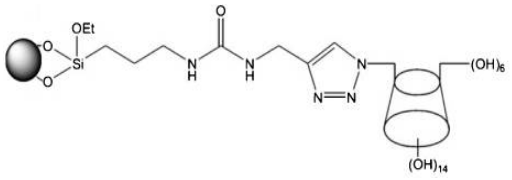
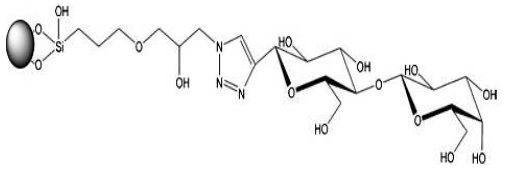
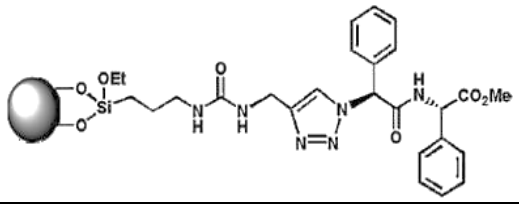
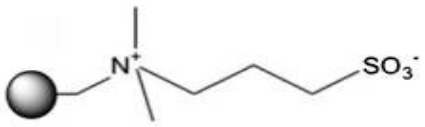
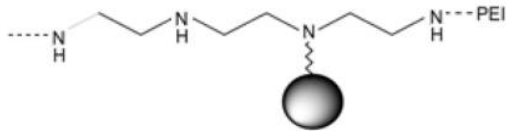
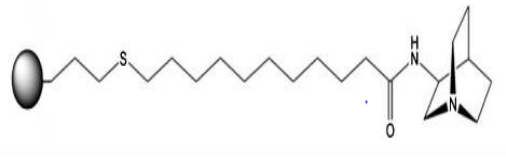
The first generation of HILIC mode separations started in 1975. Linden et al. [127] separated carbohydrates by an amino-silica phase, Bondapak (Waters, Milford, MA, USA) in a mixture of acetonotrile and water (75:25 v/v). The next generation of stationary phases for HILIC used DIOL- and amide-silica. The DIOL-silica column has mainly been used for the separation of proteins [128,129]. According to Tosoh, producer of TSKgel Amide-80, amide-silica columns have been available since at least 1985. This particular phase is described as consisting of nonionic carbamoyl groups that are chemically bonded to the silica gel, but it is commonly known as an amide-bonded silica. After Yoshida [130] applied these phases to the separation of peptides, the amide-silica phase soon found common usage in HILIC. Chemically bonded stationary phases with specific structural properties have been prepared by Buszewski et al. [131-133]. One of them contains aminopropyl ligands bonded to silica (SG–NH_2); others are an alkylamide packing phase (SG-AP) and a mixed phase (SG-MIX) containing different types of ligands (–NH_2, –CN, –Ph, –C_8, –C_{18}) bonded to the support.

Over the last 15 years, HILIC has progressed into second and third generation implementations, most of which involve mixed or multiple-interaction solid phases. Many column vendors sell both traditional HILIC and its more sophisticated relatives. Some novel separation materials for HILIC have attracted increasing attention in recent years [126,134,128,129]. Hence, the structural variations of HILIC-type stationary phases are wider than those found in reversed-phase systems. HILIC phases can be grouped into neutral polar or ionic surfaces. Table 1 shows the different structures of stationary phases that are applicable to HILIC-mode separation. These special separation materials for HILIC show good selectivity and reproducibility for the separation of polar compounds. Although the number of commercially

available columns designed especially for HILIC is growing, there is still no versatile stationary phase like C18 in RP-LC. However, different types of separation materials for HILIC have different retention characteristics and separation selectivities.

Table 4: Selected stationary phases used in HILIC separations [171]

Packing Materials	Structure of stationary phase
underivatized silica stationary phases that contain functional groups such as siloxanes, silanols with (or without) a small quantity of metals	
DIOL bonded phases	
cyano bonded phases	
amino bonded phases	
alkylamideamide	
amide bonded phases	
mix-mode	
polymeric structures of poly (succinimide) derivatives	

Packing Materials	Structure of stationary phase
polyethylene glycol/silica (HS PEG)	
β-cyclodextrin	
saccharides (maltose)	
dipeptide	
zwitterionic sulfobetaine bonded phases (ZIC-HILIC)	
cationic exchangers bonded phases	
mix-mode RP/ anionic exchangers bonded	

Unmodified bare silica gel has some advantages for HILIC, in contrast to chemically bonded stationary phases. Type A silica gels, prepared by precipitation from the solutions of silicates, are acidic because they are polluted with certain metals that activate surface silanol groups and form complexes with some chelating solutes, causing strong retention or asymmetric peaks. Type B silica gels are formed by the aggregation of silica sols in air, contain very low amounts of metals, and are more stable at intermediate and higher pH values (up to at least pH 9) than xerogel-type

materials. They generally provide better separations, especially for basic samples, because they are highly purified, less acidic “sol-gel” spherical silica particles [141]. At higher pH values, silanol groups are ionized and cation exchange plays a important role in retention, especially for positively charged basic compounds. Suppressing silanol ionization through the addition of TFA may promote the ion-pairing mechanism. Similar effects have also been observed in HILIC on monolithic silica gel columns, which offer higher permeability than the particle-packed HILIC columns [142]. Silica gel type C with a hydrosilated surface populated with nonpolar silicon hydride Si–H groups instead of silanol groups may have up to 95% of its original silanols removed, making it less polar than silica gels with higher populations of silanol groups [143]. It can be used to separate acids or bases in the HILIC mode in buffered mobile phases containing more than 50–70% organic solvent (acetonitrile).

DIOL, amino, amide and other bonded phases used in HILIC are usually prepared by chemically modifying the silica gel surface, like the C18 phases used for RP-LC [144,147]. Chemically bonded DIOL phases demonstrate high polarity and hydrogen bonding properties, and do not contain ionizable groups other than unreacted residual silanols, meaning that they are appropriate for the HILIC mode [145]. Bonded amino-silica columns are relatively often used in the HILIC mode. While basic analytes are in general strongly retained on silica gel by hydrogen bonding and ion-exchange interactions with silanol groups, acidic compounds show increased affinities to amino-silica columns, which can sometimes even lead to irreversible adsorption [147]. Chemically bonded phases with other functionalities, such as polyethylene glycol or alkyls with embedded amide or carbamate groups, are generally proposed for RP applications in water-rich mobile phases. On the other hand, when the percentage of organic solvent is high, the retention of many compounds increases with increasing concentration of acetonitrile, showing typical NP behavior [125,146,150]. Cyclodextrin-silica stationary phases that possess several linked glucopyranoside units and have chiral recognition properties are useful for HILIC chiral separations [148].

Zwitterionic sulfoalkylbetaine stationary phases have also been introduced for HILIC separations. The active layer, which is grafted onto wide-pore silica gel or a polymer support, contains both strongly acidic sulfonic acid groups and strongly basic quaternary ammonium groups separated by a short alkyl spacer. Ion-exchange interactions of the zwitterionic stationary phase are assumed. The sulfoalkylbetaine bonded phases strongly adsorb water by hydrogen bonding, and the bulk layer of water, which forms part of the stationary phase, then largely controls the retention mechanism. Zwitterionic columns are commercially available under the tradenames ZIC-HILIC (on a silica gel support) and ZIC-*p*HILIC (on a polymer support) [149].

The separation of neutral compounds on ion exchangers under typical HILIC conditions has been known about for a very long time. On both cation-exchange and anion-exchange styrene-divinylbenzene resins, only the retentions of some polar compounds (e.g., carbohydrates and related substances) increase with increasing ethanol concentration in the mobile phase. For other compounds, the opposite effects have been observed [146, 152]. Due to the presence of ion-exchange groups, a mixed-mode HILIC/ion-exchange mechanism controls the retention, which may cause specific selectivity effects. The mixed anion-exchange/cation-exchange/HILIC mechanism that occurs on silica-based, small-pore, weak ion-exchange resins was found to be useful for the analysis and purification of compounds from natural products [134].

2.2.2.1.2 Mobile phase selection

A typical mobile phase for HILIC chromatography includes water-miscible polar organic solvents such as acetonitrile with a small amount of water [125]. However, any aprotic solvent that is miscible with water (e.g., tetrahydrofuran, THF, and/or dioxane) can be used. Alcohols can also be adopted, although a higher concentration is needed to achieve the same degree of retention of the analyte relative to an aprotic solvent–water combination [126].

An eluotropic row is useful for selecting a suitable organic modifier for the mobile phase. This lists solvents according to increasing elution strength.

Relative solvent strengths in HILIC can be approximately summarized as follows:

acetone<isopropanol<propanol<acetonitrile<ethanol<dioxane<DMF< methanol<water

HILIC separations are performed either in isocratic mode with a high percentage of organic solvent or with gradients starting with a high percentage of organic solvent and ending with a high proportion of aqueous solvent [125].

It is commonly believed that in HILIC, the mobile phase forms a water-rich layer on the surface of the polar stationary phase vs. the water-deficient mobile phase, creating a liquid/liquid extraction system. The analyte is distributed between these two layers [125,151].

2.2.2.1.3 Mobile phase additives

Ionic additives, such as ammonium acetate and ammonium formate, are typically used to control the mobile phase pH and ion strength. In HILIC, they can also contribute to the polarity of the analyte, resulting in differential changes in retention. For ionizable analytes, such as aminoglycoside antibiotics, the pH must be adjusted to ensure that the analyte will be in a single ionic form. Increasing the buffer concentration decreases the retention if ion exchange controls the retention, while the opposite effect may occur, affecting the solvation, in the absence of ion exchange under HILIC conditions. If this is not done, an asymmetric peak shape, chromatographic peak “tailing,” and/or poor recovery from the stationary phase will be observed. No buffer is needed to separate neutral polar analytes (e.g., carbohydrates). The use of other salts (such as 100–300 mM sodium perchlorate) that are soluble in high organic solvent mixtures (ca. 70% acetonitrile) can be used to increase the polarity of the mobile phase in order to achieve elution. These salts are not volatile, so this technique is less useful with a mass spectrometer as a detector [125,153].

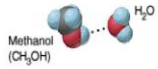
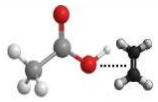
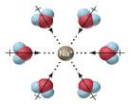
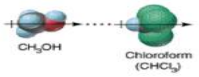
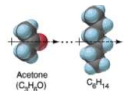
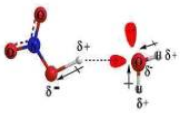
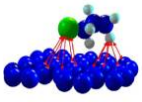
2.2.2.1.4 Separation mechanism in HILIC mode

The mechanism and theoretical description of analyte retention in HPLC has been the subject of many articles. Different research groups and scientific

schools still disagree about the most realistic retention mechanism and the best theory to describe and predict it [154,155].

There are essentially three possible ways to model the separation mechanism. The first is analyte partitioning between the mobile and stationary phases [156,157], the second is the adsorption of the analyte onto the surface of the adsorbent [158.159], the third assumes the preferential adsorption of the organic mobile phase modifier onto the adsorbent surface, followed by the partitioning of this analyte into the adsorbed layer [160]. The retention phenomenon in HPLC simultaneously depends on various types of intermolecular interactions between the solute and the stationary phase, the solute and the mobile phase, and the stationary and mobile phases. Known intermolecular interaction types are given in Table 5.

Table 5: Types of interactions between the analyte, stationary phase, and mobile phase [171]

Type of interaction		Characterization	Energy (kJ mol^{-1})	Example system
Chemical interactions	Hydrogen bonding	Formed between hydrogen of the proto-donor group and atom being hydrogen acceptor, can be formed within the same molecule, as well as between two different	- (4- 17)	
	Donor-acceptor interactions	Occurs between pairs of electron donor (Lewis base) and acceptor (Lewis acid)	- (4- 17)	
Physical interactions	Ion-dipole	Ion acts on electrically neutral molecule leads to a multicomponents	- (4- 17)	
	Dipole-dipole	Force between two electrically neutral particles, but having determined dipole moments	- (4- 17)	
	Dipole-induced dipole	Forces between the molecule possesses clear dipole moment and non-polar molecule	- (4- 17)	
	Temporary Dipole-induced dipole	Two electrically neutral particles, if they come close together, will attract on the electrostatic forces way	- (4- 17)	
Intermolecular Interactions (Van der Waals forces)		Weak forces of attraction between the fragments of individual molecules, declining rapidly with increasing distance between the interacting particles, do not lead to permanent connections	- (2- 4)	

Present theory proposes that HILIC retention is caused by partitioning. This phenomenon still lacks a thorough theoretical explanation. In this mode, the separation mechanism is based on the differential distribution of the injected analyte solute molecules between the acetonitrile-rich mobile phase and a water-enriched layer adsorbed onto the hydrophilic stationary phase [125,126] (see Fig. 3). The more hydrophilic the analyte, the more the partitioning equilibrium is shifted towards the immobilized water layer on the stationary phase, and thus, the more the analyte is retained. In other words, a separation based on the polarities of the compounds and the degree of solvation takes place. As a result of the preferential solvation of the stationary phase surface, it is very likely that the gradient of a given solvent concentration from the adsorbent surface into a bulk mobile phase is formed.

2.2.2.2 Mass Spectrometric Techniques

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

2.2.2.2.1 Basic Principle

A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-

to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

2.2.2.2.2 Instrumentation

The instrument consists of three major components:

1. **Ion Source:** For producing gaseous ions from the substance being studied.
2. **Analyzer:** For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.
3. **Detector System:** For detecting the ions and recording the relative abundance of each of the resolved ionic species.

In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

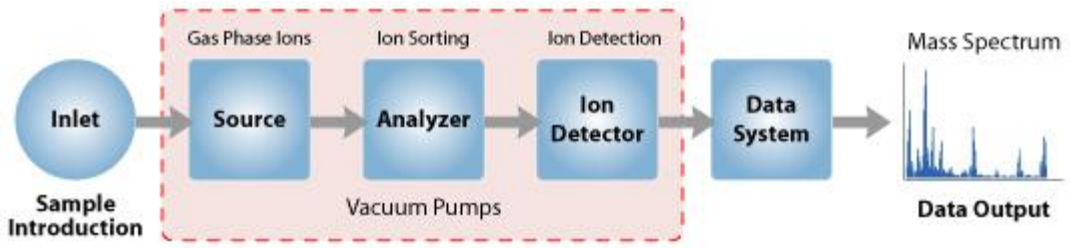


Figure 1: Components of a mass spectrometer [172].

With all the above components, a mass spectrometer should always perform the following processes:

1. Produce ions from the sample in the ionization source.

2. Separate these ions according to their mass-to-charge ratio in the mass analyzer.
3. Eventually, fragment the selected ions and analyze the fragments in a second analyzer.
4. Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.
5. Process the signals from the detector that are transmitted to the computer and control the instrument using feedback.

2.2.2.3 Liquid Chromatography tandem Mass Spectrometry

LC-MS techniques provide a universal approach applicable to the widest number of veterinary drugs and this is the reason why they have today become the technique of choice in the field of the analysis of antibacterial residues in food stuffs.

The combination of atmospheric pressure ionization tandem mass spectrometry (API-MS/MS), with liquid chromatography (LC) and ultra-performance LC (UPLC) is currently the most frequently used technique in antibacterial analysis. The most used atmospheric pressure interfaces are atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). For compounds of moderate to high polarity, ESI constitutes the most important ionization technique in MS coupled to LC for the analysis of organic contaminants, and it dominates the application area of antibacterial analysis.

Among the different mass analyzers usually applied for target analysis, triple quadrupole (QqQ) is the most widely used for measuring and quantifying residues of veterinary drugs. Hybrid quadrupole-linear ion trap (Q-LIT) system combines fully functional quadrupole and linear ion trap-MS within the same instrument and thus, apart from great sensitivity, is capable of producing MS^n spectral information, useful for structure elucidation. Q-LIT has been used in fewer applications than simple triple quadruple formats.

However, a recent trend towards the high-resolution mass spectrometry (HR-MS; i.e. time-of-flight, TOF; Orbitrap; Fourier Transform-Ion Cyclotron Resonance, FT–ICR) is undoubtedly observed. High resolution mass analyzers and hybrid mass analyzers, such as Q-TOF, LIT-Orbitrap, open a new era in food analysis, together with holistic sample preparation and retrospective analysis. Due to their high resolving power, mass accuracy, fragmentation and isotopic pattern elucidation can provide tentative identification of non-target and unknown compounds in food samples. Full scan acquisition mode and MS^n mode are useful tools of these new generation instruments.

The main source of analytical problems encountered by LC-MS users is related to matrix effect problems, particularly when studying complex samples, such as food. It represents certainly one of the main sources of pitfall for the analyst, affecting many aspects of the method performance, such as detection capability, repeatability and accuracy. Matrix effect mainly appears as ion suppression and it corresponds to the decrease of the evaporation efficiency of the ions of the analyte due to competition effects with co-extracted and co-eluted matrix components. Another proposed mechanism is the competition between analytes and interfering compounds regarding the maximal ionization efficiency of the technique [115-117]. Much less frequently and by a process not yet fully understood, the presence of endogenous compounds in the nanodroplets of the electrosprayed solution can result in an increased ion signals for the analytes compared to those of a reference standard solution.

To overcome matrix effects when quantifying, two practicable approaches can be used. The use of adequate isotope-labeled internal standards and/or analyte quantitation by matrix-matched calibration standards should eliminate the analytical systematic errors (bias) caused by ion suppression or ion enhancement [118].

2.2.2.3.1 LC-MS/MS Techniques: Advantages

Triple quadrupole MS analyzers (QqQ) present the highest sensitivity and selectivity when working in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), by selection of at least two precursor ion-to-product ion transition reactions. The fragmentation of the target compounds in order to detect only specific product ions rather than the entire molecule permits to considerably increase the signal to noise ratio of the target diagnostic signal by decreasing to a major extent the interferences due to other compounds present in the final extract with the same - or very close - molecular weight as the analyte of interest [121]. Under this condition, QqQ MS analyzers are best suited to achieve the strict tolerance levels regulated in various countries for antibacterials in different food matrices.

The large number of veterinary drugs that have to be monitored in order to ensure food safety has caused a steady increase of the number of multi-analyte analytical methods developed in recent years. The application range of MS/MS is today extremely wide, both in terms of target compounds and in terms of possible different acquisition modes. This last capability authorizes not only very sensitive and specific quantitative target measurements, but also powerful untargeted “fishing” approaches based on advanced scanning techniques like precursor ion scanning or neutral loss scanning, applicable to a class of substances with similar fragmentation patterns [119,120].

A drawback of the QqQ MS arrangement is its relatively long duty cycle (slow scan speed) that limits the number of scans that can be acquired simultaneously. As a result, SRM methods are typically limited to ~100 or 150 target analytes, depending on the chromatographic separation, resulting in a loss of sensitivity. Furthermore, for reliable quantification, two selected reaction monitoring transitions are required and some analytes present only one transition while some transitions are unspecific. In spite these disadvantages, QqQ still remains the analyzer of choice, coupled to liquid chromatography, for the determination of veterinary drugs in food matrices.

CHAPTER 3

Research objective and Scope

3.1 The analytical problem

There are three main difficulties that constitute the analytical problem in the residue analysis of veterinary drugs in food. First, there is the large number of compounds with diverse physico-chemical characteristics. In addition, the definition of ‘‘residue’’ of many contaminants includes known metabolites of toxicological interest since many drugs administered to food-producing animals are oxidized, reduced and biotransformed to water-soluble conjugates, primarily by glucuronidation, sulfatation or conjugation with glycine. Such metabolites cannot be ignored, particularly when they are even more hazardous and more persistent than the parent compounds (e.g., nitrofurans are rapidly biochemically transformed into toxic metabolites, which are highly bound to the proteins, so they are stable for longer periods in food-producing animals) [161].

The second problem is the very low concentration levels at which a veterinary drug residue should be analysed, since most of the MRLs and MRPLs established are at the ppb level (parts per billion or μg kg^{-1}). Therefore, analytical methods for the determination of veterinary drug residues in food matrices at trace levels are necessary and the procedures used for selective and quantitative extraction of the analytes, cleanup and enrichment of sample, as well as the sensitive and specific detection should meet the requirements of this challenge.

Finally, the complexity of the matrix should also be taken into consideration. Several edible tissues from food producing animals can be selected for residue surveillance including muscle, liver, kidney, skin and fat, which are normally collected at slaughter houses. In addition, further sample matrix types can be taken on-farm or at production sites, including milk, honey, eggs and fish. All these foods, except honey, are protein rich (from 3% in milk to 20% in meat), which is important for those drugs that

bind easily to proteins. They also contain significant amounts of divalent and trivalent cations that form complexes with some antibacterials, increasing their retention in different tissues. In general, many residues are present in conjugated forms and require liberation through enzymatic or chemical hydrolysis prior to extraction [60].

Due to all the aforementioned reasons and the desire of improving the cost-effectiveness of analytical procedures, the development of multiclass methods which are able to detect, confirm and quantify as many compounds as possible, has become a significant trend in the analysis of residues and contaminants in food samples. Liquid chromatography hyphenated to mass spectrometric techniques dominates in the field of multi-residue determination of veterinary drugs in complex matrices, since it permits excellent sensitivity and selectivity.

3.2 Scope

A broad range of veterinary drugs are administrated in animal husbandry in order to improve animal health but also as growth promoters for intensive animal production. The possible presence of veterinary drug residues in food of animal origin is one of the key issues for food safety. Some of the most commonly used, as growth promoters, compounds are thyreostats.

The aim of this study was the development of a novel HILIC-MS/MS methodology for the rapid simultaneous determination of six thyreostats (2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), tapazole (TAP), 2-mercaptobenzimidazole (MBI)) in bovine muscle.

Initially, an ionization study was performed in positive mode. The product ions for precursor-ion scanning were selected by studying the MS/MS fragmentation of the analytes and mass spectrometric settings was optimized.

A thorough chromatographic study was performed by testing nine different HILIC columns, consisted of different stationary phases and consequently, different multi-modal retention mechanisms. The separation finally was

accomplished by BEH Amide HILIC column with an isocratic elution at a flow rate of 0.1 mL min^{-1} of mobile phase.

Sample preparation was also optimized and the final protocol comprised of a rapid solid-liquid extraction followed by dispersive solid-phase extraction (d-SPE) clean-up with C18 sorbent and hexane partitioning. This is the first study reported to determine thyreostats using HILIC, achieving substantially low LODs and short analysis' time.

The method was validated in agreement with the guidelines of Commission Decision 2002/657/EC and satisfactory method performance characteristics were achieved. The method was applied to the analysis of unknown muscle samples.

CHAPTER 4

Instrumentation, Lab Equipment and Reagents

4.1 Instrumentation: LC-MS/MS system

A Thermo UHPLC Accela system was connected to a Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument (Thermo, San Jose, CA, USA).

Instrument control and data acquisition were carried out by using the Xcalibur software, Version 2.3, from Thermo.

Figure 2: Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument [172].

4.2 Laboratory Equipment

In the laboratory equipment used were included mobile phase solvent filtration apparatus (Millipore, XX15.04705), calibrated analytical balance with four decimal digits (Santorius-Basic), ultra-pure water apparatus 18.2MΩ / cm (Millipore Direct-Q UV), ultrasonic bath (Metason 60 Stuers), a Vortex spinner apparatus (Velp Scientifica), a centrifugation apparatus (Rotofix 32 Hettich) and a pH meter (HQ30d, HACH).

They were also used, 5, 10, 20, 50, 100 and 250 mL volumetric flasks, 100 and 250 mL beakers, 15 mL and 50 mL centrifuge tubes, 10 and 25 mL volumetric cylinders, calibrated 5 mL and 20 mL pipettes, glass and plastic pasteur pipettes, and 10 mL test tubes.

4.3 Chemicals and Reagents

All the analytes studied are presented in Table 4.1. All veterinary drug and pharmaceutical standards were of high purity grade (>90%) The vast majority of them were purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile and methanol LC–MS grade were purchased from Merck (Darmstadt, Germany) while formic acid 99% and ammonium formate from Fluka (Buchs, Switzerland). Hexane (pesticide analysis grade, 95%) was purchased from Carlo Erba (Milan, Italy) and distilled water was provided by a MilliQ purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). The ethylenediaminetetraacetic acid disodium salt (EDTA) was of analytical grade and was purchased from Panreac. RC (regenerated cellulose) syringe filters (15 mm diameter, 0.2 mm pore size) were provided from Phenomenex (Torrance, CA, USA).

4.3.1 Preparation of standard solutions

About 10 mg of each individual standard was accurately weighed and placed in a 10-mL volumetric flask and were dissolved methanol. Stock solutions of 1000 mg mL^{-1} of each compound were obtained and stored at --20 ^{o}C in brown glass to prevent the photo degradation. Intermediate standard solutions at concentration of 10 mg mL^{-1} of each compound were prepared by dilution of the stock solutions with acetonitrile. The final working solution was prepared by diluting all the compounds in acetonitrile at final concentration 1 mg mL^{-1} and it was also stored at -20 ^{o}C. New ones were prepared every month. All working solutions and calibration standards were obtained by gradient dilution of the intermediate solutions, in concentrations varying from 1 mg mL^{-1} to 1 ng mL^{-1}. Working standard solution of internal standards in a concentration of 1 mg mL^{-1} came by subsequent dilutions of their stock solutions in acetonitrile. While not in use, the working solutions were kept at -20 ^{o}C and renewed

weekly. Matrix- matched standards were prepared in the same way as the other samples.

4.3.2 Sampling and Storage

Negative bovine muscle tissue samples were used during these experiments. All samples were obtained from Ministry of Agricultural Development and Food and were confirmed to be free of targeted analyte residues by LC-MS/MS after sample preparation with the procedure developed and optimized. All tissue samples were homogenized and stored at −20 ºC until analysis.

CHAPTER 5

Development of a multi-residue methodology for the determination of thyreostats in bovine muscle tissue by HILIC-ESI-MS/MS

5.1. Ionization study of 6 thyreostatic drugs and ESI-MS/MS optimization

5.1.1 Ionization study

As the first step of the method development, the selection of the precursor and product ions were carried out. Direct infusion of individual thyreostats at concentration of 5 mg mL^{-1} in 1mM ammonium formate-formic acid 0.1%: ACN (20:80, v/v) is was achieved in positive and negative ionization mode, respectively. The mass spectra for all analytes were obtained along with analyte dependent parameters, such as collision energy and tube lens, which were optimized and calculated automatically. For each compound, the MRM transition with the highest intensity was used for quantification (quantifier), while the other transition was used for confirmation (qualifier). A quantitative data processing method was established using the most abundant SRM transition for each residue.

Table 6 gives the specific MS/MS parameters in the study. The protonated ($[M + H]^+$) molecular ions were selected as the precursor ions for all the compounds. Electrospray parameters, such as sheath gas, auxiliary gas, spray voltage and capillary temperature, were studied. The optimization was performed using flow injection analysis (FIA) with the carrier solution being the analysis' mobile phase in different proportions of aqueous/organic solvent. MS parameters were optimized in positive ionization modes mode-with variation of a single setting at a time and evaluation of the target compounds' sensitivity. Significant compromises had to be made in order to simultaneously determine all analytes with different optimum values of ESI parameters. The spray voltage was set in the maximum optimum value obtained in positive ionization mode (4000 V) and the capillary temperature

was set at 300 ^{o}C as it proved to slightly enhance the peak area of thiouracil, eluting last from the chromatographic column. Finally, the sheath and auxiliary gases' optimization revealed rather insignificant differences between the values tested. The optimum ESI parameters that were chosen for positive ionization determination are shown in Table 6.

Table 6: Optimal mass spectrometry parameters

MS settings	
Spray Voltage	3500 V
Sheath Gas Pressure	10 a.u.
Auxiliary Gas Pressure	5 a.u.
Capillary Temperature	300 ^{o}C

5.1.2 Optimization of TS ionization by testing different mobile phases

Eight mobile phases and their effect on the ionization of the six thyreostats were studied, at a ratio of aqueous/organic solvent 20/80 % (v/v), by preparing a standard solution at 5 μg/ml. A comparison was made in order to achieve the best ionization of all the analytes. Briefly, different mobile phases consisting of acetonitrile as the organic phase and water with different mobile phase additives, such as formic acid, acetic acid, ammonium acetate and ammonium formate at various concentrations were tested.

Table 7: Mobile phases tested for the ionization of 6 thyreostats

	Mobile Phases
1	1mM ammonium formate with 0.1% formic acid/ACN
2	1mM ammonium formate with 0.1% formic acid/ACN with 0.1% formic acid
3	1mM ammonium acetate with 0.1% formic acid/ACN
4	1mM ammonium acetate with 0.1% formic acid/ACN with 0.1% acetic acid
5	5mM ammonium formate with 0.1% formic acid/ACN with 0.1% formic acid
6	5mM ammonium acetate with 0.1% formic acid/ACN with 0.1% acetic acid
7	10mM ammonium formate with 0.1% formic acid/ACN with 0.1% formic acid
8	10mM ammonium acetate with 0.1% formic acid/ACN with 0.1% acetic acid

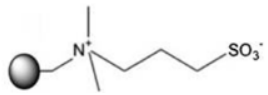
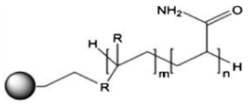
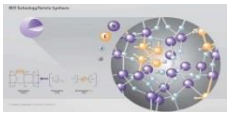
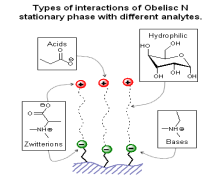
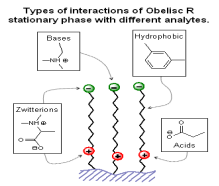
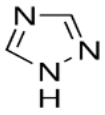
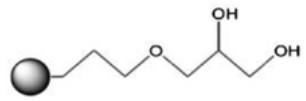
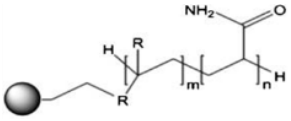
The optimum ones were chosen in terms of highest peak area and signal-to-noise ratio for the majority of the target compounds. The optimal mobile phase was 1mM ammonium formate with 0.1% formic acid/ACN with 0.1% formic acid. Furthermore, the optimal mobile phase was also tested with a ratio of aqueous/ organic solvent of 40/60 and 30/70.

5.2 Study of chromatographic analysis

5.2.1 Study of different HILIC columns

The chromatographic analysis of thyreostats based on Hydrophilic Interaction Liquid Chromatography (HILIC). In this part, nine different HILIC Columns (Table 5.4) with different retention mechanisms were studied.

Table 8: Characteristics of studied HILIC columns [171]

Column	Column Size	Functional Group	Chemical Structure
ZIC-HILIC (SeQuant)	2.1×150 mm, 3.5 µm	Sulfoalkylbetaine bonded to silica	
ACQUITY UPLC BEH Amide (Waters)	2.1×150 mm, 1.7 µm	Amide	
ACQUITY UPLC BEH HILIC (Waters)	2.1×100mm, 1.7 µm	unbonded BEH substrate	
XBridge HILIC (Waters)	150 × 2.1 mm, 3.5 µm	Silica with ethylene bridges	
Obelisc N (Sielc)	2.1×150 mm, 5 µm	positive and negative charges	
Obelisc R (Sielc)	2.1×150 mm, 5 µm	positive and negative charges 'AQ' type phases	
Cosmosil/Cosmogel (Nacalai Tesque)	2.0×150 mm	1,2,4-triazole bonded	
Fortis HILIC Diol (Fortis Technologies)	2.1× 50 mm, 1.7 µm	Diol	
TSKgel Amide-80 (TOSOH Bioscience)	2.0mm ×15cm, 3µm	Amide	

Each column was studied by getting a chromatogram of a standard solution at 300 ng/mL, prepared in 1mM ammonium formate with 0.1% formic acid/ACN-0.1% formic acid 10/90 %(v/v), in three different constitutions of mobile phase. Specifically, the constitution of three mobile phases was:

- 1mM ammonium formate with 0.1% formic acid/ACN with 0.1% formic acid with ratio 2/98

- 1mM ammonium formate with 0.1% formic acid/ACN with 0.1% formic acid with ratio 10/90
- 1mM ammonium formate with 0.1% formic acid/ACN with 0.1% formic acid with ratio 10/90

Initially, the HILIC columns were equilibrated at each mobile phase one hour at flow rate 200μL/min and one hour at a flow rate 100μL/min. Subsequently, three chromatograms of standard were taken at each constitution of mobile phase. The time of each chromatogram was 10min.

Following a thorough chromatographic comparison, the column that was selected, was Acquity BEH Amide (Waters).

5.2.2 Mobile phase optimization

5.2.2.1 Isocratic conditions

Two different mobile phases consisting of acetonitrile with formic acid 0.1% as the organic phase and water with formic acid 0.1% and ammonium formate at two concentrations (5mM and 10 mM) as a mobile phase additives, were tested, at the same ratio (10/90).

The optimum ones were chosen in terms of highest peak area and signal-to-noise ratio for the majority of the target compounds. The optimal mobile phase was 5mM ammonium formate with 0.1% formic acid/ Acetonitrile-0.1% Formic acid.

5.2.2.2 Gradient conditions

Due to the different chemical structure of the analytes, two different gradient programs were tested. The gradient elution programs for both runs are presented in Table 9. The column temperature was set at 30 °C and the full loop injection volume of the extract was set at 10 μL.

Table 9: Tested gradient programs

Gradient Program 1			
Time (min)	***A (%)***	***B (%)***	***Flow (µL/min)***
0.00	5.0	95.0	100
3.00	5.0	95.0	100
3.10	15.0	85.0	100
10.00	15.0	85.0	100
Gradient Program 2			
Time (min)	***A (%)***	***B (%)***	***Flow (µL/min)***
0.00	2.0	98.0	100
3.00	2.0	98.0	100
3.10	12.0	88.0	100
10.00	12.0	88.0	100
A (%)= 5mM A.F.-0.1% F.A. B (%)= ACN-0.1% F.A.			

The first gradient program was performed initiated from 5% of aqueous phase -in order to elute the analytes in a reasonably short time. The second gradient program starts from using 2% aqueous phase.

5.2.3 Study of retention behavior of thyreostats in HILIC

Retention characteristics of BEH Amide HILIC column were studied by testing the effect of various experimental factors on the retention of the polar stationary phase amide, such as acetonitrile content, column temperature, buffer pH, salt type and concentration in the mobile phase.

5.2.3.1 Effect of acetonitrile content

Similar to reserved-phase separation, HILIC separation commonly employs water and acetonitrile as the mobile phase, but requires much higher organic content (>60%) to ensure significant hydrophilic interaction. The level of organic solvent in the mobile phase is probably the factor that has the largest influence on retention. In this study, the effect of acetonitrile content on retention was investigated by varying the ACN's percentage - in the mobile phase. The aqueous phase consisted of water containing 5 mM of ammonium acetate concentration constant at 5 mM. Low salt concentration was

necessary to accommodate low solubility of salt in the mobile phase with high acetonitrile content (e.g., 95%) [168].

5.2.3.2 Effect of buffer concentration

Column temperature is also an important parameter that affects the retention of polar compounds in HILIC separation. The relationship between capacity factor (*k_*) and column temperature (*T*) in RPLC is often described by van't Hoff equation:

$$\ln k' = -\frac{\Delta H^{O}}{RT} + \frac{\Delta S^{O}}{R} + \ln\varphi \quad (1)$$

where ΔH^{o} and ΔS^{o} are retention enthalpy and entropy, *R* gas constant and *φ* phase ratio. If the retention of polar compounds in HILIC is through partitioning between the mostly organic mobile phase and a water-rich liquid layer on the packing surface as proposed by Alpert [163], the van't Hoff equation should apply to HILIC. In this study, the temperature effect on retention was investigated by varying column temperature from 20 to 70 ◦C, and the retention data for the model compounds were used to construct van't Hoff plots for the BEH amide column [168].

5.2.3.3 Effect of salt concentration

Many salts typically used in reversed-phase chromatography are not suitable for HILIC due to poor solubility in the mobile phase containing high level of acetonitrile. In addition to ammonium formate, other salts with relatively high solubility at high organic levels have also been used for HILIC, such as ammonium acetate and bicarbonate salts, trimethylamine phosphate and sodium perchlorate, but the last two are not compatible with MS detection.

In addition to salt type, the effect of salt concentration on the retention was also investigated by varying ammonium acetate concentration from 5 to 20mM in the mobile phase of acetonitrile/water (85/15, v/v). Further increase in the salt concentration was not possible due to solubility limitation in the mobile phase. The retention time of the six thyreostats was obtained on the BEH amide column at three concentration levels.

5.3 Sample preparation

5.3.1 Samples and quality control materials

Negative Bovine tissue samples were obtained from Ministry of Rural Development and Food. Upon arrival at the laboratory, the samples were homogenized and refrigerated at -20 °C until analysis.

One negative bovine sample was analyzed to confirm that no veterinary drugs or pharmaceuticals were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the method.

Spiked samples were prepared by adding the proper amount of a working solution containing all the analytes at the suitable concentrations, to each portion of the weighed samples. A stable concentration of the Internal Standard working solution was added at each sample to achieve a final proper concentration. For the evaluation of the different extraction procedures, blank samples were spiked at two concentration levels. Afterwards, there was a waiting period of 15 min for equilibration before starting the extraction step. Blank control samples were extracted and run with each analytical run/batch.

5.3.2 Sample preparation – extraction procedures

Finding suitable extraction conditions for the simultaneous extraction of the extremely polar thyreostats composes a great challenge in multiresidue analysis and has rarely been reported previously.

To extract thyreostats from matrices (e.g. animal tissue, excreta, plasma and organs) most often methanol is used as a solvent, but also acetonitrile and ethyl acetate have been reported. In the beginning, a mercurated affinity column was used for the clean-up of extracts nowadays solid phase extraction (SPE) gained in interest. In most cased. Silica SPE cartridges are mentioned, but anion exchange, amino-propyl and alumina cartridges have also been reported. Some even resort to the technology of matrix solid-phase

dispersion (MSPD), because homogenization, disruption, extraction and clean-up are combined in one procedure [169].

Six different sample preparation protocols were developed and tested for the extraction of thyreostats from bovine muscle tissue samples. A schematic representation of the final extraction and clean-up procedure is presented in Figure 3.

5.3.2.1 LLE with ACN/H_2O /Defatting with hexane protocol

A 5-g portion of each properly homogenized sample was weighed and placed into a 50 mL polypropylene centrifuge tube. Afterwards, spiking of the samples with appropriate volumes of the working standard mix solutions (target compounds and IS) was performed. As mentioned above, the blank samples fortified with the target compounds were used during the optimization and validation of the developed procedure. All spiked samples were allowed to stand for 10‑15 minutes before proceeding.

To extract the thyreostat residues, 10 mL of mixture containing acetonitrile and water (4:1, v/v) were added to the samples. After the addition of the solvent mixture the tube was vortex-mixed for 30 sec. The sample set in shaker plate for 20min and afterwards it was placed in ultrasonic bath at 35ºC for 20 min in order an ultrasonic-assisted extraction of TS from the matrix to take place.

Thereafter, the samples were centrifuged at 4000 rpm for 10 min and the supernatant was decanted into a new 50mL polypropylene centrifuge tube. To defatting the extracts, 5mL of n-hexane pre-saturated with ACN were added. The samples were vortexed for 1min and were again centrifuged. The hexane layer was aspirated to waste. 6mL of the final extracts were transferred in a new 15mL propylene centrifuge tube and 0,6g $MgSO_4$ and 0,125g C18 were added to clean-up the extracts. The centrifuge tubes were shaked by hand for 1min. Afterwards, a centrifugation was performed at the same settings and 4mL of the final extracts evaporated to dryness under a nitrogen stream at a temperature, not exceeding 40°C. The resulting residues were reconstituted in 400 μL of ACN/aqueous solution of 5mM ammonium

formate and formic acid, 0.1% (10:90 v/v) and then filtered through a 0.22-μm RC filter. Appropriate volumes of working multi-analyte solutions were added to blank aliquots at this step, to prepare the concentration of the matrix-matched standard required. After vortex-mixing for 10 s, each extract was then transferred into a vial, and 10 μL was injected into the LC-MS/MS system.

5.3.2.2 QuEChERS extraction with ethyl acetate protocol

Minced (thyroid) or homogenised (muscle) sample (1 g) was put into a 50-mL polypropylene centrifuge tube. An appropriate volume of IS mix was added to each sample to achieve a concentration of 80 ng g−1. Water (4 mL) was added to each sample before shaking for 5 min. The tube was placed under a fume cupboard where EDTA (10 μL, 0.1 mol L^{-1}) and 2-mercaptoethanol (10 μL) were added. Ethyl acetate (10 mL) was added, and the sample was shaken for 15 min. Next, anhydrous NaCl (1 g), anhydrous MgSO4 (4 g) with tri-sodium citrate dihydrate (1 g) and disodium hydrogen citrate sesquihydrate (0.5 g) (QuEChERS Extract Pouches, EN Method) were added and the sample was vortexed immediately for 1 min. The extract was then centrifuged at 7500 g for 10 min, and an aliquot of 7 mL of the upper layer was evaporated to dryness under a gentle stream of nitrogen. The residue was re-suspended in 200 μL of ACN/aqueous solution of 5mM ammonium formate and formic acid, 0.1% (10:90 v/v) and it was ready to be injected in the LC system. Injection volume was 10 μL [164].

5.3.2.3 LLE with ACN/QuECHERS clean-up protocol

A 5-g portion of each properly homogenized tissue sample was weighed and placed into a 50 mL polypropylene centrifuge tube. Afterwards, spiking of the samples with appropriate volumes of the working standard mix solutions (target compounds and IS) was performed. All spiked samples were allowed to stand for 10‑15 minutes before proceeding.

Afterwards, 10 mL of acetonitrile was added to the samples. After the addition of the solvent the tube was vortex-mixed for 30 sec. The sample set in shaker plate for 30min to extract thyreostats from the matrix.

Thereafter, the samples were centrifuged at 4000 rpm for 10 min and the supernatant was decanted into a new 50mL polypropylene centrifuge tube. 6mL of the final extracts were transferred in a new 15mL propylene centrifuge tube and 0,6g $MgSO_4$ and 0,125g C18 were added to clean-up the extracts. The centrifuge tubes were shaked by hand for 1min. Afterwards, a centrifugation was performed at the same settings and 4mL of the final extracts evaporated to dryness under a nitrogen stream at a temperature, not exceeding 40°C. The resulting residues were reconstituted in 400 µL of ACN/aqueous solution of 5mM ammonium formate and formic acid, 0.1% (20:80 v/v) and then filtered through a 0.22-µm RC filter. Each extract was then transferred into a vial, and 10 µL was injected into the LC-MS/MS system.

5.3.2.4 Extraction with methanol protocol

1 g aliquot of minced and mixed muscle was transferred to the tube and 5 mL of a mixture of methanol and Britton–Robinson buffer, pH 8.0 (30:20 (v/v)) was added. 10 µL of internal standard (DMTU) at a concentration of 1 µg mL^{-1} was added to the sample. The homogenization step was performed next and thereafter the tube was centrifuged at 10,000 rpm in −20 ˚C (±2 ˚C) for 20 min. After centrifugation supernatant collected in glass tubes was placed in the water bath for 20 min at 60 ◦C (±2 ˚C) to allow the proteins from muscle opportunity for denaturation. After this step the tube was centrifuged at the same settings as previously used. The supernatant was removed and a degreasing step using two 4 mL of petroleum ether performed. The methanol phase was collected in the glass tube and evaporated to dryness under a nitrogen stream at 40 ˚C (±2 ◦C) to the half content of the tube. Each extract was then transferred into a vial, and 10 µL was injected into the LC-MS/MS system [165].

5.3.2.5 LLE and SPE clean-up protocol

A 5.0-g portion of bovine muscle was weighed into a 50-mL polypropylene centrifuge tube and 100 µL of the IS working solution were added. Spiked

samples were constructed at 0.5 × VL, 1 × VL and 1.5 × VL concentration levels and for prohibited compounds 1 × VL, 1.5 × VL and 2 × VL as indicated in European Commission 2002/657/EC.

When fortified, the samples are vortex-mixed for 30 s and allowed to stand for 10–15 min. After addition of 10 mL of ACN the samples are vortexed for 1 min and shaken for 30 min using a mechanical shaker. Then, the sample tube is centrifuged at 4000 rpm (4300 rcf) for 5 min and the supernatant is decanted in a glass tube. The ACN extract is evaporated to final volume 1.0 mL under a stream of nitrogen at 30 °C. A volume of 20 mL of an aqueous extraction solvent is subsequently added to the sample. This extraction solvent, consisting of 10 mM ammonium acetate, 0.4 mM EDTA, 1% NaCl (w/v) and 2% TCA (w/v) in H2O, has been previously reported in literature to be adequate for aminoglycosides' extraction [53]. The samples are vortexed for 1 min and shaken for 60 min using a mechanical shaker. Afterwards, the sample tube is centrifuged at 4000 rpm for 5 min and the supernatant is decanted in a new polypropylene tube. The sample extract is adjusted to pH 6.5 by adding approximately 6 drops of NaOH 30% (w/v). The value was verified using a pH-meter device. Then, the extract is loaded onto an OASIS HLB (200 mg, 6 mL) cartridge, previously conditioned sequentially with 6 mL of MeOH and 6 mL of H2O. The sample is passed through the cartridge at a flow no faster than 1 drop/s and, then, it is vacuum-dried for approximately 15 min. The elution of the analytes was carried out with 2 × 0.5 mL of aqueous formic acid 10% (v/v) and 3 × 1 mL of ACN. The eluate is collected and combined with the 1-mL ACN extract. The sample weight/final extract volume ratio equals to 1 g/mL meaning that no dilution or preconcentration of the analytes is performed with the proposed methodology. At this step proper volumes of working solutions were added to blank aliquots, to prepare the range of matrix-matched standards required. Finally, 500 μL of the combined extract were filtered through 0.22 μm regenerated cellulose filters, transferred in a vial and 10 μL were injected into the HILIC–MS/MS system [166].

5.2.2.6 d-SPE clean-up protocol

10 mL of ACN/water (4/1, v/v) to 2 g homogenized sample in 50 mL polypropylene (PP) tubes was added. The tubes were shaken for 15 min and centrifuged at 25 ◦C for 5 min at 4000rpm. Afterwards, the extract was transferred to a 50 mL centrifuge tube containing 500 mg of end-capped C18 sorbent. The tube vortexed for 1min and 10 mL of hexane pre-saturated with MeCN was added. After this addition the tube was shaking for 30 s and a centrifugation at the same settings was performed. The hexane layer was aspirated to waste and 5mL of the extract was evaporated under stream nitrogen at 45 ◦C to dryness. The residues were reconstituted in 400 μL of ACN/aqueous solution of 5mM ammonium formate and formic acid, 0.1% (10:90 v/v) and then the final extracts filtered through a 0.22-μm RC filter and transferred into a vial. 10 μL was injected into the LC-MS/MS system [167].

5.3.3 Final extraction procedure optimization

Following a thorough study of the six sample preparation methods mentioned above, the protocol that was selected, is described in Section 5.2.2.6. The protocol was optimized concerning the preconcentration factor so that high recoveries sensitivity in lower concentrations is achieved, with as low as possible matrix effect.

Three preconcentration studies were carried out: (1) 2g-portion of sample was weighted and reconstituted to 1000Ml (preconcentration factor: 1), (2) 4g-portion of sample was weighted and reconstituted to 1000μL (preconcentration factor: 2), (3) 4g-portion of sample was weighted and reconstituted to 500μL (preconcentration factor: 4).

After the comparison of the three tests protocols, the 3rd protocol test was selected and a four times preconcentration was reached.

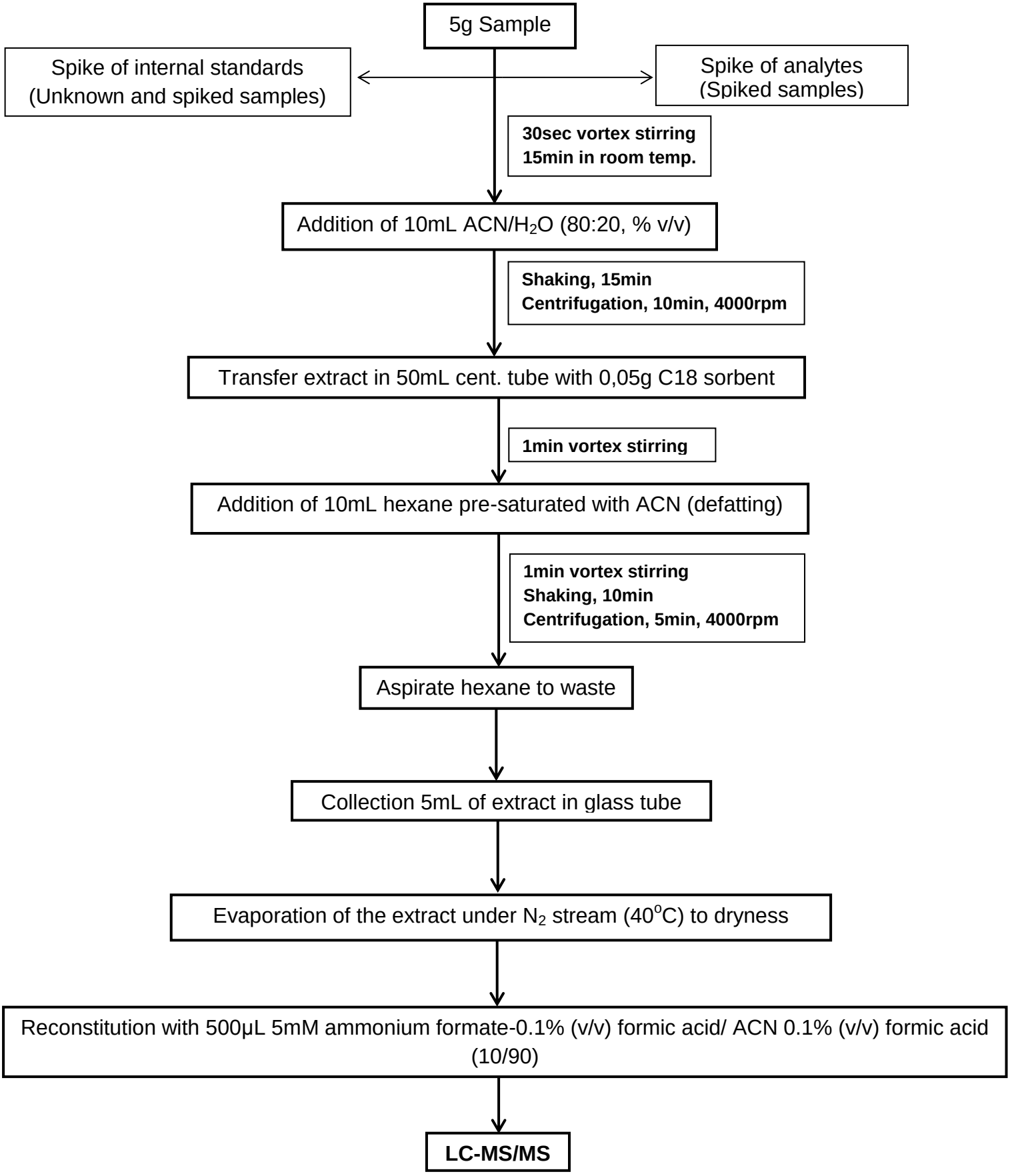


Figure 3: Schematic diagram of the final sample preparation method.

5.4 Method validation

The applicability of the developed method was tested following the accepted criteria for analytical method validation, as indicated in the Commission

Decision 2002/657/EC for quantitative screening methods [170]. Method detection limit (LOD), quantitation limit (LOQ), precision, selectivity, decision limit (CCα) and detection capability (CCβ) and precision were determined for all compounds in bovine muscle tissue.

5.4.1 Identification

An analyte was considered as positively identified and confirmed in a sample when the criteria established in the EU Commission Decision 2002/657/EC were met:

- the ratio of the relative (to the IS) retention time of the analyte to that of the same analyte in standard solution was within ± 2.5 % tolerance
- the presence of a signal at each of the two SRMs for the analyte was achieved (the use of two selected precursor-product ion transition per compound counts for four identification points, which fulfill the EU identification points requirement)
- the signal intensity ratios of the two MS/MS transitions (quantifier and qualifier) with those obtained using fortified blank samples were within the tolerance defined [170].

5.4.2 Selectivity/ Specificity

The selectivity of the method was evaluated by the analysis of 20 control blank samples from the same matrix. The absence of any signal at the same elution time as the target thyreostats indicated the absence of chemical or matrix interferences that may give a false positive signal.

5.4.3 Linearity

The linearity of calibration curves was assessed by using a six-point calibration curve of standards in pure solvent as well as in blank bovine

muscle tissue at six different concentrations (2.5 to 40 ng mL^{-1}). This number of levels was chosen in order to achieve the optimal concentration range for each target analyte, considering the large differences in sensitivity between the single substances. Each calibration standard was injected in each batch in duplicate. Peak area and peak area ratio of the analyte/IS were used as the analytical response versus concentration in all cases. Calibration curves were obtained by least-squares linear regression analysis and acceptable linear regression R^2 values were obtained for all compounds over the concentration ranges.

5.4.4 Precision

The precision of this method was demonstrated in term of repeatability (intra-day precision) and within-laboratory reproducibility (inter-day precision). Repeatability and reproducibility were expressed as the %RSD values of set of 9 replicate analysis for repeatability and 6 replicate for reproducibility at the 3 concentration levels examined (0.5, 1 and 1.5 times the VL).

5.4.5 Trueness

The trueness of the method was estimated through recovery studies. Average recoveries of each analyte at the Validation Level (10 µg kg^{-1}) were calculated performing the analysis in 6 replicates for each matrix (Table 16).

5.4.6 LODs & LOQs

LODs and LOQs were calculated by analyzing blank samples spiked at 0.1, 0.5, 2 or 10 µg kg^{-1}, according to each analyte's sensitivity, as described in the Experimental section. For instrumental LODs and LOQs standard solutions in the same concentrations were analyzed in quintuplicate. Results are shown in Chapter 6.

5.4.7 Matrix Effect

When complex samples, such as milk, muscle or egg are analyzed, LC–MS/MS measurements, especially in the ESI mode, might significantly be influenced by matrix effects. Matrix effects derive from various physical and

chemical processes and may be difficult or impossible to eliminate. They relate to the concentrations and protonation levels of co-extracted components and can be variable and unpredictable in occurrence. Matrix effects are co-dependent and can affect the ionization efficiency of the analytes, leading to suppression or enhancement of the signal depending on the analyte/matrix combination. Obviously, this affects the quantification, unless matrix effects are minimized or compensated [68, 236]. The best way to compensate the matrix effect is the use of isotope labeled internal standards (ILIS). However, these compounds are not available for many veterinary drugs, they increase severely the cost of the analysis and it is well known that an adequate correction is assured only when the own ILIS is used [237]. The use of analogue ILIS is not always satisfactory [236, 237]. Therefore, other approaches such as matrix-matched calibration or standard addition method can be used for proper quantification of the samples [68, 78, 87, 236].

To evaluate matrix effect, the slopes obtained in the matrix-matched calibration curves were compared with those obtained with solvent standards. Matrix Effects (ME%) were calculated by subtracting 1 from the ratio between the standard solution calibration curve slope in matrix extracts (B) and in pure solvent (C) for each compound, and then multiplying by 100:

$$ME\ (\%) = ((B/C) - 1) \times 100$$

The signal is enhanced if the value is positive, whereas it is suppressed if the value is negative. A signal enhancement or suppression effect is considered as acceptable if the matrix effect values range from -20% to +20%.

5.5 Application to Real Samples

To evaluate the applicability of the proposed method, 12 bovine muscle tissue muscle samples were extracted. The retention time, quantification and confirmation transitions and relative ion intensities of the detected ions in unknown samples were compared to those of corresponding spiked samples and matrix-matched calibration standards in the same batch to confirm the

identity of the detected analytes using the criteria established by Decision Commission 657/2002/EC.

CHAPTER 6

Results and Discussion

6.1 Ionization study of Thyreostats ESI-MS/MS optimization

The ultimate goal of this study was the development of a multi-residue method for the determination of polar thyreostats which have rarely been included in multi-residue/multi-class analytical methods. Since thyreostats' different physicochemical properties render their simultaneous determination with other veterinary drugs quite problematic, an extended investigation of the chromatographic behavior of these antibiotics was performed in order to increase their sensitivity and make their simultaneous chromatographic detection with other drugs efficient.

Initially, experiments for the selection of the precursor and product ions for the 6 thyreostats under HILIC conditions were carried out. Direct infusion of individual standards of each compound in mobile phases was performed in positive and negative ionization mode. The solvents that were tested are presented in Table 7.

The mass spectra for all thyreostats were obtained in full-scan MS mode and the abundance of precursor ions was compared at the different mobile phases. The mass spectra of TS revealed monoprotonated ions $[M+H]^+$ as base peaks.

Taking sensitivity into consideration, the mass spectra of TS obtained in positive ionization mode showed higher relative abundances of the target analytes than in negative ionization mode. Therefore, the study based was performed in positive mode. Indicatively, the mass spectra of MBI are presented in both ionization modes (Figure 4).

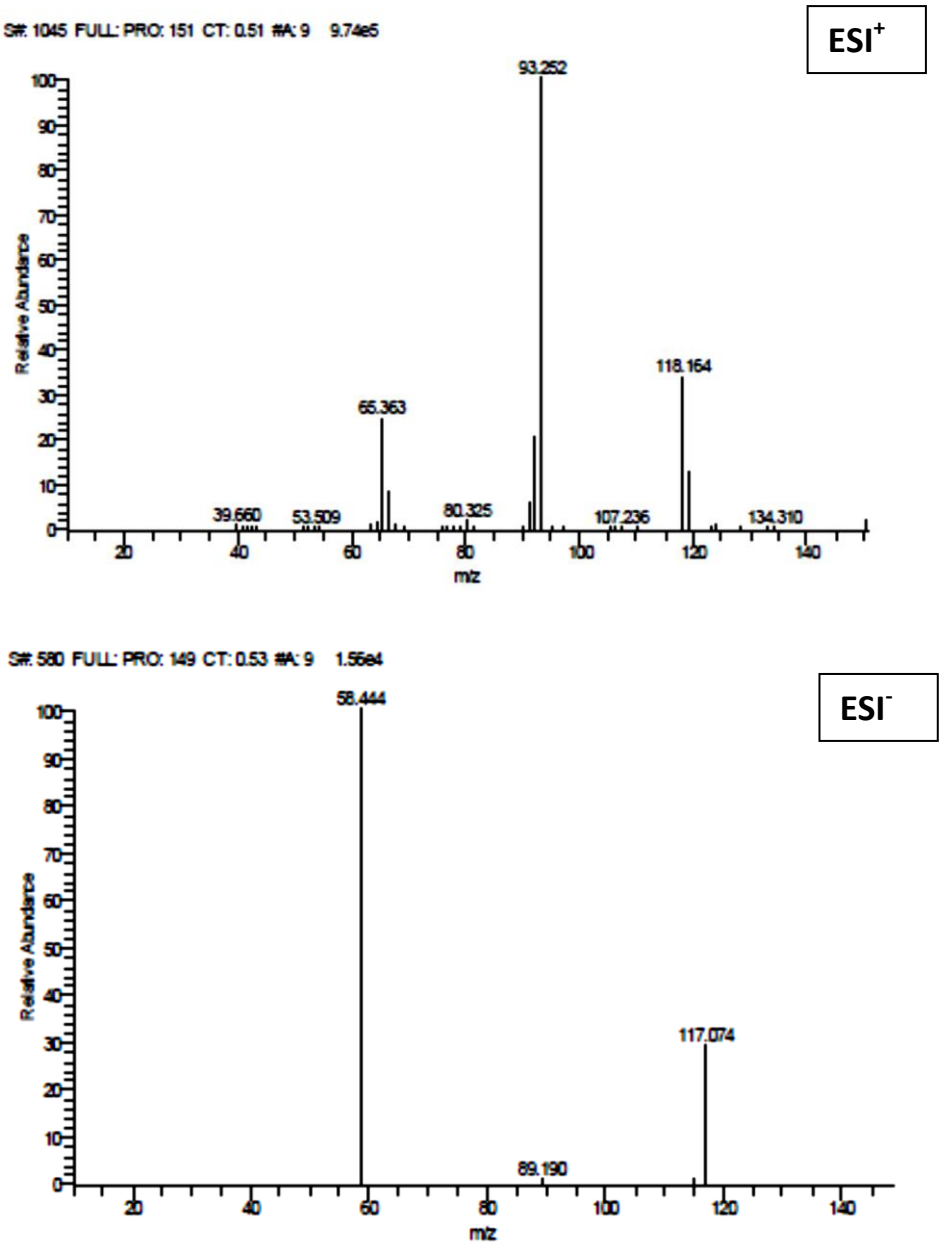


Figure 4: MS/MS spectrum of MBI in positive and negative mode.

Among all analytes, thiouracil gave the lower relative sensitivity. Consequently, further optimization of the ionization efficiency of thiouracil was held by direct infusion of TU standard prepared in various solvents. The abundance of precursor ions was compared. The aqueous/organic ratio of in all solvents tested was 90/10(v/v) to match the eluting conditions of the analytes. Organic phase consisted of acetonitrile and the aqueous phase of

water with different mobile phase additives, such as formic acid, acetic acid, ammonium acetate and ammonium formate at various concentrations.

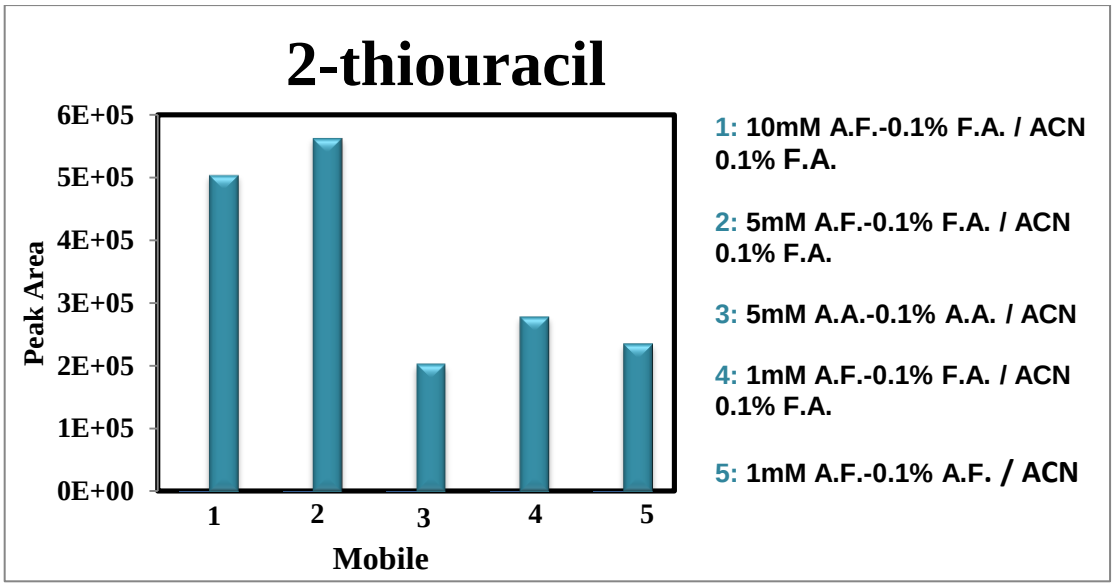


Figure 5: Intensity study of TU precursor ion in various mobile phases with ratio 20:80.

The solvent composition that presented the highest intensity of the precursor ion of TS was ACN/aqueous ammonium formate 5mM with 0.1 % formic acid (20/80, v/v).

Considering the above study (Figure 5), the ionization of 6 thyreostats was performed by infusing a standard solution prepared in the optimal constitution of mobile phase. The results are presented in the following table. The protonated ($[M+H]^+$) molecular ions were selected as the precursor ions for all the compounds.

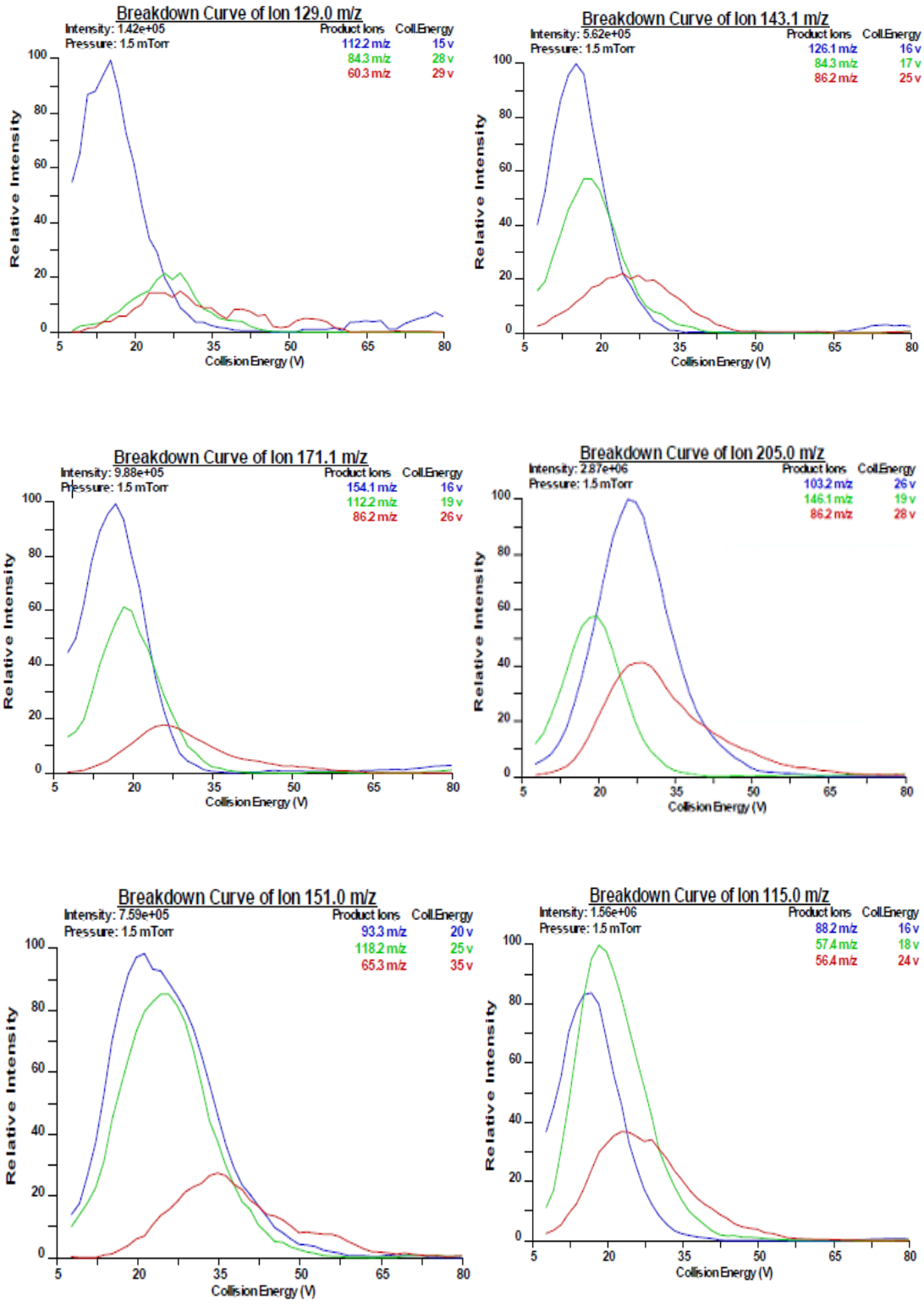


Figure 6: Breakdown curves for precursor ions of TS.

Table 10: Product ions and ionization parameters of 6 thyreostats

Standard	***Precursor Ion* ($[M+H]^+$)**	***Product Ions ****	***Collision Energy (eV)***	***Tube Lens (V)***
2-thiouracil (TU)	129	**112.2**	15	66.83
		84.3	28	
6-methyl-2-thiouracil (MTU)	143,1	**126.1**	16	67.58
		84.3	17	
6-propyl-2-thiouracil (PTU)	171,1	154.1	16	75.34
		112.2	19	
6-phenyl-2-thiouracil (PhTU)	205	**103.2**	26	77.59
		146.1	19	
2-mercaptobenzimidazole (MBI)	151	**93.3**	20	74.58
		118.2	25	
methimazole (TAP)	115	**57.4**	18	63.07
		88.2	16	
Internal Standard				
5,6-dimethyl-2-thiouracil (DMTU)	157	**140.1**	16	74.33
		98.2	18	
metzimazole-d3 (TAP-d3)	118	**91.2**	17	62.57
		60.4	20	
6-propyl-2-thiouracil-d5 (PTU-d5)	176	**117.2**	19	79.34
		159.1	18	

*** Quantifiers are in bold**

The optimal mass spectrometry settings are presented in **Table 11.**

Table 11: Mass spectrometric settings during the ionization study

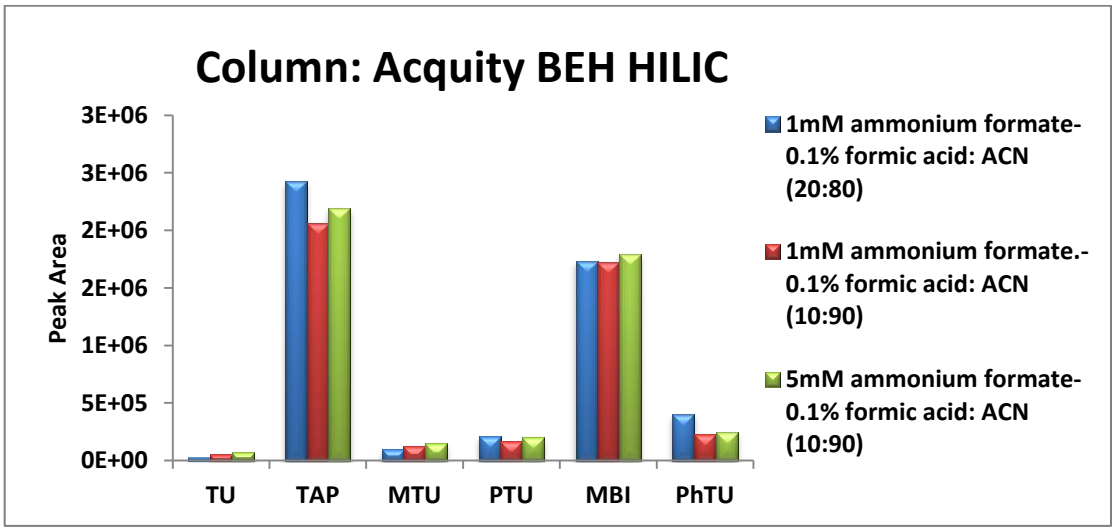
MS parameters	
Spray Voltage	3500 V
Sheath Gas Pressure	10 a.u.
Auxiliary Gas Pressure	5 a.u.
Capillary Temperature	300 ^{o}C
Probe	C

6.2 Chromatographic analysis

6.2.1 Separation study of TS in different HILIC columns

The aim of this study was the determination of thyreostats in short analysis time, obtaining symmetrical peaks and achieving high sensitivity, A standard solution of all analytes (300 µg mL^{-1}) was analyzed in triplicate in nine different HILIC columns and the retention times and peak areas and the peak shape of the most abundant SRM transition was compared.

In the following figures, the results of the analysis in each HILIC column are presented.



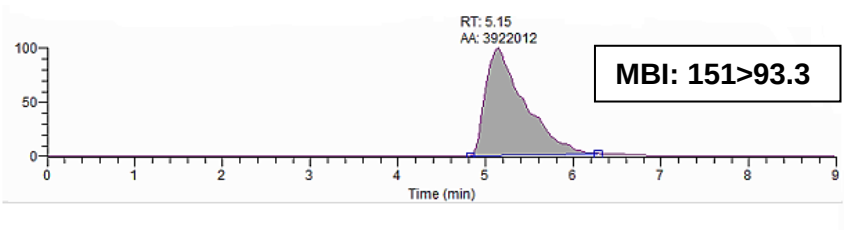
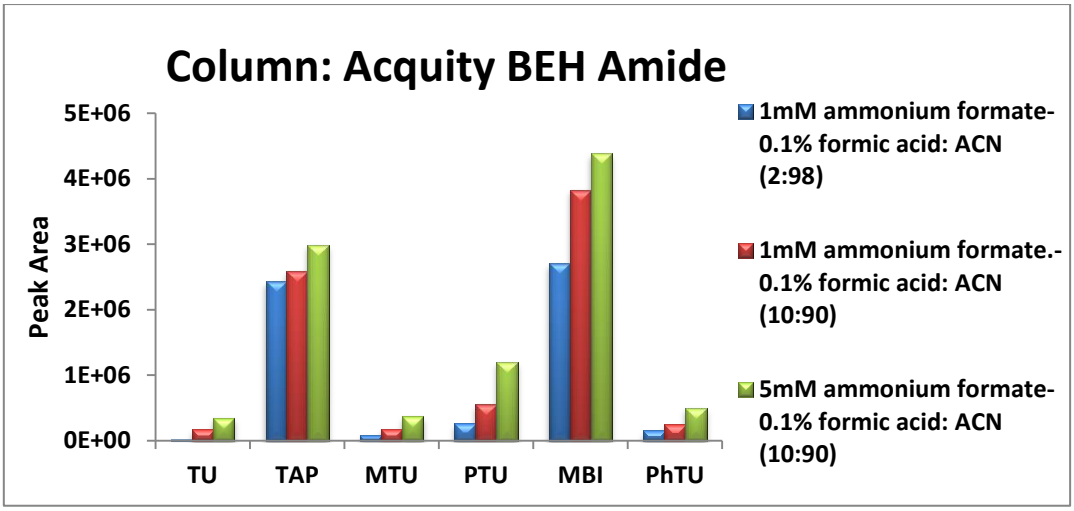


Figure 7: Chromatographic study of MBI in Acquity BEH HILIC column.



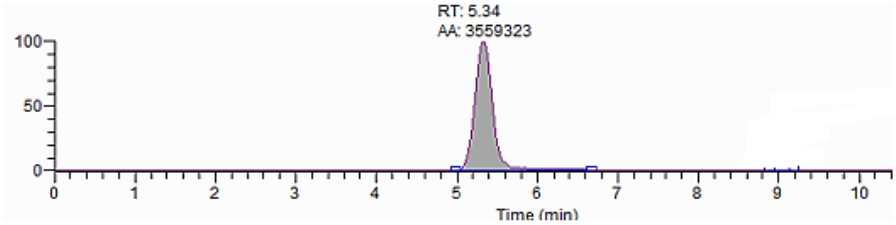
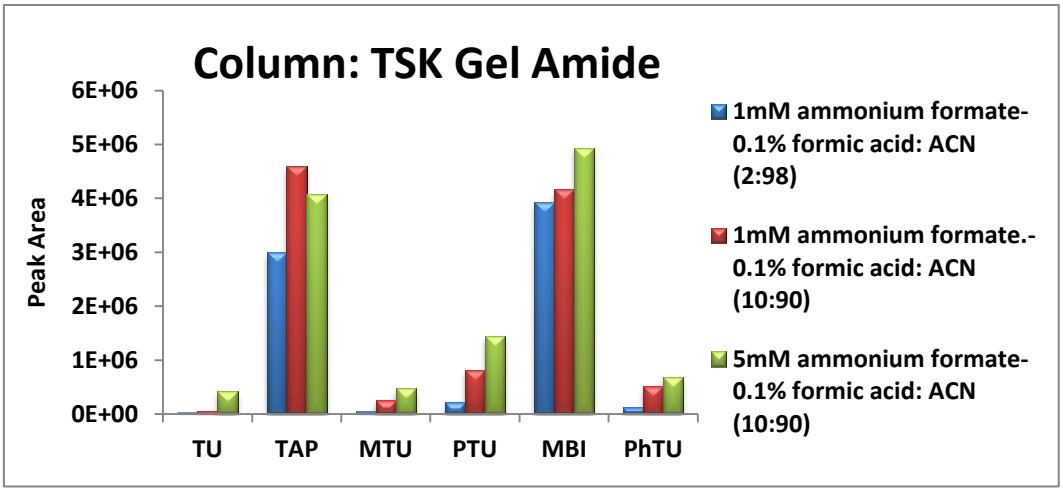


Figure 8: Chromatographic study of MBI in Acquity BEH Amide column.



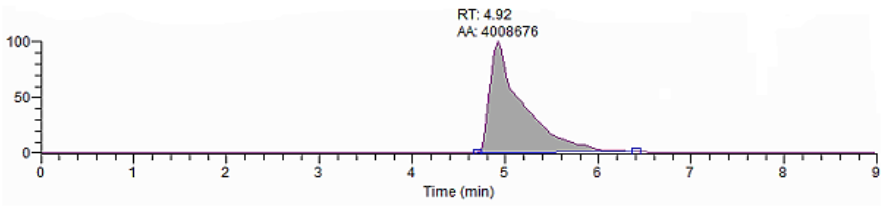


Figure 9: Chromatographic study of MBI in TSK Gel Amide column.

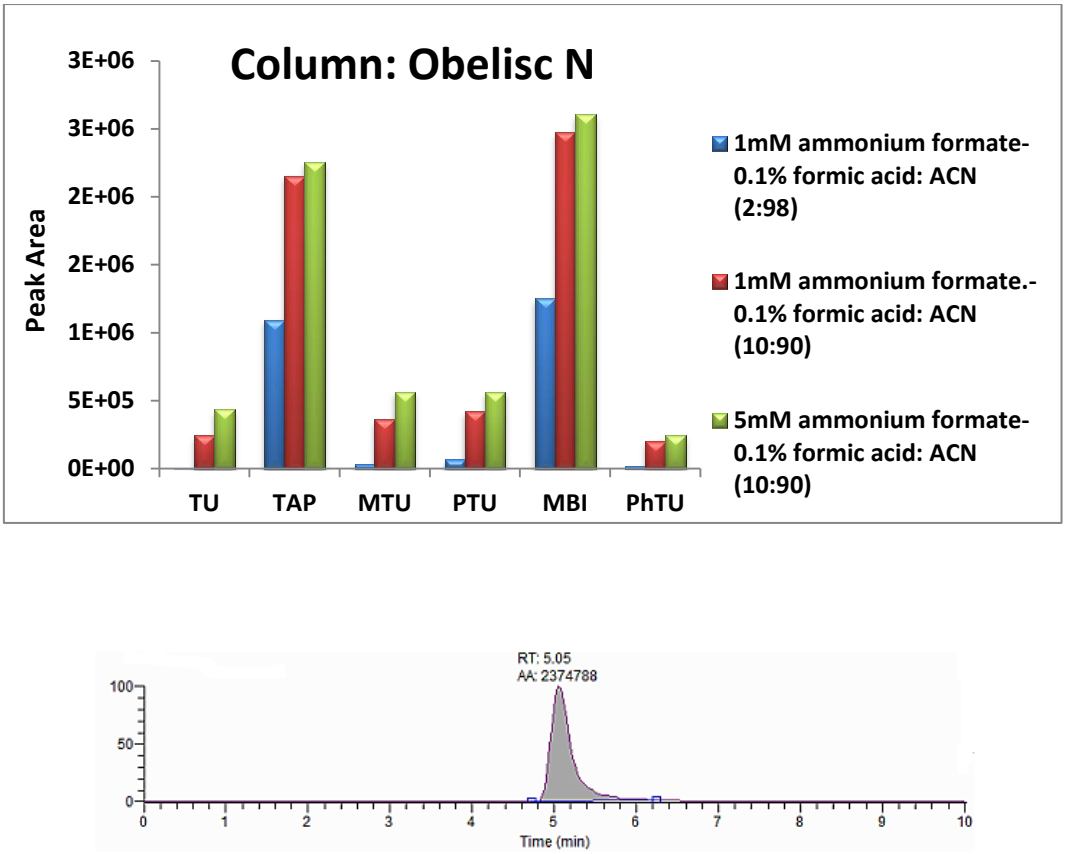


Figure 10: Chromatographic study of MBI in Obelisc N column.

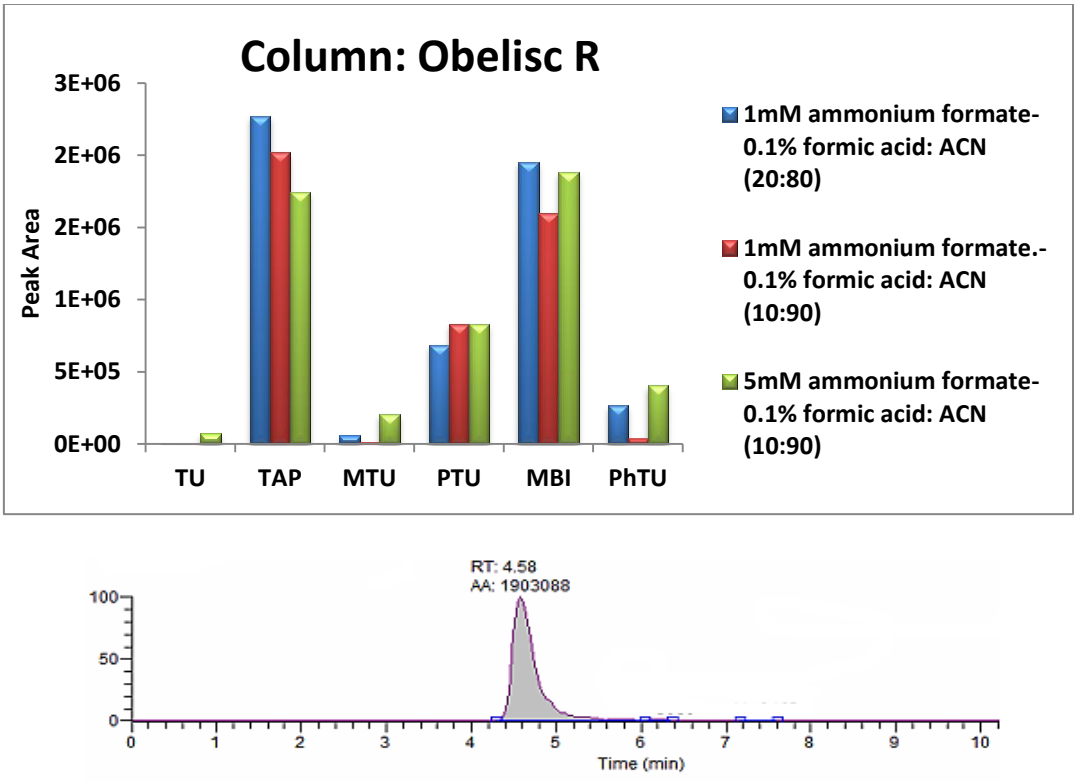
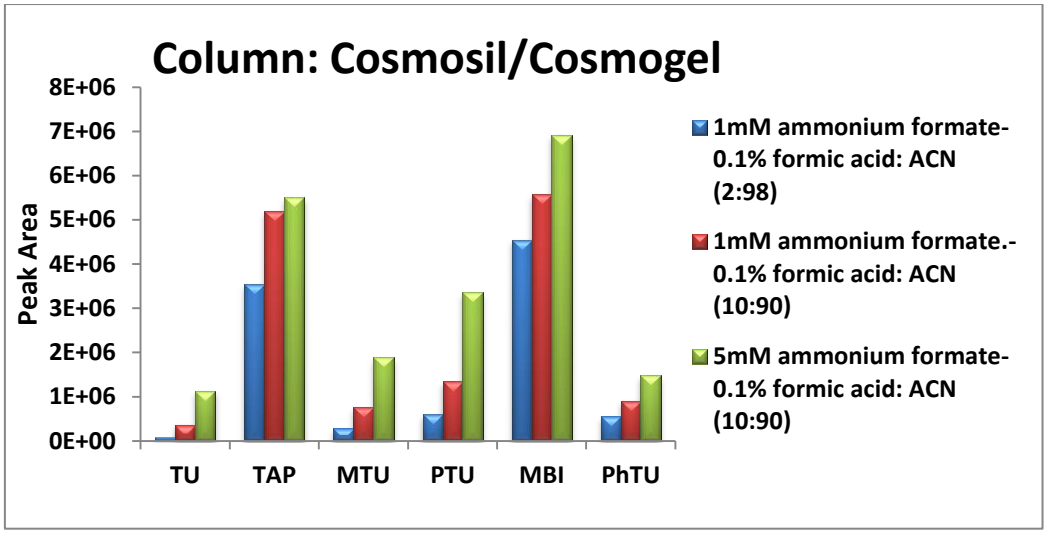


Figure 11: Chromatographic study of MBI in Obelisc R column.



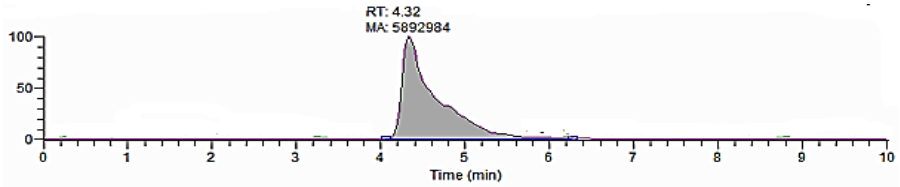
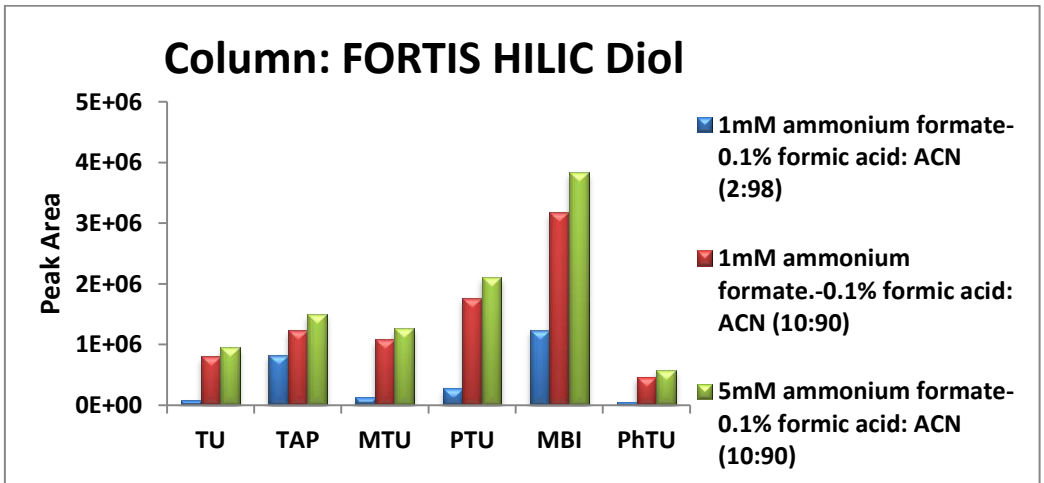


Figure 12: Chromatographic study of MBI in Cosmosil/ Cosmogel column.



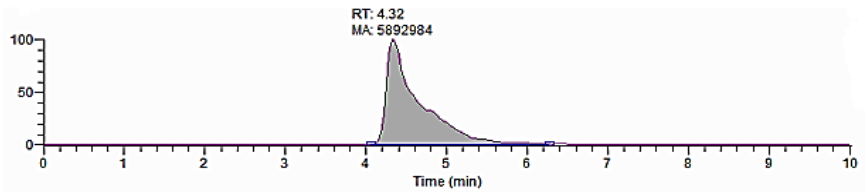
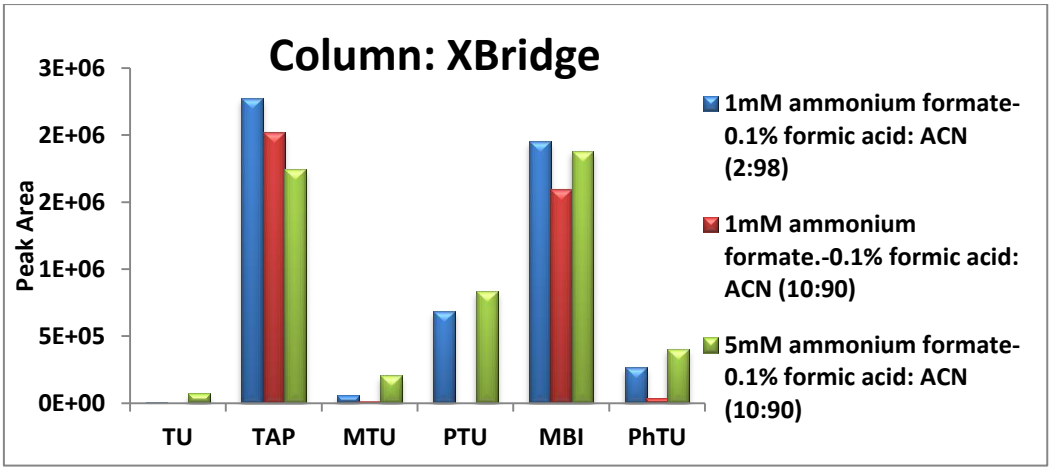


Figure 13: Chromatographic study of MBI in FORTIS HILIC Diol column.



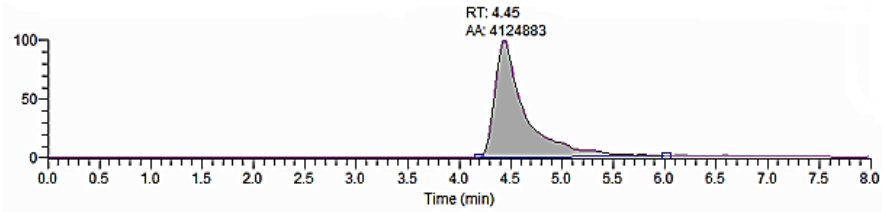
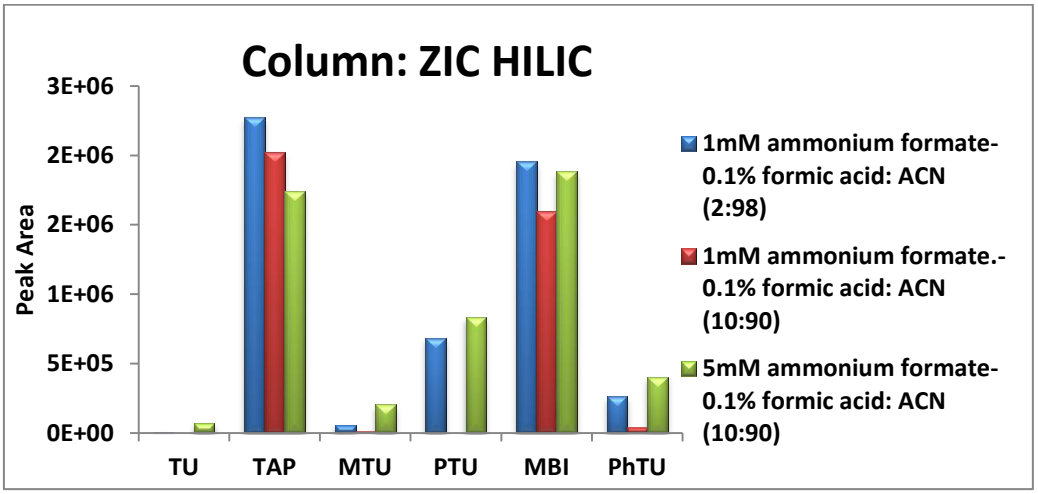


Figure 14: Chromatographic study of MBI in XBridge HILIC column.



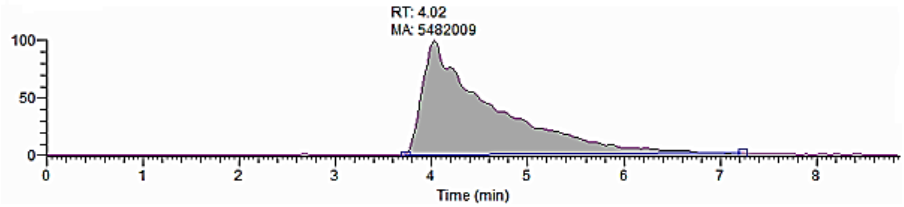


Figure 15: Chromatographic study of MBI in ZIC HILIC Diol column.

6.2.2 Mobile phase optimization

As can be seen from the above figures, BEH Amide column presented the best chromatographic behavior (Figure 12). Specifically, the satisfied peak areas for all TS, the short retention time and the shape of each peak have contributed to this outcome. ZIC HILIC has the worst response due to the tailing in most of the peaks.

As it described in paragraph 5.2.2 a last optimization was performed by testing isocratic conditions and two gradient programs. No significant difference was observed between isocratic and gradient conditions as far as retention times are concerned. Due to the large re-equilibration time required in HILIC determinations, isocratic elution was selected using a final mobile phase of 5Mm ammonium formate-0.1%formic acid/Acetonitrile 0.1% formic acid. All analytes were separated and eluted from 4.4 min (phenylthiouracil) to 5.6 min (mercaptobenzimidazole) at the final conditions.

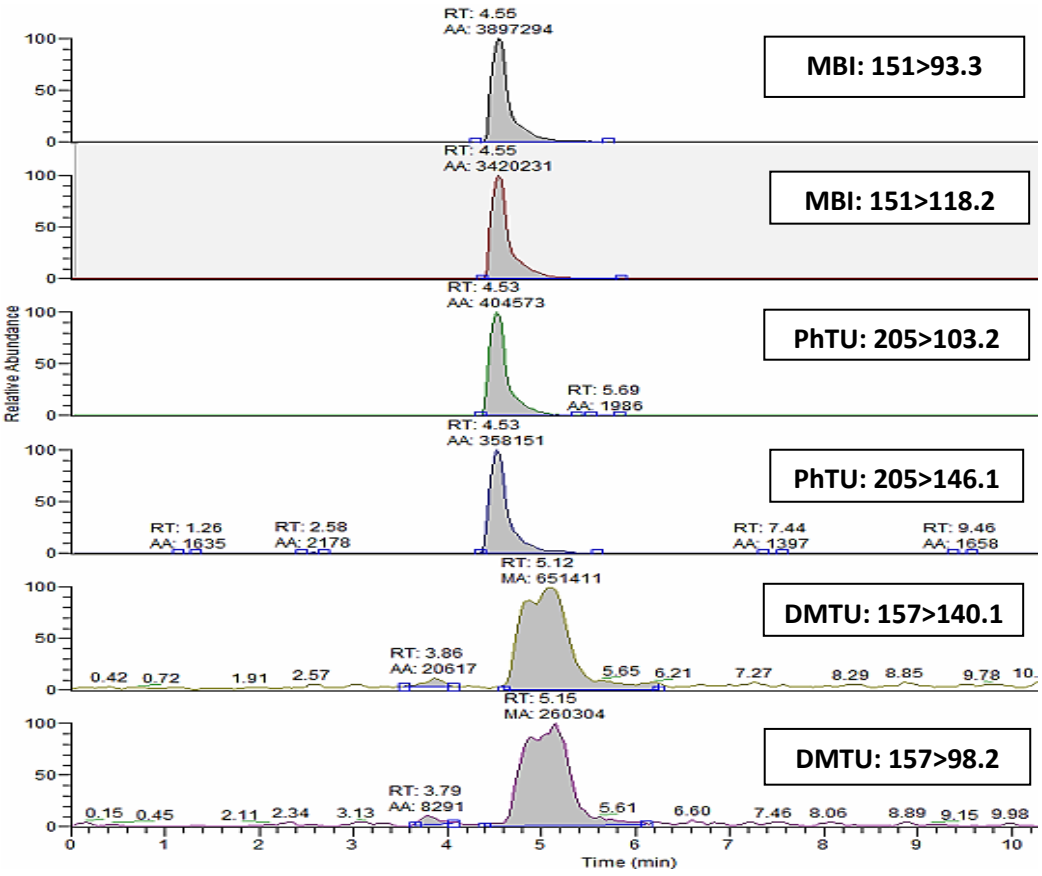


Figure 16: SRM Chromatograms of MBI, PhTU and DMTU (IS).

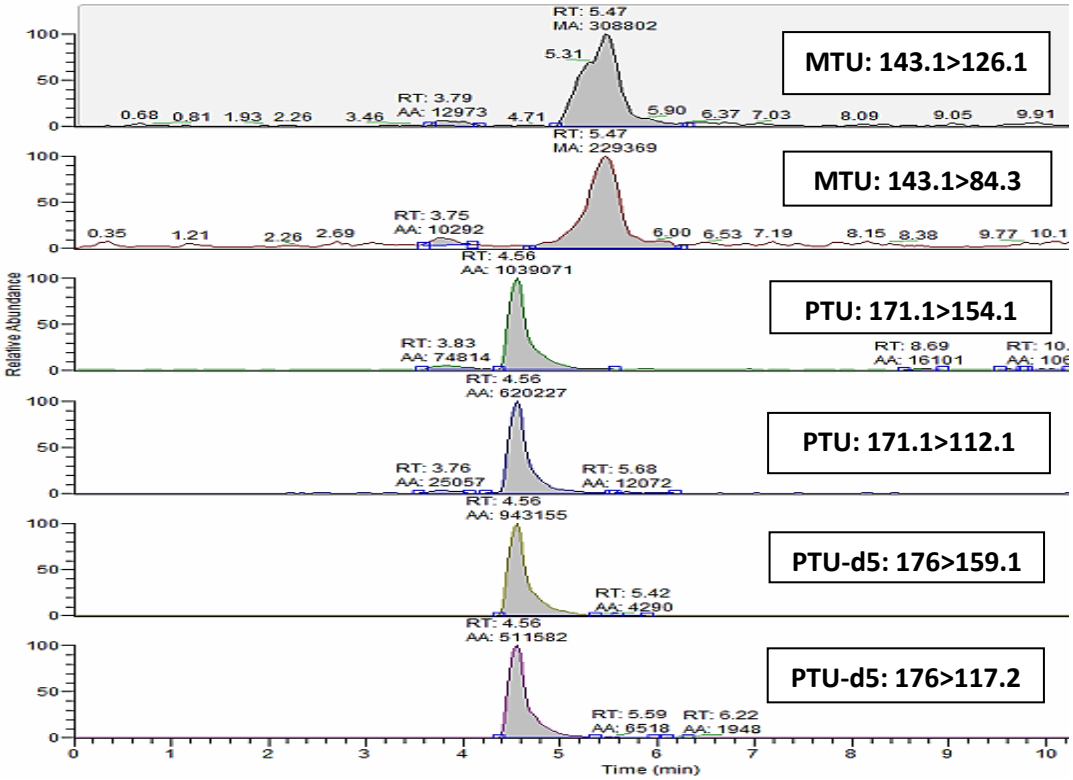


Figure 17: SRM Chromatograms of MTU, PTU, PTU-d5 (IS).

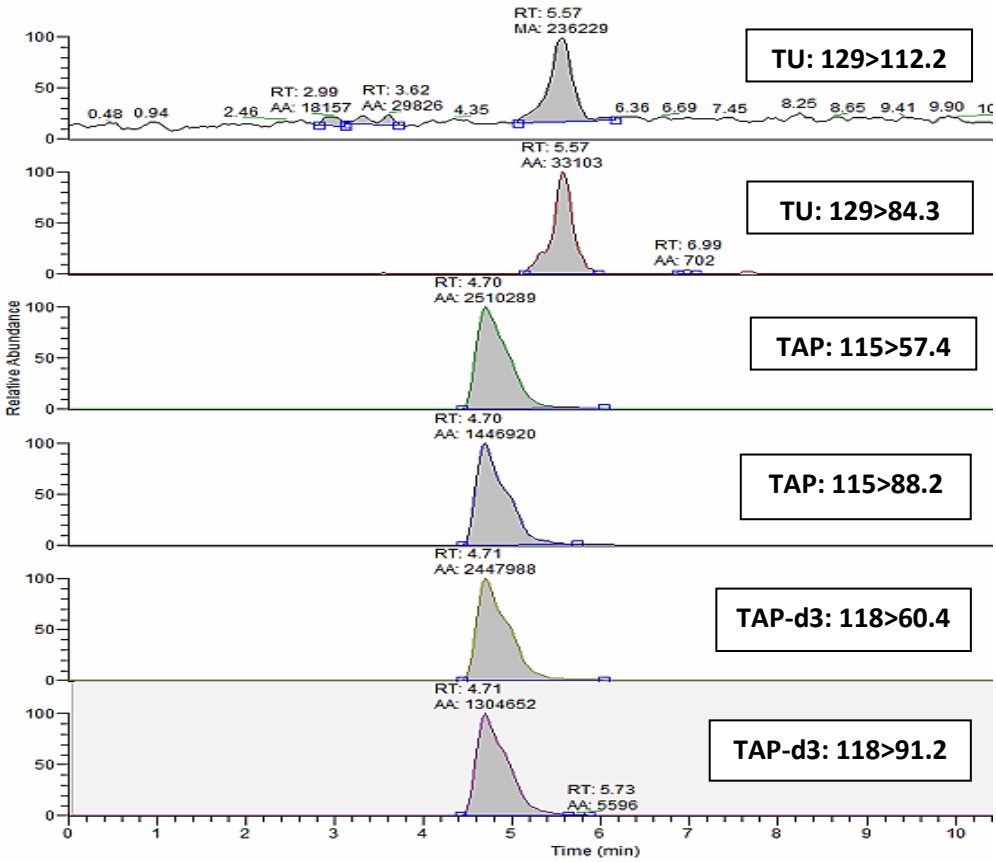


Figure 18: SRM Chromatograms of TU, TAP, TAP-d3 (IS).

6.3 Retention behavior of TS in BEH Amide

6.3.1 Effect of temperature

It was shown that with temperature increasing 3 of 6 compounds behave at the same way (as shown in the Figure 19). Retention behavior of thiouracil appears to be unaffected by this parameter. However, based on the slope of the van't Hoff plots, the retention enthalpy values were calculated for six compounds on the BEH amide column as presented in Table 12 and it was demonstrated that the reaction performed within the analytical column is exothermic for TS with ΔH ranging with the PTU occupying the largest negative value (Table 12).

Table 12: Calculated parameters of Van't Hoff equation

	Slope (-ΔH/R)	***ΔH^o(KJ/mol)***	***Process***	***R^2***
TU	135.52	-1126.71	Exothermic	0.9996
MTU	793.51	-6597.24	Exothermic	0.9949
PTU	855.61	-7113.54	Exothermic	0.9789
PhTU	730.2	-6070.88	Exothermic	0.9393
TAP	477.82	-3972.60	Exothermic	0.9060
MBI	779.08	-6477.27	Exothermic	0.9526

As shown in Table 12, all compounds exhibited negative retention enthalpy, indicating an exothermic process of transferring solutes from the mobile phase to the stationary phase.

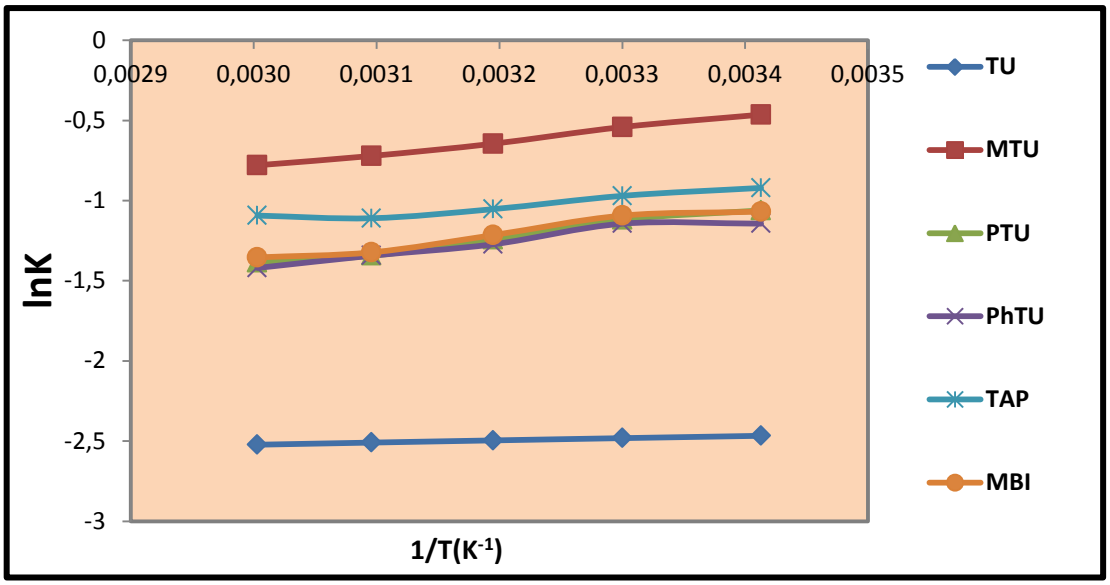


Figure 19: Effect of temperature in retention behavior.

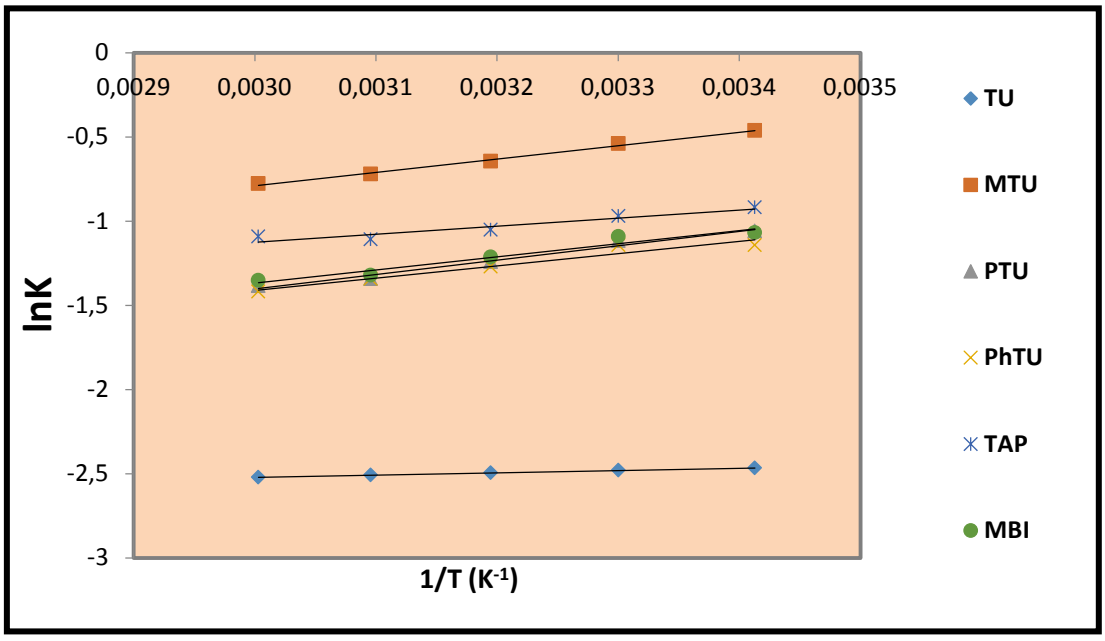


Figure 20: Van't Hoff plot for TU, MTU, PTU, PhTU, TAP and MBI.

6.3.2 Effect of acetonitrile content

The logarithmic capacity factors of six compounds were plotted against the water content and the logarithmic water content in the mobile phase of the columns, as shown in Figure 21. It was proved by the regression analysis and the value of R^2 that graph shown Figure 21 (a), is more linear for thiouracil

(R^2= 0.9879) and methylthiouracil (R^2= 0.9901) than in Figure 22 (b). However, the linearity for propylthiouracil (R^2= 0.9879), phenylthiouracil (R^2= 0.9879) and mercartobenzimidazole (R^2= 0.9879) was better in Figure 21 (a). Regarding to methimazole does not display linearity on either of the two graphs. Thus, the retention mechanism for TU and MTU is partitioning, for PTU, PhTU and MBI is adsorption and TAP appears to have to linear behavior in both graphs implying a change in retention mechanism or multiple forces, contributing to retention.

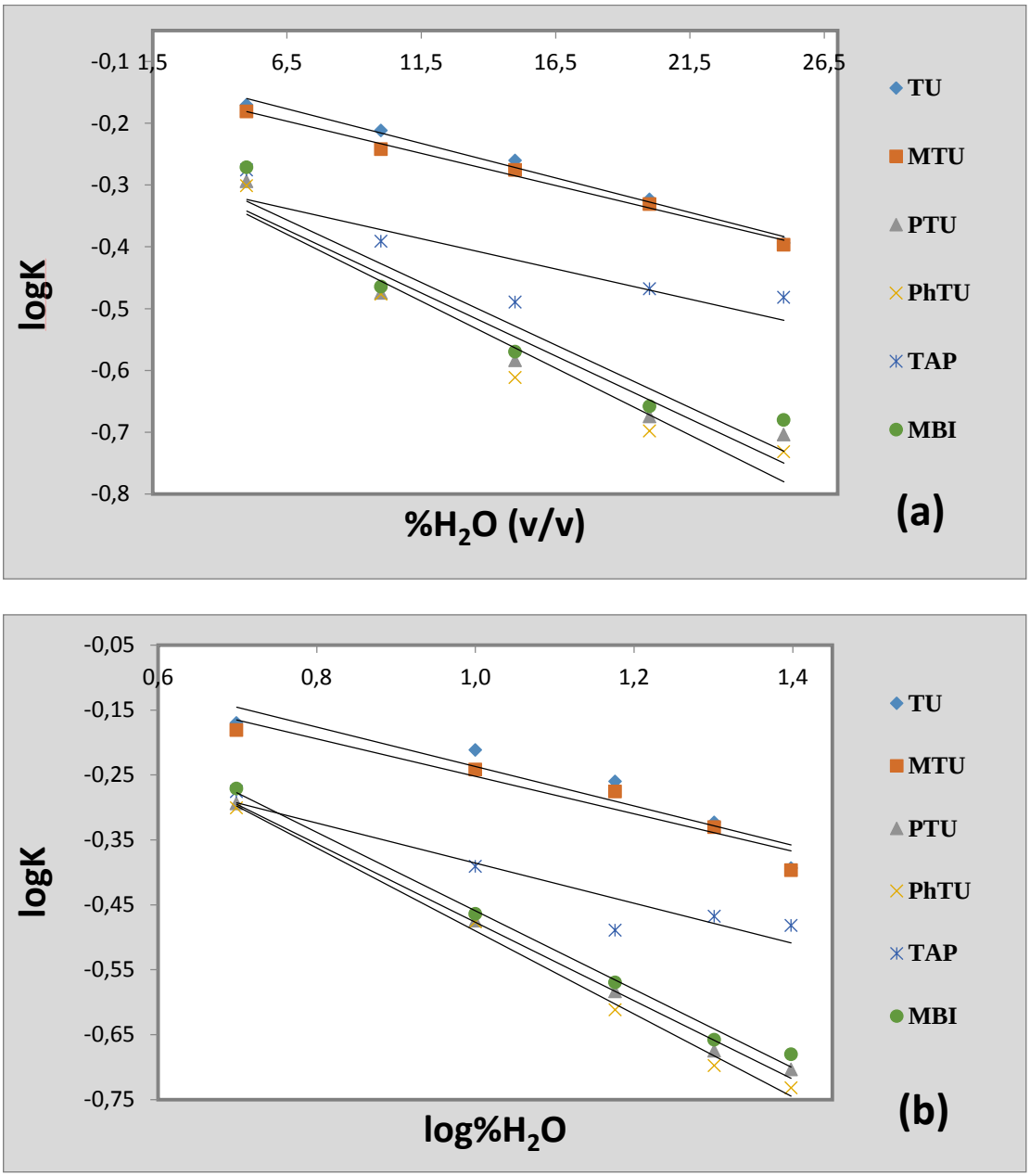


Figure 21: Effect of water content in retention time of TU, MTU, PTU, PhTU, MBI and TAP.

6.3.3 Effect of salt concentration

In addition to salt type, the effect of salt concentration on the retention was also investigated by varying ammonium formate concentration from 5 to 20mM in the mobile phase of acetonitrile/water (90/10, v/v). Further increase in the salt concentration was not possible due to solubility limitation in the mobile phase.

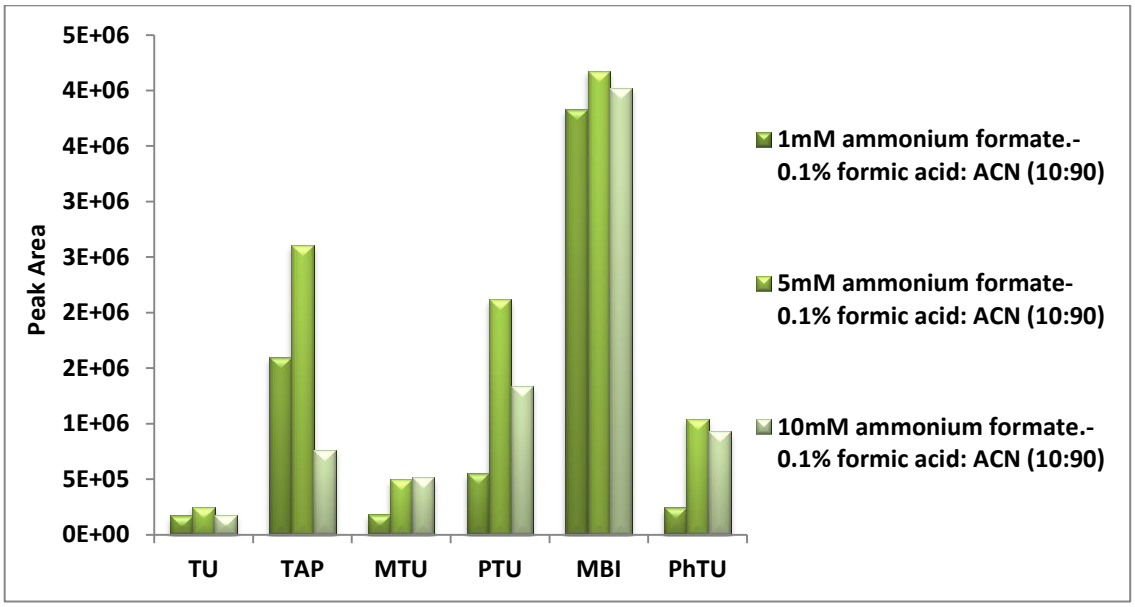


Figure 22: Effect of salt concentration in of TU, MTU, PTU, PhTU, MBI and TAP.

6.4 Sample preparation

After a thorough comparison of the recoveries obtained from each sample pretreatment, the preparation with the highest recovery for the majority of the compounds was selected. A pre-concentration test then was performed comparing the recovery and the matrix effect for each thyreostatic drug. The results obtained are shown in the Table 13. The pretreatment containing 4 times pre-concentration gave satisfactory recoveries and low matrix effect.

Table 13: Recoveries and matrix effect for TS

Pre-concentration(x)	*Recovery (%)*	*Matrix Factor (%)*	*Matrix Effect(%)*
thiouracil			
1x	88.1	72.4	27.6
2x	89.7	67.8	32.2
4x	91.5	69.9	30.1
6-methyl-2-thiouracil			
1x	58.8	145	-45.4
2x	108	73.5	-26.5
4x	92.4	53.7	-46.3
6-propyl-2-thouracil			
1x	119	79,4	-20.6
2x	62.1	104	4,10
4x	103	59.7	-20.3
6-phenyl-2-thiouracil			
1x	97.6	30.2	-69.8
2x	79.3	15.3	-84.7
4x	100	65.7	-34.3
methimazole			
1x	110	106	5.80
2x	109	106	6.00
4x	102	99.1	0.80
mercaptobenzimidazole			
1x	82.0	38.9	-61.1
2x	64.0	65.0	-45.0
4x	86.0	71.7	-28.3

6.5 Method Validation

6.5.1 Linearity

The linearity of calibration curves was assessed using a six-point standard solution calibration curve in pure solvents as well as in blank bovine muscle tissue extracts at different concentrations (0.25 to 4×VL for each target compound). The linear regression analysis was carried out by plotting the peak area versus the analyte concentrations for compounds with no corresponding IS and the peak area ratio of the analyte and I.S. versus the analyte concentrations. when an IS correction was used. The regression line of the form y = bx + a. the standard deviation of the intercept Sa, the standard deviation of the slope Sb and the correlation coefficients R^2, for standard solutions were determined. Figure 23 shows indicatively the calibration curve

for the standard solution of mercaptobenzimidazole and Figure 24 shows the calibration curve of the same compound in matrix extract.

For instrument linearity, the calibration parameters showed good linearity since correlation coefficients were >0.99 for all analytes. R^2 ranged from 0.9911(mercaptobenzimidazole) to 0.9996 (thiouracil) for standard solution curves.

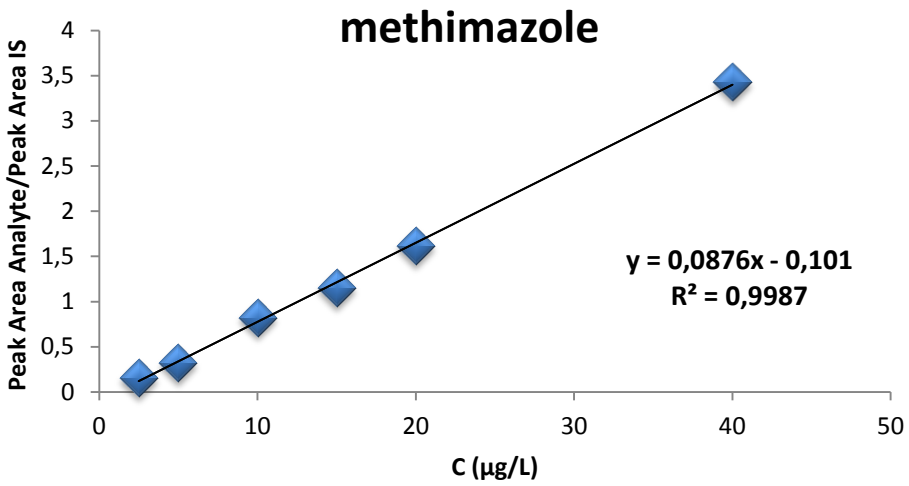


Figure 23: Calibration curve of methimazole standard solutions.

Table 14: Regression lines for standard solutions of examined TS

N=6	Working Range of Standard Solutions (µg/L): 2.5-40	
	Linear Equation	**R^2**
2-Thiouracil	y= (27.06 ± 0.27)×10^2 x+ (11.3 ± 5.4)× 10^2	0.9996
6-methyl-2-thiouracil	y= (64.01 ± 0.073)×10^{-3} x+ (-2.18 ± 1.5)× 10^{-2}	0.9995
6-propyl-2-thiouracil	y= (171.34 ± 5.8)×10^{-3} x+ (1.12 ± 1.5)× 10^{-1}	0.995
6-phenyl-2-thiouracil	y= (62.40 ± 3.3)×10^2 x+ (1.20 ± 6.6)× 10^3	0.989
methimazole	y= (87.5 ± 1.6)×10^{-3} x+ (10.09 ± 3.2)× 10^{-2}	0.9990
2-mercaptobenzimidazole	y= (19.21 ± 0.91)×10^2 x+ (15.33 ± 18)× 10^2	0.991

Calibration parameters were also proved that linearity is equally good in the case of spiked samples. R^2 ranged from 0.991 (mercaptobenzimidazole) to 0.9996 (thiouracil) in matrix extracts.

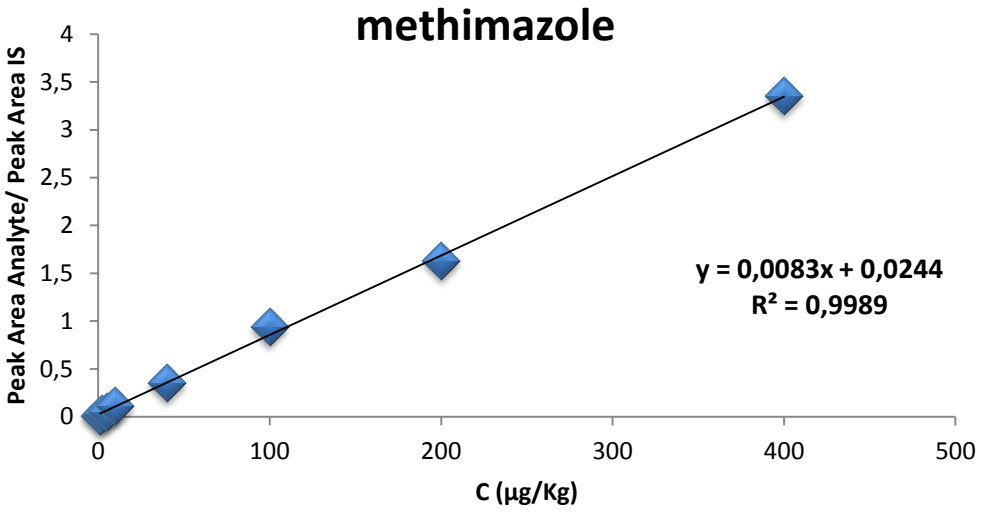


Figure 24: Calibration curve of methimazole in spiked tissue.

Table 15: Regression lines for examined TS in spiked muscle tissues

N=6	Working Range of spiked matrices (µg/Kg): 1-400	
	Linear Equation	**R^2**
2-thiouracil	y= (11.58 ± 0.11)×10^2 x+ (24.63 ± 18)× 10^2	0.9994
6-methyl-2-thiouracil	y= (102.19 ± 3.1)×10^{-4} x+ (-1.34 ± 4.9)× 10^{-2}	0.995
6-propyl-2-thiouracil	y= (149.03 ± 3.4)×10^{-4} x+ (9.23 ± 5.6)× 10^{-2}	0.997
6-phenyl-2-thiouracil	y= (72.44 ± 0.11)×10^2 x+ (16.38 ± 4.8)× 10^2	0.9990
methimazole	y= (83.06 ± 1.1)×10^{-4} x+ (24.41 ± 18)× 10^{-3}	0.9990
2-mercaptobenzimidazole	y= (197.84 ± 2.9)×10^{-4} x+ (16.38 ± 4.8)× 10^{-2}	0.9990

6.5.2 Precision

The precision of this method was calculated as intra-day precision (repeatability) and inter-day precision (within-laboratory reproducibility),

It can be observed that relative standard deviations were always lower than 20 for all thyreostats. Moreover, the obtained RSD values of the within-laboratory reproducibility did not exceed in any case the acceptable values calculated from the Horwitz equation. These results indicate the good precision and reliability of the developed method. Precision results for all compounds in all concentration levels are presented in Table 16.

Table 16: Precision values for target thyreostats

Repeatability	**Level A (0.5xVL)**			**Level B (1xVL)**			**Level C (1.5xVL)**		
n=9 for each level	**Average %Rec.**	**SD**	**%RSD**	**Average %Rec.**	**SD**	**%RSD**	**Average %Rec.**	**SD**	**%RSD**
2-thiouracil	84.5	8.0	9.5	82.8	7.1	8.6	88.7	4.9	5.5
6-methyl-2-thiouracil	89.2	9.1	10	93.6	13	14	91.7	7.8	8.5
6-propyl-2-thiouracil	98.9	6.6	6.7	103	7.5	7.3	98.5	11	11
6-phenyl-2-thiouracil	80.7	8.3	10.3	79.5	11	14	76.8	6.8	8.8
methimazole	96.2	6.6	6.9	100	6.0	6.0	102	5.8	5.7
2-mercaptobenzimidazole	80.2	10	12	84.7	11	13	76.6	7.4	9.7
Reproducibility	**Level A (0,5xVL)**			**Level B (1xVL)**			**Level C (1,5xVL)**		
n=6 for each level	**Average %Rec.**	**SD**	**%RSD**	**Average %Rec.**	**SD**	**%RSD**	**Average %Rec.**	**SD**	**%RSD**
2-thiouracil	81.8	6.6	8.1	82.7	11	14	87.0	7.5	8.6
6-methyl-2-thiouracil	87.1	17	19	91.3	13	14	108	11	10
6-propyl-2-thiouracil	96.6	7.9	8.2	102	4.4	4.3	97.3	8.0	8.3
6-phenyl-2-thiouracil	80.1	8.9	11	82.7	11	14	77.8	8.4	11
methimazole	87.0	14	16	96.3	8.9	9.2	98.4	7.0	7.1
2-mercaptobenzimidazole	77.4	9.5	12	76.7	10	13	80.7	10	13

6.5.3 Accuracy

The accuracy of the method was estimated through recovery studies Average recoveries of each analyte were calculated performing the analysis in 18 replicates at each validation level in three different days (6 samples per day per validation level). These results of the recovery study are given in Table 6.6. Recoveries at the 0.5×VL varied from 77.4% (mercaptobenzimidazole) to 96.6% (propyl-thiouracil). In spite that some compounds present recovery values not close to 100%, they are considered acceptable since they were reproducible.

6.5.4 LODs & LOQs

LODs and LOQs were evaluated as described in the Experimental Section, showing the obtained results in Table 17. LOQs ranged from 4.6 μg kg^{-1} (thiouracil) to 9.9 μg kg^{-1} (methylthiouracil) and were in all cases lower than the corresponding VL where one established.

For some compounds lowest method LODs than instrumental LODs were obtained due to the severe matrix enhancement of these compound to the matrices examined.

Table 17: LOD and LOQ values of instrument and method for target thyreostats

Instrument	**Limit of Detection LOD (μg L^{-1})**	**Limit of Quantification LOQ (μg L^{-1})**
2-Thiouracil	1.5	4.6
6-methyl-2-thiouracil	3.3	9.9
6-propyl-2-thiouracil	2.1	6.4
6-phenyl-2-thiouracil	2.4	7.4
methimazole	2.8	8.5
2-mercaptobenzimidazole	2.7	8.2
Method	**Limit of Detection LOD (μg kg^{-1})**	**Limit of Quantification LOQ (μg kg^{-1})**
2-Thiouracil	4.1	9.6
6-methyl-2-thiouracil	2.9	9.4
6-propyl-2-thiouracil	3.3	7.1
6-phenyl-2-thiouracil	2.8	8.3
methimazole	2.9	8.7
2-mercaptobenzimidazole	3.8	9.2

6.5.5 Decision limit (CCα) and Detection capability (CCβ)

All the compounds that do not have established MRLs were treated as banned compounds and the CCα and CCβ were calculated through the calibration curve procedure. Decision limits ranged from 3.7 µg kg^{-1} (thiouracil) to 9,7 µg kg^{-1} (mercaptobenzimidazole) and detection capability from 4.8 µg kg^{-1} to 11 µg kg^{-1}.

Table 18: CCα and CCβ values for target thyreostats

	Decision Limit CCα (µg kg^{-1})	Detection capability CCβ (µg kg^{-1})
2-thiouracil	3.7	4.8
6-methyl-2-thiouracil	5.2	8.1
6-propyl-2-thiouracil	8.0	9.3
6-phenyl-2-thiouracil	7.5	9.0
methimazole	6.1	8.4
2-mercaptobenzimidazole	9.7	11

6.5.6 Selectivity/Specificity

The selectivity of the method was evaluated extracting and analyzing 20 control blank bovine muscle tissue samples. No background peaks, above a signal-to-noise ratio, were present at the same elution time as the target thyreostats. This shows that the method is free of endogenous interferences.

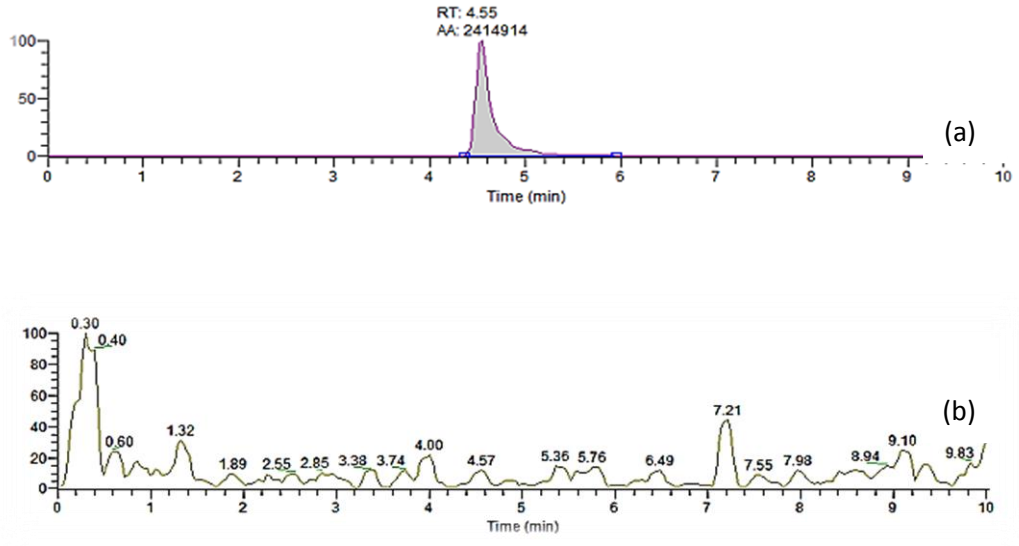


Figure 25: SRM chromatogram of MBI in standard solution (a) and in blank sample (b).

6.5.7 Matrix Effect

% Matrix effects higher than 20% or lower than -20% indicate a strong matrix effect. It can be observed that a significant signal enhancement was noticed for phenylthiouracil. Thiouracil presented insignificant matrix effect.

Table 19: Matrix effect for 6 thyreostatic drugs.

Compound	% Matrix Effect
2-Thiouracil	16.1
6-methyl-2-thiouracil	-32.5
6-propyl-2-thiouracil	-21.7
6-phenyl-2-thiouracil	-17.8
methimazole	-6.30
2-mercaptobenzimidazole	-38.6

6.6 Conclusions

A broad range of veterinary drugs are administrated in animal husbandry in order to improve animal health, but also as growth promoters for intensive animal production. The possible presence of veterinary drug residues in food of animal origin is one of the key issues for food safety.

A rapid, sensitive and efficient multiresidue analytical method for the simultaneous determination of 6 TS in bovine muscle tissue by HILIC–MS/MS has been developed. The method includes rapid solid-liquid extraction followed by dispersive solid-phase extraction (d-SPE) clean-up with C18 sorbent and hexane partitioning. HILIC-MS/MS determination was performed after a thorough chromatographic study in BEH Amide column.

This is the first study reported to determine thyreostats, using HILIC, achieving substantially low LODs and short-time analysis. The method was validated in agreement with the guidelines of Commission Decision 2002/657/EC and was applied to the analysis of unknown meat samples.

ABBREVIATIONS – ACRONYMS

ACN	Acetonitrile
CRLs	Community Reference Laboratories
DMTU	dimethylthiouracil
d-SPE	dispersive-Solid Phase Extraction
EC	European Council
ESI	Electrospray Ionization
EU	European Union
EURL	Entreprise Unipersonelle à Responsibilité Limitée
FDA	Food and Drugs Administration
HILIC	Hydrophilic Interaction Liquid Chromatography
IS	Internal standard
LC-MS/MS	Liquid Chromatography – tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MBI	Mercaptobenzimidazole
ME	Matrix Effect
MeOH	Methanol
MLOD	Method Limit of detection
MLOQ	Method limit of Quantification
MRL	Maximum Residue Limit
MRPL	Minimum required performance limit
MS/MS	Tandem Mass Spectrometry
MSPD	Matrix Solid Phase Extraction
MTU	methylthiouracil
PhTU	phenylthiouracil
PTU	propylthiouracil
QqQ	Triple quadrupole
Rec	Recovery
RP	Reversed-phase

RSD	Relative Standard Deviation
RT	Retention time
SD	Standard Deviation
SPE	Solid Phase Extraction
TAP	methimazole
TU	thiouracil
UHPLC	Ultra-high performance liquid chromatography

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