DEVELOPMENT OF NON-TARGETED STRATEGIES FOR THE ANALYSIS OF TRACE ORGANIC CONTAMINANTS IN HONEY

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Non-targeted analysis of contaminants in honey

ABSTRACT

Due to the constant increase of contaminants and toxins reported in food, the world of food analysis is shifting towards the detection and identification of currently unknown or unexpected contaminants using non-targeted analysis. As it does not rely on the initial use of analytical standards, the non-targeted approach opens the door for new applications in the field of food authentication and food safety. Although the number of non-targeted studies being used and reported has increased in recent years, the chemical risk assessment community has highlighted the need to further develop non-targeted methods to better characterize human exposure to chemicals, and to identify potential risk compounds in food matrices. The overall objective of this work was the development of a non-targeted method for the analysis of trace organic contaminants in honey. Each step of the non-targeted workflow was studied and optimized to allow for the screening of a variety of contaminant families in this complex matrix. First, a fast screening and quantification method was successfully developed and validated for the targeted analysis of 7 veterinary drug residues in honey, using direct injection high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS). The selected veterinary drug residues were detected at levels approximately 20 to 100 times lower than the actual regulatory limits, with acceptable recoveries, linearity and repeatability, for a total analysis time of 45 min per sample. A data-independent All-Ions MS/MS mode was used to continuously record MS and MS/MS data at four different collision energies, and allowed for the confirmation of the identity of the target analytes, showing the non-targeted potential of the method. Next, the data pre-treatment steps for the non-targeted identification of trace organic contaminants in honey were studied using the same 7 veterinary drugs as case study. The impact of 7 parameters on the correct identification of the target compounds was assessed, and only the expansion window for chromatogram extraction and the average scans included in the spectra influenced the identification results significantly. These findings confirmed that data pre-treatment parameters can affect the identification of trace contaminants in food. The optimized identification workflow was used to screen 55 honey samples from the Canadian market using a

library of 43 honey-related compounds, including veterinary drugs, pesticides and other contaminants, which led to the detection of tylosin A and 5-hydroxymethylfurfural (HMF) among these samples. Then, the optimized non-targeted workflow was applied to the screening of plastic-related compounds in 104 honey samples from the Canadian market, and a total of 662 compounds were tentatively detected using a library of leachable and extractable compounds. The identity of two of these compounds, namely bis(ethylhexyl) adipate (DEHA) and tris (2-butoxyethyl) phosphate (TBOEP), was further confirmed with pure analytical standards, and their estimated daily intake was found to be far below the current tolerable daily intake. The chemical burden in honey samples sold in either glass or plastic jars was compared using 3 data treatment approaches, each of which resulted in a different list of relevant contaminants. These findings showed that some of the most commonly used data treatments in metabolomics need to be carefully selected when it comes to identifying trace contaminants in food. Among the compounds discovered based on the differential frequency of detection, 6 were unique to honey samples sold in plastic jars and 3 were unique to honey samples sold in glass jars. Finally, the degradation of the veterinary drug tylosin A in water, spiked honey and incurred honey after different thermal treatments was studied using the optimized non-targeted method. The results, in terms of rates of degradation of tylosin A and increase of tylosin B (the only degradation product reported so far) were in agreement with the literature. However, the non-targeted approach used for this study led to the tentative identification of two new degradation products, namely 5-O-mycaminosyltylonolide (OMT) and lactenocin. Although the thermal treatment applied was the same, the degradation products identified in water, spiked honey and incurred honey appeared to be different, reinforcing the conclusion that relying only on the water model or spiked food matrix is not sufficient to understand the thermal degradation of antibiotics in food matrices. The possibility of a semi-quantification of tylosin B in honey using tylosin A, its parent compound, was also assessed. The present results proved that a semi-quantification using the parent compound to quantify its degradation compounds can be a suitable strategy when using non-targeted analysis. Overall, this research demonstrated that non-targeted analysis can

improve the characterization of contaminant occurrence and fate in food, using honey as a key example.

RÉSUMÉ

Des nouveaux contaminants et toxines sont découverts en permanence dans les aliments. Aussi, le monde de l'analyse des aliments s'intéresse de plus en plus aux outils permettant la détection et l'identification des contaminants inconnus ou inattendus. Ce type d'analyse, que l'on appelle non-ciblée, ne s'appuie pas sur un étalonnage à partir du composé chimique pur, ce qui ouvre la porte à des nouvelles applications en termes d'authentification et de sécurité alimentaire. Bien que le nombre d'études utilisant l'analyse non-ciblée ait augmenté dans la littérature scientifique ces dernières années, les experts en évaluation des risques chimiques ont souligné le besoin urgent de développer davantage ces méthodes pour (i) mieux caractériser l'exposition humaine aux produits chimiques, et (ii) identifier les composés qui peuvent poser un risque dans les aliments. L'objectif de cette thèse était le développement d'une méthode d'analyse non-ciblée pour l'analyse des contaminants organiques traces dans le miel. Toutes les étapes du workflow non-ciblé ont été étudiées et optimisées pour permettre le criblage de différentes familles de contaminants dans cette matrice complexe. Tout d'abord, une méthode rapide, basée sur l'injection directe en HPLC-QTOF-MS a été développée et validée pour l'analyse ciblée de 7 médicaments vétérinaires dans le miel. Les médicaments vétérinaires sélectionnés ont été détectés à des niveaux 20-100 fois plus bas que les limites légales actuelles, avec des résultats excellents en termes de recouvrement, linéarité et répétabilité, et ceci pour un temps total d'analyse de 45 min par échantillon. Le mode d'acquisition indépendante des données *All-Ions MS/MS* a été utilisé pour enregistrer en continu des données MS et MS/MS pour quatre énergies de collision différentes. Ces données ont permis la confirmation de l'identité des analytes ciblés et ont démontré le potentiel non-ciblée de l'approche. Dans une seconde étude, l'impact du prétraitement des données sur l'identification non-ciblée des traces de contaminants organiques dans le miel a été étudié, ceci en utilisant les même 7 résidus vétérinaires comme composés modèles. L'impact de 7 paramètres a été évalué, et seule la fenêtre d'expansion pour l'extraction des chromatogrammes et la moyenne des scans inclus dans les spectres ont influencé de façon significative les résultats. Ces résultats montrent que les paramètres de prétraitement des

données peuvent affecter l'identification des contaminants traces dans les aliments. Le workflow d'identification optimisé fut ensuite appliqué pour cribler 55 échantillons de miel du marché canadien à l'aide d'une bibliothèque de 43 composés en lien avec le miel (incluant des médicaments vétérinaires, des pesticides et d'autres contaminants). La tylosine A et l'hydroxyméthylfurfural furent positivement détectés dans ces échantillons de miel. Par la suite, le workflow non-ciblé développé a été appliqué au criblage de composés associés aux plastiques dans 104 échantillons de miel du marché canadien. Au total, 662 composés possibles, issus d'une bibliothèque de composés associés aux plastiques, ont été détectés dans le miel. L'identité de deux de ces substances, à savoir l'adipate de bis(2-éthylhexyle) et le phosphate de tris(2-butoxyéthyl), a été confirmée avec étalons des composés purs. La consommation quotidienne estimée de ces substances à travers le miel fut calculée comme nettement en dessous des doses journalières tolérables. Les compositions chimiques des échantillons de miel vendus dans des pots en verre ou en plastique furent comparées à travers 3 approches de traitement de données différentes. Ces approches menèrent à différentes listes des composés pertinents. Ce résultat montre que les méthodes de traitements des données couramment utilisées en métabolomique par exemple doivent être sélectionnées prudemment quand elles sont appliquées à l'identification de contaminants traces dans les aliments. Parmi les composés isolés par fréquence de détection différentielle, six étaient uniques aux échantillons vendus dans des pots en plastique et trois étaient uniques aux échantillons vendus dans des pots en verre. Enfin, la dégradation thermale du médicament vétérinaire tylosine A a été étudiée avec la méthode non-ciblée optimisée, pour des solutions aqueuses, du miel dopé et du miel naturellement contaminé. Les résultats, en termes de taux de dégradation de la tylosine A et de production de tylosin B (le seul produit de dégradation identifié préalablement) correspondirent à ceux décrits dans la littérature. Pourtant, grâce à l'approche non-ciblée de cette étude, deux nouveaux produits de dégradation potentiels, à savoir OMT et lactenocine, ont été identifiés. Bien que le traitement thermal ait été le même dans toutes les expériences, les produits de dégradation dans les solutions aqueuses, le miel dopé et le miel naturellement contaminé étaient différents. Ceci confirme que les études des modèles aqueux ou les matrices alimentaires dopées ne sont pas

suffisantes pour comprendre la dégradation thermale des antibiotiques dans des matrices alimentaires. La possibilité de semi-quantification de la tylosine B dans le miel en utilisant la tylosine A, sa substance mère, comme référence a été aussi évaluée. Les résultats ont montré que la semi-quantification des produits de dégradation à partir de la substance mère est une stratégie raisonnable dans le cadre de l'analyse non-ciblée. En conclusion, cette recherche a montré, à travers le miel comme matrice d'étude, que l'approche non-ciblée améliore la caractérisation des contaminants chimiques et de leur devenir dans les aliments.

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When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

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CONTRIBUTION OF AUTHORS

This thesis is presented in a manuscript form and consists of seven chapters. Chapter 1 presents a general introduction to the field of non-targeted analysis applied to food and the research objectives of the thesis. In Chapter 2 a current and in-depth literature review is presented, which covers the various aspects of non-targeted analysis as well as the world of honey and its contaminants. Then, Chapters 3 through 6 are based on manuscripts and are bridged logically and sequentially through connecting paragraphs. Out of these four manuscripts, Chapter 3 has been accepted for publication in the Journal of Food and Drug Analysis, and Chapter 4 has been accepted for publication in the Journal of the American Society for Mass Spectrometry. Chapter 5 will be submitted for publication in the Journal of Agricultural and Food Chemistry, and Chapter 6 will be submitted for publication in Food Research International. Finally, Chapter 7 presents an overall conclusion of the thesis, along with contributions of this investigation to knowledge and recommendations for future research. This dissertation is in accordance with guidelines for thesis preparation as published by the Faculty of Graduate Studies and Research of McGill University.

The present author was responsible for the concepts, design of experiments, experimental work, data acquisition, data treatment and manuscript preparation in all the manuscripts. Dr Stéphane Bayen, the thesis supervisor, had direct advisory input into the work as it progressed and as manuscript co-author critically edited the dissertation prior to its submission. In Chapter 3 “Direct injection high-performance liquid chromatography coupled to data independent acquisition mass spectrometry for the screening of antibiotics in honey”, Daniel Furlong and Samareh Arooni collaborated with the sampling and sample preparation, and Fred Butterworth provided the honey samples from CFIA. Jean-François Roy and Dr. Jerry Zweigenbaum helped with the interpretation of data. All of them co-authored Chapter 3. Swaroopini Ramachandran co-authored Chapter 5 “Suspected-target screening for the assessment of plastic-related chemicals in honey” and helped with the sampling and sample extraction.

PUBLICATIONS

- A. von Eyken, S. Bayen; Optimization of the data treatment steps of a non-targeted LC-MS based workflow for the identification of trace chemical residues in honey. Accepted for publication in the Journal of the American Society for Mass Spectrometry (2019).

- A. von Eyken, D. Furlong, S. Arooni, F. Butterworth, J. F. Roy, J. Zweigenbaum, S. Bayen; Direct injection high-performance liquid chromatography coupled to data independent acquisition mass spectrometry for the screening of antibiotics in honey. Accepted for publication in the Journal of Food and Drug Analysis (2019).

CONFERENCE PRESENTATIONS

- A. von Eyken, S. Ramachandran, S. Bayen; Can good things come in small (plastic) packages? – Non-targeted multivariate comparison of honey in glass and plastic jars using direct injection-HPLC-QTOF-MS. 13th Montreal Post-ASMS Mass Spec Symposium. Pointe Claire, Canada, 16th October 2018. (Poster presentation)
- A. von Eyken, S. Bayen; Can good things come in small (plastic) packages? – Non-targeted multivariate comparison of honey in glass and plastic jars using HPLC-QTOF-MS/MS. 62nd International Conference on Analytical Sciences and Spectroscopy. Toronto, Canada, 11th – 14th June 2018. (Oral communication)
- A. von Eyken, S. Bayen; Non-targeted screening and identification of organic contaminants in honey using HPLC-QTOF-MS/MS. 62nd International Conference on Analytical Sciences and Spectroscopy. Toronto, Canada, 11th – 14th June 2018. (Poster)
- A. von Eyken, A. Baesu, S. Bayen; Analyse non ciblée de contaminants chimiques: comment caractériser l'inconnu? Colloque TOXEN. Montreal, Canada, 16th May 2018. (Oral communication)
- A. von Eyken, S. Bayen; Determination of the thermal degradation products of tylosin A in honey using an MS based foodomics approach. 8th International Symposium on Recent Advances in Food Analysis. Prague, Czech Republic, 7th – 10th November 2017. (Oral communication)
- A. von Eyken, R. Claudio, J. Li, S. Bayen; Comparison of extraction procedures to explore the fate of nicarbazin residues in eggs after cooking by using a non-targeted analysis workflow. 8th International Symposium on Recent Advances in Food Analysis. Prague, Czech Republic, 7th – 10th November 2017. (Poster)
- A. von Eyken, S. Bayen; Semi-quantification of tylosin residues in honey in a non-targeted workflow: using the known to quantify the unknown. 15th Instrumental Analysis Conference. Barcelona, Spain, 3rd – 5th October 2017. (Oral communication)

- A. von Eyken, J. F. Roy, J. Zweigenbaum, S. Bayen; Optimization of the data treatment steps of a non-targeted LC-MS based workflow for the identification of veterinary drugs in honey. 15th Instrumental Analysis Conference. Barcelona, Spain, 3rd – 5th October 2017. (Poster)
- A. von Eyken, S. Arooni, D. Furlong, J. F. Roy, J. Zweigenbaum, S. Bayen; Development of a fast screening and quantification method of seven antimicrobials in honey using direct injection in HPLC-QTOF-MS/MS. 29th Annual Tandem Mass Spectrometry Workshop. Lake Louise, Canada, 30th Nov – 3rd Dec 2016. (Poster)

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ABBREVIATIONS

AFB	American foulbrood
ANOVA	Analysis of variance
A_w	Water activity
BBzP	N-butyl benzyl phthalate
BPA	Bisphenol A
BPAF	Bisphenol AF
BPE	Bisphenol E
BPF	Bisphenol F
BPS	Bisphenol S
BPZ	Bisphenol Z
C_{AQ}	Concentration obtained through absolute quantification
CCD	Colony collapse disorder
CFIA	Canadian Food Inspection Agency
C_{SQ}	Concentration obtained through semi-quantification
DBP	Di-n-butyl phthalate
DCHP	Dicyclohexyl phthalate
DEET	Diethyltoluamide
DEHA	Bis(ethylhexyl) adipate
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DIBP	Diisobutyl phthalate
DIBP	Diisobutyl phthalate
DIPP	Diisopropyl phthalate
DMP	Dimethyl phthalate
EFB	European foulbrood

ESI	Electrospray ionization
FHWM	Full width at half maximum
GCxGC-EI-TOF-MS	Bi-dimensional gas chromatography coupled to electron ionization time-of-flight mass spectrometry
HMDB	Human metabolome database
HMF	5-hydroxymethylfurfural
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography coupled to diode array detector
HRMS	High resolution mass spectrometry
IDL	Instrument detection limit
IPs	Identification points
IT	Ion trap
LC-ESI-MS	Liquid chromatography coupled to electrospray ionization mass spectrometry
LC-ESI-MS/MS	Liquid chromatography coupled to electrospray ionization tandem mass spectrometry
LC-ESI-QTOF-MS	Liquid chromatography coupled to electrospray ionization quadrupole-time-of-flight mass spectrometry
LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LC-QTOF-MS	Liquid chromatography coupled to quadrupole time-of-flight mass spectrometry
LC-TOF-MS	Liquid chromatography coupled to time-of-flight mass spectrometry
LLE	Liquid-liquid extraction

LOD	Limit of detection
Log D or Log K_{OW}	Octanol-water partition coefficient
LOQ	Limit of quantification
MDL	Method detection limit
ME	Matrix effect
MRL	Maximum residue limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multiple-mass fragmentations/Multiple mass spectrometry
NCRMP	National Chemical Residue Monitoring Program
ND	Non-detected
NIAS	Non-intentionally added substances
NMR	Nuclear magnetic resonance
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PCDL	Personal compound database and library
PET	Polyethylene terephthalate
PPCPs	Pharmaceutical and personal care products
PRCs	Plastic-related compounds
PSA	Secondary amine exchange material
PVC	Polyvinyl chloride
QA	Quality assurance
QC	Quality control
QqLIT	Quadrupole linear ion trap
QqQ	Triple quadrupole
Q-TOF	Quadrupole time-of-flight

QuEChERS	"Quick, easy, cheap, effective, rugged and safe"
RE	Recovery
RF	Response factor
RfD	Reference dose
RSD	Relative standard deviation
RT	Retention time
S/N	Signal-to-noise ratio
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring
TBOEP	Tris(2-butoxyethyl) phosphate
TDI	Tolerable daily intake
TOF	Time-of-flight
TP	Transformation product
UHPLC	Ultrahigh-performance liquid chromatography
WRL	Working residue limit

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

The list of contaminants and toxins reported in food is continuously increasing, including new agrochemicals, emerging environmental pollutants and new food-contact material residues [1-3]. In this context, the single-analyte method approach relying on the use of pure analytical standards for each analyte has become obsolete for the broad surveillance of chemical contaminants in the food supply chain. To address this issue, a novel approach, called non-targeted analysis, has emerged with the objective to detect and identify multiple unknown or unexpected contaminants in food [4].

As it does not rely on the initial use of analytical standards, the non-targeted approach opens the door for new applications in the field of food authentication, such as the determination of botanical origin of samples [5], the verification of the production process (e.g. organic vs. conventional farming) [6], or the detection of food adulteration and chemical identity of food [7]. It also brings new perspectives in the field of food safety, such as the screening of new contaminants [8], the retrospective examination of data to detect additional compounds [9], or the determination of the degradation compounds of known contaminants [10], among others.

In view of the potential of the non-targeted approach, the chemical risk assessment community has highlighted the specific need to further develop non-targeted methods to better characterize human exposure to chemicals [11] and to identify potential risk compounds in food matrices [4]. In the field of food contaminants, we have identified that further knowledge is required to better optimize the steps of the non-targeted workflow, especially in terms of data pre-treatment, and to explore the wide range of data treatment available and their applicability to food contaminants.

The non-targeted approach can be virtually applied to any type of food matrix. Honey has been shown to contain a wide range of agricultural residues and environmental

contaminants at trace levels (in the order of $\mu g.g^{-1}$ to $ng.g^{-1}$) [12-14]. Since honey is produced and consumed worldwide, the monitoring of organic contaminants in honey can provide results relevant to both food safety and environmental pollution [15]. Recently, a few authors have reported the use of non-targeted approaches for honey analysis in applications such as the determination of botanical origin or the detection of contaminants [5, 16]. However, there are still several aspects of the non-targeted workflow that need to be further developed and optimized in order to overcome the challenges that working with trace organic contaminants in a complex food matrix represent. For example, we identified that the application of direct injection to liquid chromatography coupled to mass spectrometry (LC-MS) using a data-independent MS/MS acquisition for the non-targeted analysis of trace organic contaminants in honey has not been evaluated. Additionally, the application of non-targeted approaches for the study of the degradation of known honey contaminants during food processing has not been investigated. A semi-quantification approach to obtain quantitative information of such degradation products using their parent compounds as a reference has been suggested in other matrices [17], but not validated so far.

1.2 RESEARCH OBJECTIVES

The objective of this research was to develop non-targeted methods for the analysis of trace organic contaminants in honey and explore various applications of this approach for the surveillance of chemical residues in honey. Honey was used as a case study to improve our knowledge of the non-targeted analysis approach in food science. The specific objectives of this study were:

1. To develop and validate a method based on direct injection high-performance liquid chromatography coupled to data independent acquisition mass spectrometry for the targeted and non-targeted screening of veterinary drugs in honey.

2. To optimize the data pre-treatment steps for the non-targeted identification of veterinary drugs in honey, and to apply the optimized workflow to the screening of veterinary drugs, pesticides and other contaminants in honey.

3. To apply the previously developed non-targeted method to screen plastic-related compounds in honey and investigate the differences among honey products sold in glass and plastic jars, while providing recommendation on the application of a non-targeted data treatment to the study of trace chemicals in food matrices.

4. To characterize the behaviour of tylosin A in honey during heating and storage, to identify its degradation products using a non-targeted approach, and to assess the potential of non-targeted MS data for the semi-quantification of its degradation products.

CHAPTER 2: LITERATURE REVIEW

2.1 NON-TARGETED ANALYSIS OF FOOD CONTAMINANTS

New contaminants and toxins are constantly being reported in food, including new agrochemicals, emerging environmental pollutants and food-contact material residues [1-3]. In this context, the classical analytical design of one method and one standard for each analyte has become obsolete, the new trends being the detection and identification of currently unknown or unexpected contaminants through the use of non-targeted analysis. The chemical risk assessment community has highlighted the need to further develop non-targeted methods to better characterize human exposure to chemicals [11], and to identify potential risk compounds in food matrices [4]. In this section, the non-targeted analysis will be introduced and its application to the detection and the identification of organic contaminants in food, including their metabolites and transformation products, will be discussed.

2.1.1 Foodomics

Cifuentes *et al.* defined foodomics as "a discipline that studies the food and nutrition domains through the application of advanced omics technologies to improve consumer's well-being, health and knowledge" [18]. Cifuentes *et al.* introduced this new approach as the application of postgenomic tools, such as epigenomics, genomics, transcriptomics, proteomics and metabolomics, to solve the old and new problems of food analysis [18]. Although foodomics has many advantages and opens an infinity of new possibilities to study, it still needs to overcome some limitations. The analytical techniques used in foodomics have to face important difficulties, mainly derived from the high complexity of food matrices, the large number of bioactive food compounds, their wide range of concentrations, and the physico-chemical behavior of the various target analytes. The two main techniques currently used in foodomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) [19].

NMR is a non-destructive analysis method that provides fast, highly reproducible and quantitative detection of a broad range of products with ^{1}H atoms or any other atom with NMR active nuclei, such as ^{31}P, ^{15}N and ^{13}C. NMR can provide both qualitative and quantitative information which can be converted into biological information using multivariate data analysis [20]. For example, NMR can be used for the analysis of food toxicants, such as organophosphorus pesticides, and it has promising foodomics applications, e.g. in the field of phospholipidomics, a fast-growing area of research [21]. However, NMR has lower sensitivity compared to MS-based techniques, and it allows for the detection of only the most abundant compounds, while trace substances can remain undetected [22]. For this reason, MS provides several key advantages and is currently the leading technique for the analysis of organic contaminants in food matrices. The following sections will focus on the application of MS-based non-targeted methods.

2.1.2 Non-targeted analysis

Depending on the use of standards and the information known about the analytes, two types of analysis can be differentiated: targeted analysis and non-targeted analysis. The conventional targeted analysis approach relies on the development of a method based on analytical standards prior to the analysis of real samples [23]. This approach requires the definition of a list of selected compounds and is strongly dependent on the availability of pure standards of the targets. On the other hand, non-targeted analysis does not rely on standards. This approach, however, has to make use of advanced analytical techniques (e.g. MS) for the detection and the identification of the compounds [24]. Non-targeted analysis can be divided into suspect analysis and analysis of unknowns [25]. Both types of analysis are performed in the same way, the only difference between them being the fact that in suspect analysis the identity of the analyte is known, and so in many cases standards can be used for confirmatory purposes, while in unknown analysis the structures of the analytes are not defined.

Non-targeted approaches in food analysis are used in two main fields: food authentication and food safety. As the food supply chain becomes increasingly complex and global, food authentication is becoming one of the most growing fields in food science [26]. In this field, foodomics can be used in several applications such as the determination of geographical origin of samples, verification of the production process (e.g. organic vs. conventional farming), or the detection of food adulteration and overall chemical identity of food [5-7, 27]. In food safety, non-targeted analysis is used for the screening of contaminants, retrospective examination of data to detect additional compounds and determination of degradation compounds of known contaminants, among others [9, 10]. **Table 2.1.** summarizes some examples of applications of non-targeted approaches to food analysis in different matrices.

Table 2.1. Examples of applications of non-targeted approaches to food analysis.

Field of application	Food matrix	Description	Reference
Food authentication	Olive oil	Metabolic profiling to determine phenolic compounds of virgin olive oil	[27]
	Human milk	Detection of human milk adulterants	[7]
	Honey	Botanical discrimination of unifloral honeys	[5]
	Olive oil	Discrimination between olive oils from organic and conventional production	[6]
Food safety	Mussels	Determination of thermal degradation products of a veterinary drug	[10]
	Fruits and vegetables	Retrospective screening of pesticide metabolites	[9]
	Milk and fish	Screening of 143 veterinary drugs and pharmaceuticals	[8]

2.1.3 Non-targeted analysis workflow

Non-targeted analysis workflow can be divided into four main steps: sample preparation, instrumental analysis, data pre-treatment and data treatment (**Figure 2.1**). The following sections focus on the characteristics and challenges of each of these four steps.

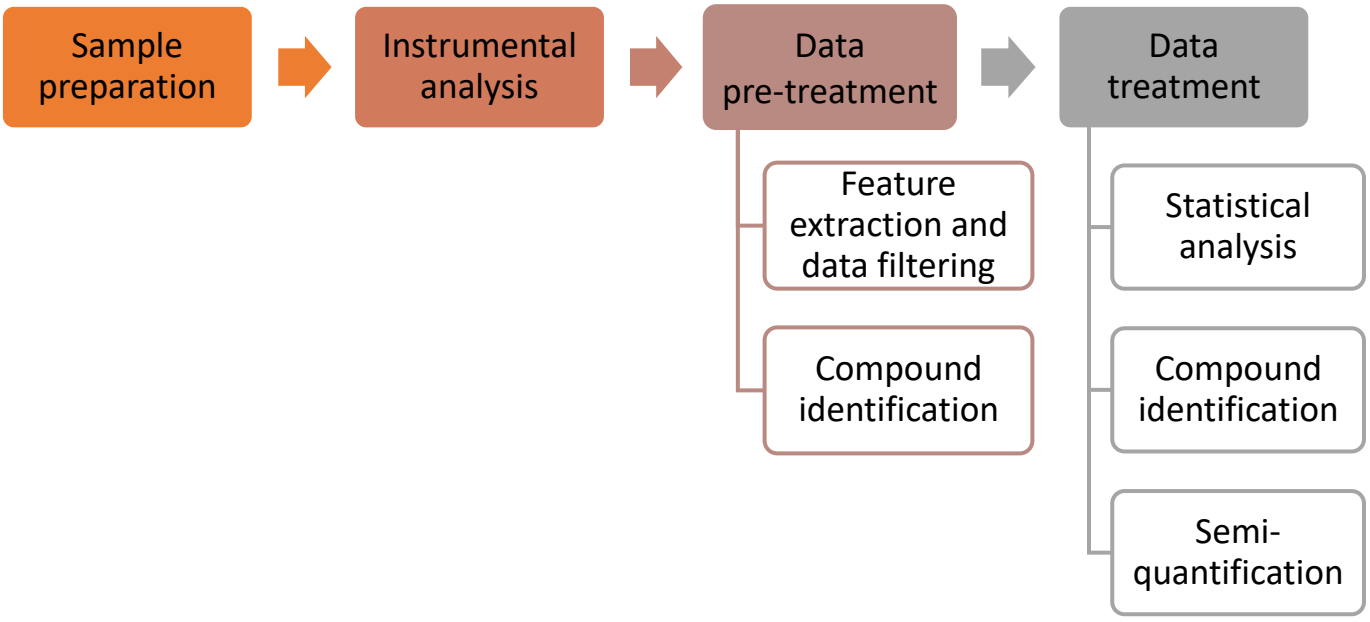

Figure 2.1. Non-targeted analysis workflow

2.1.3.1 Sample preparation

Traditionally, sample preparation in targeted analysis involves a series of extraction and clean-up steps designed to separate the target analytes from interferences [28]. These steps are generally time-consuming and resource intensive. Besides, these steps are likely to eliminate other compounds whose analysis could prove decisive in current or future assessment of the food sample (e.g. presence of other contaminants, chemical tracers, metabolites, etc.). For this reason, non-targeted analysis tends to eliminate or minimize the sample preparation in order to increase the number of potential analytes.

Since no standards are used, and in many cases the analytes are unknown, the goal of sample extraction optimization in non-targeted analysis is to be able to extract the maximum number of analytes possible. For example, Theodoridis *et al.* developed a non-targeted LC-MS based method for global metabolite profiling of grapes, using a combination of liquid-liquid extraction (LLE) and solid-phase extraction (SPE) with highly cross-linked

hydroxylated polystyrene-divinylbenzene copolymer cartridges [29]. For the determination of the optimal solvent mixture for the LLE, an experimental design consisting of 22 points with different composition of water, methanol and chloroform was built, with the goal of determining the optimal solvent mixture in terms of the number of features extracted after data processing, robustness and extraction efficiency [29]. Although this example was done in the field of food metabolomics, the same approach to extraction optimization would be applied in non-targeted analysis of food contaminants.

A type of sample preparation that has gained popularity in food analysis in recent years is the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach. These procedures usually include single-phase extraction of sample with a suitable solvent, followed by the removal of water and further clean up and purification [4]. QuEChERS methods have the combined advantage of having the ability to recover a broad range of chemicals while being simple and cost effective [30]. Because they can extract analytes with very different physico-chemical properties, these approaches have become a common sample treatment method in non-targeted analysis. An example of the application of QuEChERS methods for the non-targeted analysis of food is presented in the screening of pesticides in fruits and vegetables by Mezcua *et al.* [31].

Another sample preparation approach introduced for the fast analysis of trace compounds in environmental and food matrices, which solves the problem of analyte removal during the clean-up, is direct injection. This sample preparation strategy, also known as “dilute and shoot”, is based on the direct introduction of the sample into the instrument, with only a previous dilution in solvent depending on the nature of the sample. For example, Bayen *et al.* applied direct injection to liquid-chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) for the analysis of pharmaceuticals and endocrine-disrupting chemicals in mussels and clams [32]. This “cleanup-free” approach, which relied on a simple solvent extraction with acetonitrile/methanol (50:50), was made

possible using isotopically labeled surrogates to correct for matrix effects. A post-column switch on the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) system that diverted the first minutes of elute to the waste was used to remove potential interferences [32]. Olmo-García *et al.* developed a method for metabolic profiling of phenolic compounds in olive oil using direct injection LC-ESI-MS [27]. In this case, the sample preparation was reduced to the dilution of olive oil in acetone, and the method was successfully validated and applied to the quantification of 21 phenolic compounds without any other extraction step. "Dilute and shoot" approaches have also been used in honey for the targeted determination of pesticides, veterinary drugs and other trace contaminants in honey prior to liquid chromatography coupled with time-of-flight MS (LC-TOF-MS), Orbitrap MS, and triple quadrupole MS [33-38]. This approach has the advantage of being much faster and less resource intensive than other extraction methods, while avoiding the removal of any potential analytes.

2.1.3.2 Instrumental analysis

In the last decades LC-MS/MS has become one of the fundamental techniques for the analysis of food toxicants. As described below, various mass analyzers can be used to determine organic contaminants in food matrices. For this type of application, full-scan high sensitivity, high resolution, accurate-mass measurements and multiple-mass fragmentations (MS^n) are needed [36].

Low resolution mass analysers have been defined as those with a resolving power below 10,000 with a mass accuracy above 5 ppm, while high resolution MS have resolving power above 10,000 with a mass accuracy below 5 ppm [37]. Kellmann *et al.* studied the effect of using different resolving power settings on the analysis of several contaminants in honey and animal feed [38]. The authors spiked their samples with 151 pesticides, veterinary drugs, mycotoxins and plant toxins at levels ranging from 10 to 250 $ng.g^{-1}$, and the analyses were performed using a single-stage Orbitrap with resolving power from 10,000 to 100,000.

According to their results, in the case of honey a resolving power of 25,000 was sufficient to obtain a mass assignment error close to the typical instrument mass accuracy (< 2ppm) even at the lowest levels of concentration. However, during the analysis of animal feed, which are more complex matrices, a higher resolving power (> 50,000) was required. In this case, it was found that at lower resolving power the error in the assignment of mass increased due to the co-elution of analytes with interferences at the same nominal mass. In the same line, Nielen *et al.* proposed that, during the analysis of veterinary drugs, for the confirmation of postulated structures of unknown hormones and β-agonists a resolution of at least 70,000 was required [39]. According to the authors, this resolution provides reliable elemental compositions of product ions differing in one CO, C_2H_4 or N_2 substructure up to *m/z* 400. Both studies demonstrate that, for this type of analysis, high resolution techniques are needed.

A summary and comparison of mass analyzers used to determine food contaminants and their metabolites and degradation products, including their pros and cons as well as applications as reported by Picó *et al.*, is presented in **Table 2.2**. The conclusion of this comparison is that the only MS techniques suitable for the non-targeted analysis of known and unknown food contaminants, that is, with enough mass accuracy, mass resolution, full-scan sensitivity and compatible with multiple MS^n, are quadrupole-time-of-flight (Q-TOF) and Orbitrap [36].

Among the ionization sources available for MS, electrospray ionization (ESI) is the method of choice for interfacing LC to the mass spectrometer [40]. Its popularity is due to its high sensitivity and ability to analyze large, non-volatile, chargeable molecules such as proteins and nucleic acids. However, it is also widely used for the analysis of small molecules such as drugs, natural products, pesticides and carbohydrates [40]. Ions are generated in ESI by application of a potential to an ESI emitter through which a solution containing the analytes is infused [41]. The solution emerges from the capillary as a fine spray with excess charge

evenly distributed on the surface of the droplets and, as the droplets travel towards the entrance of the mass spectrometer, the solvent evaporates causing an increase in the surface charge density on the droplet. Then, as evaporation occurs, the surface charge density increases to the point at which Coulombic fission occurs and charged offspring droplets are generated [41]. Finally, according to the desorption model proposed by Iribarne *et al.*, the electric field at the droplet surface provides the work required to lift the analyte ion from the droplet surface to form a gas-phase ion that will enter the mass spectrometer [42]. When ESI is used, the ions most commonly detected are the pseudomolecular ions $[M+H]^+$ and $[M-H]^-$ in positive mode and negative mode, respectively [43]. However, the formation of other adducts such as $[M+Na]^+$, $[M+K]^+$ or $[M+NH_4]^+$ is possible depending on the analyte and the solvent in which it is dissolved [33, 44].

Table 2.2. Comparison of mass analyzers used to determine metabolites and degradation products of food contaminants [36].

System	Pros	Cons	Applications
QqQ	• High sensitivity in single reaction monitoring (SRM) • Wide linear dynamic range	• Incompatible with high-speed separation • Difficult to determine a large number of compounds simultaneously (over 100) • Low sensitivity when using full-scan modes	• Unambiguous identification and quantification of target metabolites and degradation products of food contaminants in complex matrix samples
IT	• MS^n capabilities • Full-scan high sensitivity	• Low accurate nominal mass • Low mass resolution • Medium sensitivity in SRM • Narrow linear dynamic range	• Screening of a large number of targets without loss of sensitivity • Identification of unknown peaks based on structural information obtained from the fragmentation patterns • Data can be reprocessed a posteriori

QqLIT	• MS^n capabilities with high sensitivity in SRM • Full-scan high sensitivity • Wide linear dynamic range	• Low accurate nominal mass • Low mass resolution	• Unambiguous identification and quantification of target metabolites of food contaminants in complex matrix samples • Screening of large number of targets without loss of sensitivity • Identification of unknown peaks based on structural information obtained from the fragmentation patterns • Data can be processed a posteriori, if acquired in full scan
TOF	• High mass accuracy (1-3 ppm) • High mass resolution (10,000 at full width at half maximum (FHWM))	• Small linear dynamic range	• Screening of a large number of targets without loss of sensitivity • Identification of unknown peaks based on accurate mass and isotopic profile evaluation • Data can be reprocessed a posteriori

QTOF	• High mass accuracy (5 ppm) • High mass resolution (45,000 FHWM) [45] • Full-scan high sensitivity • Compatible with high speed separations • MS^2 capabilities	• Relatively small linear dynamic range	• Screening of a large number of targets without loss of sensitivity • Identification of unknown peaks base on accurate-mass and isotopic profile evaluation • Data can be reprocessed a posteriori • Elimination of structural ambiguities of the compounds
Orbitrap	• Superior mass accuracy (1-2 ppm) • Outstanding mass resolution (200,000 FHWM) • Wide linear dynamic range • Compatible with high-speed separations • MS^n capabilities	• Very expensive	• Screening of a large number of targets without loss of sensitivity • Identification of unknown peaks based on accurate-mass and isotopic profile evaluation • Data can be reprocessed a posteriori • Definitive structural characterization • Reliable elemental compositions of product ions differing in one CO, C_2H_4 or N_2

2.1.3.2.1 Q-TOF in food analysis

Due to its high mass accuracy and mass resolution, as well as full-scan high sensitivity and MS^2 capabilities, liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) is one of the major LC-MS platforms for non-targeted analysis and characterization of unknowns, including parent contaminants or their transformation products in food [46]. An example of the application of this technique was reported in 2012 by Díaz *et al.* in the study of organic contaminants, residues and illicit substances in waste water, human urine and food samples [47]. In order to increase the identification capabilities of this technique, an MS^E mode consisting in two acquisitions functions (a low energy function at 4 V and a high energy function with a collision energy ramp ranging from 15 to 40 V) was used. By using this configuration, the protonated (or deprotonated) molecules can be obtained with the low energy function, while the high energy function promotes fragmentation, improving the identification of the compound. As a result of this non-targeted screening, two fungicides were identified in orange samples, one fungicide and one insecticide were identified in banana peel samples, and two mycotoxins were identified in corn samples [47].

More recently, Dasenaki *et al.* reported another example of the application of LC-QTOF-MS, in this case in a screening method for 143 veterinary drugs and pharmaceuticals in milk and fish tissue [8]. This method also worked with two different collision energies but, in this case, the high collision energy was 25 V. According to the authors, at low collision energy all the ions from the preselected mass range, which in this case was *m/z* 40-1000, are heading toward the flight tube without isolation at the quadrupole, and thus there is no collision-induced dissociation at the collision cell. At high collision energy, no isolation is taking place at the quadrupole, and the ions from the preselected mass range are fragmented in the collision cell, creating the MS/MS spectrum [8]. When identifying the non-targeted compounds, the low collision energy experiment provided the authors with

the MS spectra and chromatograms, and thus it was used for the retention time and the molecular formula and mass accuracy of the precursor ions and adducts. Then, the high collision energy experiment provided the MS/MS spectra and chromatograms were obtained, therefore information on the molecular formula and mass accuracy of the fragment ions were obtained and used for the final identification of the analytes [8].

In addition, this technique allows for the retrospective analysis of contaminants using already acquired data without the need for additional sample injection. Herrera-López *et al.* monitored the transformation products (TPs) of several organic contaminants in waste water using liquid chromatography coupled to electrospray ionization quadrupole-time-of-flight mass spectrometry (LC-ESI-QTOF-MS) as well as bi-dimensional gas chromatography coupled with electron ionization time-of-flight mass spectrometry (GCxGC-EI-TOF-MS) [48]. Four omeprazole metabolites were successfully identified, proving that QTOF, together with an adequate data treatment, is an effective tool for the determination of TPs of organic compounds [48].

2.1.3.2.2 Orbitrap in food analysis

Despite being such an expensive technique, Orbital trap mass detectors, also known as Orbitrap, have been used in a wide range of food analysis applications. It has been reported in monitoring of pesticide residues, veterinary drug residues, natural toxins (including mycotoxins), process contaminants, environmental contaminants, food allergens and also in the identification of adulteration in food and beverages [49].

Several examples of the application of this technique in the field of food contaminants can be found. Jia *et al.* developed an analytical method for simultaneous analysis of 333 pesticide and veterinary drug residues in baby food using Ultrahigh-performance liquid chromatography and electrospray ionization quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-ESI-Q-Orbitrap) [50]. In this study, 258 pesticides and 75 veterinary

drugs were selected, including gestagens, macrolides, androgens, quinolones, non-steroidal anti-inflammatory drugs, tetracyclines, ionophores, sulphonamides, corticoids, avermectines, tranquilisers, nitroimidazoles, amphenicols, coccidiostats, β-agonists and penicillins. For the quantitative part of the study, data were acquired using full MS scan mode. For confirmatory purposes data-dependent MS/MS scans were performed. In this mode, precursor ions for the target compounds were sent to the collision cell of the Q-Orbitrap, where mass resolution was set at 17,500 FWHM (*m/*z 200), and they were fragmented with a normalized collision energy of 35% to obtain MS/MS spectra [50]. According to Jia *et al.*, using this configuration coeluting compounds from the matrix and noisy peaks can be easily excluded, thus facilitating the identification and quantification of both known and new analytes in a single run analysis.

2.1.3.3 Data pre-treatment

Non-targeted analysis of food matrices usually results in relatively large datasets which require a data pre-treatment step in order to reduce the list of peaks and be able to identify the generally small signals of the compounds of interest [51]. This is a critical step of the non-targeted workflow, because any errors at this stage would change the list of features detected and available for data treatment, which could potentially have a great impact in the final results of the non-targeted analysis. The data pre-treatment consists mainly in a feature extraction and data filtering step which can be followed by an identification of compounds depending on the nature of the study and availability of spectral libraries.

2.1.3.3.1 Feature extraction and data filtering

The feature extraction LC- or GC-MS non-targeted analysis data, performed previous to data treatment, is a multistep process. First, the whole chromatogram needs to be examined in order to create a compound list of all peaks that represent real molecules [52]. During this process, detected ions are assigned a monoisotopic peak with a corresponding *m/z* value,

and an isotopic distribution analysis is performed to identify isotopic clusters that might be characteristic of certain elemental compositions. Next, the presence of adducts that may be associated with the eluting compounds is assessed based on a list of potential adducts specified beforehand [53]. Finally, if the mass accuracy is sufficient and there is a minimal isotopic distribution error, the correct molecular formula can be generated for the compounds of interest [54, 55].

An example of data filtering in non-targeted analysis of food contaminants is the work of Herrera-López *et al.*, which studied the TPs of six structurally different compounds selected from a list of 122 chemicals, including PPCPs, pesticides and PAHs [49]. In the case of omeprazole, for example, a wastewater sample contained originally 7,643 features that, thanks to the application of a mass filter around the mass of the parent compound of 5 and 10 ppm for MS and MS/MS data, respectively, were reduced to 567. Further data treatment led to only 43 compounds considered for further identification based on elemental composition simulation and interpretation of the MS/MS spectra. At the end, four omeprazole metabolites were successfully identified [49]. Thus, this paper illustrates the need for a data-filtration step in non-targeted analysis in order to reduce the large list of signals obtained initially to a more manageable number of features.

Data filtering includes a succession of steps with several parameters affecting the results of each step. Examples of these parameters are exact mass tolerance, *m/z* extraction window, retention time tolerance and peak height filter [56]. In metabolomics, the need for identifying the parameters that have the main impact on number and quality of reported metabolites has been already recognized [57]. However, to-date there has been no systematic assessment of the influence of any other parameters of data filtering processes other than the exact mass tolerance on the identification of trace contaminants in food.

2.1.3.3.2 Compound identification

The data filtering steps take the large list of signals recorded for all the samples and reduce it to a list of the molecular formulas of the compounds of interest. Then, if a compound database is available, further identification of these compounds can be carried out. To do so, mass spectral similarity between an experimental mass spectrum and each mass spectrum in a reference library is calculated based on various parameters [58]. However, even in a specific database, several compounds can be associated with a single molecular formula. To overcome this issue, acquisition methods using data-dependent or data-independent MS/MS can provide some information (exact mass, isotopic patterns) for both the parent ions and their fragments that can be used to further confirm the correct identification of the target compounds [59]. The use of MS/MS spectra for identification of compounds has, however, some limitations. Generally speaking, fragments present lower intensity and accurate mass than the protonated molecule (parent compound). In the field of trace contaminant analysis, where the intensity of the signal of the parent compound is already very low, the fragments obtained can end up being too small to provide reliable information for identification [52]. In addition, different types of HRMS instruments (i.e. QTOF vs. Orbitrap) can present differences in performance that affect several of the parameters used for identification, such as the accuracy of the relative isotopic distribution at a given monoisotopic peak intensity [54]. Ultimately, this could affect the non-targeted identification of compounds if the instrument used to build the database is different from the instrument used during the analysis.

Another parameter that can be used when screening against a compound database, besides the MS or MS/MS spectra information, is retention time. Several examples of the use of retention time in database screening exist in the literature [8, 31, 44]. Retention time, however, depends strongly on the specific chromatography conditions of the experiment, so small differences between the chromatographic method used to build the database and

the one used during the non-targeted analysis can have a great impact in the correct identification of compounds. For this reason, although it can be a very useful parameter when using an in-house built library, it is not recommended to rely on retention time when using a commercial library.

Although in many cases in-house or commercial databases are used, online mass spectral libraries are also useful tool for small molecule identification in environmental monitoring, forensic science, food analysis and metabolomics, among others [49]. These repositories allow investigators to compare their MS/MS data to those recorded using pure standards and catalogued in the database. The use of these libraries improves the speed, efficiency and cost effectiveness of non-targeted analysis [49]. The online free MS databases being currently most used are MassBank, Human Metabolome Database (HMDB) and METLIN [60-62]. MassBank is the public repository of mass spectra of chemical compounds smaller than 3000 Da analyzed using optimized up-to-date chemical methods. Each spectrum is complemented by a description of the instrument used as well as its analytical parameters, such as the method of ionization, the type of ion analyzer, the ionization voltage, the matrix in which each compound is analyzed and the collision energy conditions for the MS^n measurement. The information presented for each compound includes a peak list *m/z* data in high and/or low resolution and its relative or real intensities [60].

HMDB is a database that contains information about small molecule metabolites found in the human body, as well as drugs and drug metabolites, toxins and environmental pollutants, food components and food additives [61]. This library presents quantitative chemical, physical, clinical and biological data about more than 40,000 compounds, which are either *detected* metabolites (those with measured concentrations or experimental confirmation of their existence) or *expected* metabolites (those for which biochemical pathways are known or human intake/exposure is frequent, but the compound has yet to be detected in the human body) [61].

METLIN is also a metabolite database [62]. It is principally intended to be used in metabolomics approaches, although it is not limited to this field. METLIN contains over 64,000 structures of pharmaceuticals and their metabolites, pesticides, human and animal endogenous compounds, polyphenols and plant metabolites, among others, and it incorporates more than 59,000 high-resolution MS/MS spectra [63].

Depending on the nature of the study, compound identification can be performed as part of the data pre-treatment in order to reduce the list of compounds to consider for the data treatment to a specific family of contaminants. However, compound identification is not exclusive to pre-treatment steps. It can also be done later in the data treatment to identify a compound that has been found to be relevant for the study. In that case, pre-treatment generally consists only in data filtering to obtain molecular formulas, and the identification workflow is applied once the compound of interest has been found. The general description of the identification process presented in this section is therefore also applicable for the identification of compounds during the data treatment.

2.1.3.4 Data treatment

As seen in **Table 2.1.**, non-targeted analysis has a wide range of applications in food analysis, and each of them will require a different data treatment approach depending on the objectives of the study. In most cases, data treatment will include some type of statistical analysis in order to determine the compounds relevant to the study. Then, these compounds will be identified, if this was not previously done during the data pre-treatment, and in some cases a semi-quantification approach will be applied in order to obtain some quantitative information.

2.1.3.4.1 Statistical analysis

The great amount of data generated in foodomics often becomes too big to be effectively evaluated by conventional univariate approaches and requires multivariate analysis, which are pattern recognition methods that use all measured variables simultaneously and are able to identify underlying latent factors that might carry important information [22]. These methods are very sensitive to the noise and non-sample related variations that could lead to misinterpretations of the results, reason why a previous data filtering step is needed.

Once the data has been filtered, the chemometric analysis begins. There are several multivariate analysis methods that can be applied to data sets, and in all of them the main objective is to make use of all the variables present in the samples simultaneously and establish the relationship amongst them. Some of the multivariate data analysis used in foodomics include unsupervised data analysis, such as Principal Component Analysis (PCA) and Hierarchical Cluster Analysis, and supervised methods such as Partial Least Squares Regression, Partial Least Squares Discrimination Analysis, Soft Independent Modelling of Class Analogies and Extended Canonical Variables Analysis. Additionally, other more advanced data analysis methods can be used, such as ANOVA Simultaneous Component Analysis (ASCA), Multi-block Analysis, Sparse PCA and Parallel Factor Analysis [22].

Some other examples of widely used data treatment strategies in non-targeted analysis of food, besides multivariate analysis, include unique entity analysis and volcano plot. Unique entity analysis usually searches for compounds that are present in 100% of the samples of one group and absent in 100% of the samples of the other group [64]. The volcano plot is a widely used statistical tool in metabolomics to detect compounds whose intensities are significantly different among two groups of samples by combining a Student t-test and a fold change calculation [7, 65].

2.1.3.4.2 Quantification in non-targeted analysis

Although great advances have been done in the identification of non-targeted substances in food matrices, to date providing quantitative information in a non-targeted workflow in the absence of a standard remains a challenge because of the lack of a known response factor of the analyte [66, 67]. In this context, the concept of semi-quantification is introduced as a way of obtaining some approximate quantitative values in the absence of the standards needed for a true quantification. The three main approaches adopted by different authors are semi-quantification based on *in silico* ionization prediction [68, 69], semi-quantification based on chemical similarity [6, 70, 71] and semi-quantification based on similar chromatographic behaviour [72].

The ionization process in ESI-MS is dependent on many physicochemical and thermodynamic properties of the analytes, such as their nonpolar surface area, free energy of solvation, octanol-water partition coefficient (log *D*), LC retention time and gas-phase proton affinities [40, 41, 73-75]. For this reason, some authors made the hypothesis that the response factor of the analytes could be predicted by these properties, which could then be used to *semi-quantify* analytes in non-targeted studies. Chalcraft *et al.* built a model to predict the relative response factor of low-abundance metabolites based on their molecular volume, log *D* and absolute mobility [68]. Later, Wu *et al.* developed a quantitative structure-ion intensity relationship in LC-MS for the semi-quantification of 25 organic acids by incorporating both the intrinsic structural properties and solvent factors as parameters in the model [69]. Regarding the semi-quantification approach using chemical similarity, Wang *et al.* developed a semi-quantification method for alkaloids in plant extracts based on the idea that molecules with common chemical moieties would produce common fragment ions in MS/MS [70]. Alkaloids were classified into two groups based on their structure and then they were quantified using a reference standard corresponding to each group, achieving a semi-quantification accuracy ranging 103.36 to 105.01%. Along the

same lines, Bu *et al.* semi-quantified organic contaminants in river sediments using a representative standard for each group of compounds, and calculated concentrations differed from the actual measurements by a maximum factor of 4 for most of the analytes [71]. Kalogiouri *et al.* successfully developed a model for the semi-quantification of phenolic compounds in olive oil identifying the most appropriate standard using chemical similarity analysis [6]. Following a semi-quantitative approach using similarity of chromatographic behaviour, Pieke *et al.* compared two strategies to select internal standards for the non-targeted analysis of a wide range of contaminants leaching from food contact material, based on either similar retention time or similar exact mass, and found that the quantification error was minimized when using retention time to choose the standard for quantification [72].

In the case of degradation products of known contaminants, some authors support the idea of a semi-quantification using the parent compound as the internal standard in the quantification of its degradation products. This method has been applied for the quantification of the degradation products of chlortetracycline and demeclocycline in agricultural soils and drainage waters [17]. Although this approach seems to give satisfactory results, to date no validation of this semi-quantification approach for degradation products of organic contaminants has been reported.

2.2 HONEY

According to the Codex Alimentarius Commission, honey is “the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transformed by combination of specific substances of their own, deposited, dehydrated, stored and left in the honey comb to ripen and mature” [76]. It is a natural sweetener produced and consumed worldwide, used as a food and medical product since the earliest times [77]. The following sections present an overview of the history, manufacturing process, chemical composition, physicochemical and medical properties of honey, before focusing on the main families of organic contaminants that are found in this food matrix and their analysis.

2.2.1 Honey then and now

As the only available natural sweetener, honey was an important food for *Homo sapiens* from his very beginnings [77]. The oldest record interpreting a man harvesting honey was found in Bicorp (Spain), dating back to 7000 BC, and it depicts a man using a ladder to reach a hive, carrying a container to hold combs, and workers buzzing around his head [78]. Those primitive honey hunters would eat the comb as it was broken off from the nest, containing wax, honey, pollen and bee brood, which would provide them with a sweet and nutritious mixture of carbohydrates and proteins [79]. Thousands of years later began the development of beekeeping and controlled honey production. The earliest known record of keeping bees in hives and harvesting honey from them dates from 2400 BC, in Egypt. These early beekeepers would use techniques similar to honey hunting, with the exception that the bees were in mobile hives that the beekeeper could move and place wherever it was more suitable [79]. Honey played an important role in Ancient Egypt, as part of their spiritual, social and economic life. Carvings found in temples and sarcophagi, and papyri

show that most medicines from that era contained honey, and that Ancient Egyptians commonly drank beer made of wheat, barley and honey [78]. The first written reference to honey, recorded on a Sumerian tablet dating back to 2100-2000 BC, mentions the use of honey as a drug and an ointment [80]. Honey appears also in Hebrew literature, which describes the promised land as a place flowing with milk and honey [78]. Indeed, there are several mentions of honey in the Old and New Testaments, such as the quote from King Solomon "Eat honey my son, because it is good" (Old Testament, proverb 24:13). Honey was also highly valued by the Ancient Greeks, who believed that it abolished fatigue and used to give it to athletes before major events [78]. During the Middle Ages, in Central and Northern Europe honey was recognized as the "elixir of life", it was attributed miraculous properties and was used in surgery and burn therapy because of its healing powers [78]. Until the general spreading of sugar production and consumption in Europe in the 18th century, honey remained the main sweetener and therefore it was widely harvested and commercialized both as a food and for its medicinal properties [79].

Despite the worldwide decline in bee colonies observed in the recent years, the beekeeping sector in Canada has increased over the past decade, reaching the record of 789,598 bee colonies and 10,544 beekeepers in 2017 [81]. Most of Canadian honey is produced in the Prairies, Alberta being the most important honey producer with 43% of the total Canadian production, followed by Saskatchewan (24%) and Manitoba (19%). In terms of beekeepers, however, Ontario leads the ranking with 32% of Canadian beekeepers, followed by British Columbia (25%) and Alberta (13%) [81]. The estimated yearly average consumption of honey per person in Canada was 0.85 kg in 2017 [81].

In terms of trade, around 40% of Canada's honey production is exported. The major Canadian honey importer is the United States, which represents 79% of the Canadian honey exports, followed by Japan (16%) and China (3%) [81]. Canada also imports honey from other countries for a total value of 41 million Canadian dollars in 2017, Brazil (18%), Spain

(11%) and Mexico (10%) being the main sources of honey imports in terms of quantity of honey [81].

Regarding the honey sector worldwide, China, New Zealand and Argentina are the top 3 exporters of honey, while the United States, Germany and Japan are the top 3 importers of honey [81].

2.2.2 The process of honey production

The production of honey starts with the foraging honey bee flying out of the colony to collect nectar, which is the source of carbohydrates for the bees and basic ingredient of honey [82]. Nectar is a sweet material produced by flowering plants which is mainly composed of sugars. When the forager finds the nectar of the flower, she takes it and stores it in her honey sac, a widened part of her alimentary canal which contains a valve to prevent the nectar passing through the digestive system [79]. The foraging bee will then visit other flowers to keep collecting nectar until she fills her honey sac, which can contain a load of nectar of 25 to 70 mg, in a foraging trip that can last from 25 min to 2.5 h and that usually covers a distance of 1 to 3 km from the hive [78]. In one day, the forager will generally do between 3 and 24 trips depending on the weather and the nectar flow and can fly a distance of up to 12 km from the hive if necessary. As a general rule, bees forage on specific plant species and less than 3% of bees carry mixed pollen or nectar back to the hive, although the foraging range widens as pollen and nectar become scarce [82]. Weather conditions have a direct impact on the foraging activities. Bees will generally not forage for nectar at temperatures below 13˚C or above 43˚C, although at higher temperatures they will still go out for water. Regarding wind, the foraging bees will stop gathering nectar at winds of about 24 km/h, the speed at which they normally fly [82].

Inside the honey sac, the nectar is diluted with saliva containing secretions from several glands, especially the hypopharyngeal glands, which contribute the enzymes used later

during the elaboration of honey: invertase, diastase and glucose oxidase [79]. Once the foraging bee arrives at the hive, she will retain her nectar load until she encounters one or more house bees willing to take the food from her. She then regurgitates the nectar load and passes it to the home bees that will ingest it, cleans her proboscis from any nectar residues and stays inside the hive for some minutes to eat and rest before flying out to collect more nectar [78]. The home bee now moves to a less crowded area of the hive and begins a series of manipulations with her mandibles and proboscis to actively evaporate moisture and blend the nectar with the enzyme invertase in a process that can last up to 20 min. After that, she deposits the unripened honey into a cell by crawling in, regurgitating the contents of her honey sac and painting this processed nectar on the upper side of the cell by using her proboscis as a brush [78]. This honey, which can initially have a moisture content of up to 95%, rests inside the open cell until the moisture drops to 20%, when worker bees will seal the cell with a thin layer of wax, providing it with an air-tight cover. The speed of the process, which generally takes from 2 to 5 days but can be delayed to more than 21 days in some cases, depends on the degree to which the honey-containing cells are filled, the initial moisture of the nectar and the ventilation of the hive [79].

The process of honey manufacture, from the hive to the final product, depends largely on the scale of the operations (i.e. small quantities of honey for personal consumption of the beekeeper vs. large scale bottling in industries). There are however a number of basic steps that are common to all scenarios. First, the honeycombs are extracted trying to minimize the impact on the hive [78]. In some cases of honey with a particularly high moisture content (e.g. rape or chestnut honey), a dehumidification step is needed to lower the humidity to 18.5-18.0%. In this case, honeycombs are placed in a very dry and warm room in order to lower the moisture content of honey before extraction [83]. Then, the layer of wax that seals every cell in the honeycomb is removed by a process called uncapping, which can be done either manually with a heated knife in small-scale operations or with partially automated uncapping machines in larger-scale facilities [78]. Uncapped combs are then

placed in the honey extractor, where honey is removed by centrifugation and collected at the bottom of the extractor. This raw honey is then transferred to a heated tank, usually at a temperature between 37 and 49˚C, where wax residues and other extraneous matter will float to the surface and will be easily removed [78]. At this point, honey can be directly filtered and bottled or, alternatively, an additional heating process can be applied before filtration. If no heating step is used and honey is directly filtered and bottled, the final product is raw or unheated honey [83]. Otherwise, there are several options for this after-extraction heating process that will depend on the scale of production and the type of final product aimed to obtain [78]. One of the most common heating processes is honey pasteurization, which takes place when temperature is raised up to 72˚C, maintained for about 120 s, and then rapidly cooled [84]. The goal of honey pasteurization, contrary to the majority of food products, is not for food safety purposes but to avoid crystallization and prolong the liquid state of honey [83]. The full process of honey production, from the nectar to the bottles, is summarized in **Figure 2.2**. Additionally, it should be noted that, in Canada, the production of organic honey differs from that of regular honey in that no synthetic veterinary drugs can be used on the bees, the crops honey bees feed from need to be free of synthetic pesticides and genetically modified organisms, and the environment in which the honey bees live and feed should be as free from environmental pollution as possible [85].

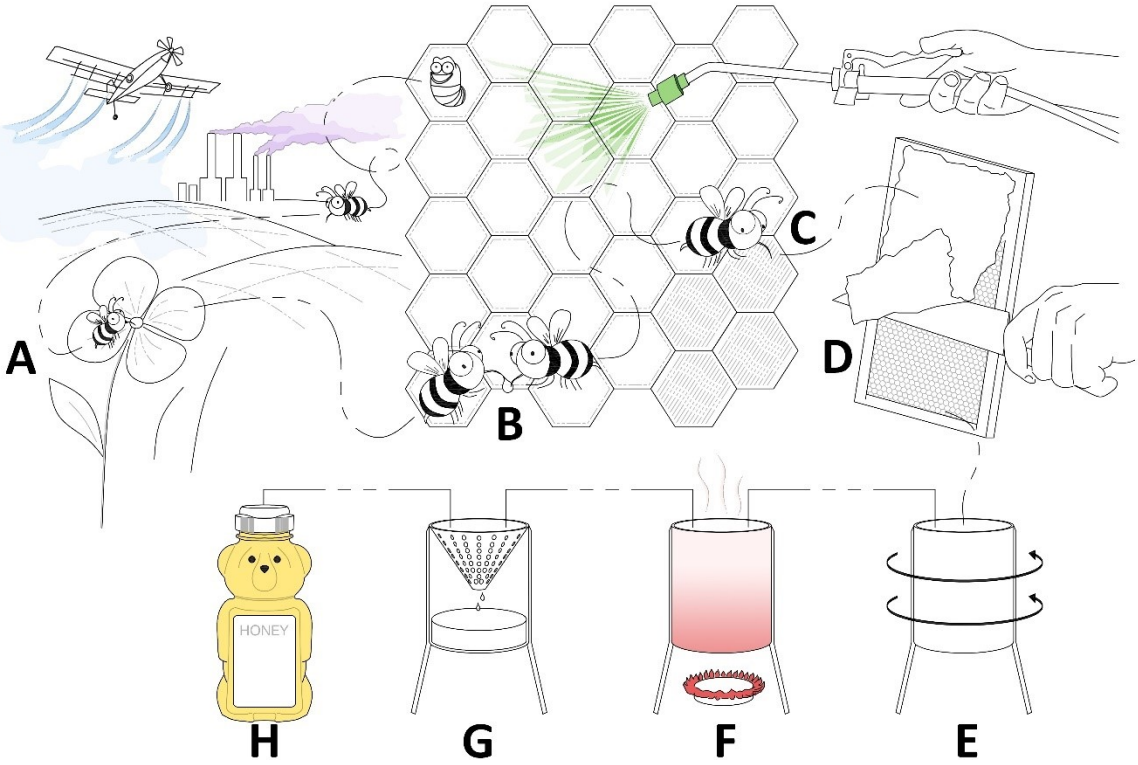

Figure 2.2. Diagram of the honey production steps along with different sources of organic contaminants that play a role in each steps. (A: nectar collection by the foraging bees, B: transfer of the nectar from the foraging bee to the home bee, C: storage of unripened honey inside the comb cells, D: uncapping of honey, E: centrifugation, F: heating, G: filtration, H: bottling of the final honey product).

The amount of nectar needed to produce 1 kg of honey depends on the type of flower and its moisture content. In the case of plants containing concentrated nectars, such as acacia, the initial nectar collected by the foraging bees can contain over 60% sugar so little water needs to be evaporated to produce the honey [79]. Clover nectar, on the other hand, is generally more diluted and more than 4 kg of nectar can be needed to produce only 1 kg of honey. Nectars with high water content (i.e. above 87%) are hardly worth harvesting because of the amount of work that producing the honey will involve, so they are usually

not collected by the bees except in early spring, when they need water to dilute stored honey [79].

Honey bees also forage for pollen, water and propolis. Pollen is collected generally by younger bees and is used as a source of proteins, fats, vitamins and minerals. Once inside the hive, pollen pellets are packed into cells, where pollen undergoes a type of fermentation and keeps without spoiling [82]. Water is collected by foraging bees when nectar is not available or during hot weather, when it is necessary to cool the interior of the hive. Contrary to nectar and pollen, water is not stored inside the hive but some home bees will keep it in their honey sac and thus serve as a water reservoir, distributing it to others when needed [78]. Finally, propolis is produced by a few foraging bees in each colony, especially during hot weather, and is used to repair the hive, smooth out the internal walls and as a protective barrier against external invaders and weathering threats [86].

Honey is not the only bee product being commonly commercialized. Bee pollen, collected by the foraging bees and stored in the hive cells, is also commercialized because of its high protein and vitamin contents [79]. Propolis has been reported to have many beneficial properties such as antibacterial, antiviral, anti-inflammatory, analgesic, tissue regenerative, antioxidant, cytostatic and hepatoprotective properties, reason why it is sold for human consumption [87-90]. Another bee product commercialized worldwide is beeswax, which is used in beekeeping but also in a variety of purposes such as candle making, lipsticks, pill coatings, crayons, chewing gums and inks [78]. Inside the hive, beeswax is secreted by the honey bees through their wax glands and it is used for comb building and repairing [79]. Royal jelly, also called bee's milk, is secreted by young bee workers through their hypopharyngeal glands and used to feed larval workers and the queen. It is also harvested and sold as a dietary supplement for its high content in vitamins B, C and D, and because it contains 10-hydroxydecanoic acid, a substance with antibiotic properties [82]. Finally, bee venom is harvested because of its pharmacological properties [79].

Two types of honey can be differentiated based on the source of nectar that the honey bees use: blossom honey and honeydew honey. Blossom or floral honey is produced by bees from nectar contained in flowers or blossoming plants, while honeydew honey is obtained from secretions produced by plants such as those in genera *Pinus*, *Abies*, *Castanea* and *Quercus*, or from excretions from plant-sucking insects, mainly from the family *Aphididae*, on the living part of plants [91]. Most of the physicochemical and bioactive properties of honey, such as aroma, taste, electric conductivity, specific rotation, pH, and antioxidant and antibacterial properties will differ in blossom and honeydew honeys [91].

2.2.3 Composition of honey

As most food matrices, honey is a complex mixture of components from multiple origins. It is estimated that honey contains about 200 substances including sugars, water and other components such as proteins, organic acids, vitamins, minerals, pigments, phenolic compounds, volatile compounds and solid particles derived from honey harvesting [92-95]. Sugar is the main component of honey and, although its composition depends on the honey's botanical and geographical origin as well as climate, processing and storage, the average concentration of sugars in honey is around 80% [78, 96, 97]. Monosaccharides represent 75% of the sugars found in honey, fructose and glucose being the two major components. In most types of honey, the content of fructose is higher than that of glucose, with some exceptions such as rape and dandelion honey, in which the fraction of glucose may be higher than the fraction of fructose [97]. One of the main responsible agents for the high content in fructose and glucose is the enzyme invertase, segregated by the bees and mixed with the nectar inside their honey sacs. Invertase converts the sucrose present in the nectar into glucose and fructose, thus allowing the production of a highly concentrated solution of sugars that would not be possible with only sucrose [79]. Other sugars found in honey, apart from fructose, glucose and sucrose, include rhamnose, trehalose, nigerobiose, isomaltose, maltose, maltotetraose, maltotriose, maltulose, melezitose, melibiose,

nigerose, palatinose, raffinose and erlose, among others [98]. A consequence of the presence of sugars in honey is the formation of undesirable compounds such as furfural and 5-hydroxymethylfurfural (HMF) through non-enzymatic reactions (i.e. Maillard reaction) after heating or storing the honey for a long time [96].

The protein content of honey varies depending on the species of the honey bees; while *Apis cerana* honey contains between 0.1 and 3.3% protein, its content in *Apis mellifera* honey is in the range of 0.2-1.6% [99]. The sources of proteins and amino acids in honey can be both from animal and vegetal origin, pollen being the main source [100].

Organic acids account for approximately 0.57% of the composition of honey, acetic, butyric, citric, formic, gluconic, lactic, malic, pyroglutamic and succinic being the main acids found [79]. These acids, which are also used to discriminate the honeys according to their botanical and/or geographical origin, are related to the color and flavor of honey as well as some of its chemical properties such as acidity, pH and electrical conductivity [101].

Honey also contains small amounts of vitamins, mainly the vitamin B complex (i.e. vitamins B1, B2, B3, B5, B6, B8 and B9) as well as vitamin C. These vitamins are well preserved in honey because of its generally low pH [102]. However, the commercial filtration of honey can cause a reduction in its vitamin content because of the almost complete removal of pollen, and vitamins can also be oxidized by the hydrogen peroxide produced by the glucose oxidase present in honey [94].

The mineral content depends strongly on the botanical and geographical origin of honey, and ranges from 0.04% in light honeys to 0.2% in dark honeys [93]. The most abundant element found in honey is potassium, which generally corresponds to one third of the total mineral content, and smaller quantities of sodium, iron, copper, silicon, manganese, calcium and magnesium are also commonly found [93]. Regarding the presence of heavy metals, such as arsenic, lead, mercury and cadmium, an increased concentration has been

observed in honey samples near industrial areas. However, to date no maximum residue limits (MRL) have been established in honey [103].

Polyphenols are another important group of compounds regarding the appearance and functional properties of honey. The phenolic profile of honey comprises compounds such as vanillinic acid, caffeic acid, syringic acid, coumaric acid, ferulic acid, quercetin, kaempferol, myricetin, pinobanksin or luteolin [77]. They are often used as an indicator of floral origin, since the distribution of the three main phenolic families (benzoic acids, cinnamic acids and flavonoids) shows different profiles in honeys from different floral origins [77]. Flavonoids are the most common polyphenols in honey, with an average concentration of approximately 20 $mg.kg^{-1}$ [104]. These compounds can significantly contribute to the total antioxidant activity of honey, thus bringing beneficial effects for human health [77].

Finally, the aroma profile of honey strongly depends on its botanical origin. Indeed, more than 500 volatile compounds have been identified in different types of honey [105]. In addition, volatile compounds in honey can have their origin on the processing conditions and storage of the honey, and they can also be produced by the honey bees from plant constituents [100]. Some aroma compounds of different types of honey include 3,9-epoxy-1-p-mentadiene, t-8-p-menthan-oxide-1,2-diol and cis-rose in lemon honey, diketones, sulfur compounds and alkanes in eucalyptus honey, and hexanal and heptanal in lavender honeys [106, 107].

2.2.4 Physicochemical properties of honey

The physicochemical parameters most commonly measured in honey, which are related to its identity and quality, include moisture, water activity (A_w), pH, free acidity, electrical conductivity, colour and diastase activity [100]. Water is the second largest constituent of honey, and the moisture content of honey is one of its most important characteristics,

influencing other properties such as viscosity, crystallization, color, flavour, taste, specific gravity, solubility and conservation [92]. The moisture content of honey is usually between 15 to 21%, and the Codex Alimentarius stipulates that the maximum allowed moisture content of honey is 20% [76]. The percentage of moisture in honey depends on its botanical origin, climate, level of maturity achieved in the hive, processing techniques and storage conditions [5, 108]. A_w of honey is usually between 0.50 and 0.65, 0.60 being the threshold above which microbial stability can be compromised. Contrary to moisture content, there are no legal limits for the value of A_w. However, controlling the A_w is of great importance because honey contains osmophilic yeasts that can cause fermentation, forming ethyl alcohol and carbon dioxide, that can change the quality of the honey [92, 96, 108].

Honey is a relatively acidic food, with a pH generally between 3.2 and 4.5 [5]. Although there is no pH limit described by the Regulatory Committees, the optimum pH for most organisms is between 7.2 and 7.4, so the acidity of honey naturally inhibits the growth of micro-organisms [109]. pH can be used as a marker of honey adulteration with high fructose corn syrup, which leads to a significant increase in pH compared to pure honey [110]. Free acidity, characterized by the presence of organic acids in equilibrium with lactone, internal esters and some inorganic ions such as phosphates, sulfates and chlorides, is another important parameter to measure in honey, as it is related to its deterioration [100, 111]. For this reason, the Codex Alimentarius allows a maximum value of 50 $meq.kg^{-1}$ for free acidity in honey [76]. Values higher than this may be indicative of fermentation of sugars into organic acids, which is not desirable in honey [96].

The electrical conductivity of honey is related to its mineral content and acidity; the higher the honey content in ions, organic acids and proteins, the higher the resulting conductivity [108]. This parameter can be used to distinguish blossom honeys from honeydew honeys, since the latter are usually characterized by higher values of electrical conductivity [91]. In addition, there is usually a positive correlation between the electrical conductivity and the

strength of the colour and flavour of the honey [5, 92]. The Codex Alimentarius recommends a maximum value of 800.00 $mS.cm^{-1}$ for honey [76].

Colour, which is a very important parameter for honey commercialization, is one of the properties that varies the most and it is strongly influenced by the botanical origin of the honey [100]. Other factors that can influence the colour of honey are the mineral content, the temperature at which the honey remains in the hive and storage time [112]. The Codex Alimentarius stipulates that the colour of honey should be from nearly colourless to dark brown [76].

Finally, diastase activity, similar to HMF, can be used as an indicator of the aging of honey. Diastases (α- and β-amylases) are enzymes naturally present in honey whose function is to digest the starch molecule into a mixture of maltose and maltotriose. Because they are thermolabile, their content can act as an indicator of possible overheating of the honey [108]. The diastatic activity in honey corresponds to the activity of the enzyme present in 1 g of honey which can hydrolyze 0.01 g of starch in 1 h at 40˚C, expressed as the diastase number in Göthe units [113]. The Codex Alimentarius stipulates a minimum value of 8.00 Göthe units for honey, with the exception of honeys with a naturally lower diastase activity, in which case the minimum tolerable is 3 Göthe units if the HMF content is up to 15 $mg.kg^{-1}$ [76]. The natural diastase content of honey can vary depending on the age of the bees, the nectar collection period, the physiological period of the colony, the quantity of nectar flow and its sugar content, because a high flow of concentrated nectar leads to a lower enzyme content [114].

2.2.5 Honey and human health

Honey has been used as a medical product since the earliest times. As seen before, throughout history honey has been associated with health benefits and it has been used for a wide variety of medicinal applications [78]. Nowadays, the known medicinal properties of

honey include antibacterial, antioxidant, antimutagenic, antitumor and anti-inflammatory activity, and cardiovascular effects, among others [77].

The antimicrobial activity of honey is mainly due to its high osmolarity, acidity and particularly its hydrogen peroxide content [115]. Other factors such as lysozyme, phenolic acids and flavonoids also contribute to the antimicrobial activity of honey, which is commonly known as non-peroxide antibacterial activity [116]. Although it has been suggested that the main part of the non-peroxide antibacterial activity might be of honey bee origin, part of it may be of plant origin, which would explain the differences in antibacterial activity of different unifloral honeys [115]. Because of being more resistant to heat and light, non-peroxide antibacterial activity has been found by some authors to be more important than hydrogen peroxide in terms of antibacterial effects of honey, as it remains intact after long periods of storage [116]. However, other authors suggest that the contribution to antibacterial properties of non-peroxide antibacterial activity may be smaller than that of hydrogen peroxide [117]. Therefore, to optimize its overall antibacterial activity, honey should be stored in a cool, dark place and storage time should be minimized [77]. To-date, the unifloral honey with the greatest known antimicrobial activity is manuka honey, originating from the East Cape region of the North Island of New Zealand [117].

The antioxidant activity of honey can be defined as the ability and potential to reduce oxidative reactions within the food systems and human health [77]. Several components in honey are responsible for its antioxidant activity, such as flavonoids, phenolic compounds, some enzymes (e.g. glucose oxidase, catalase), ascorbic acid, amino acids and proteins, among others [77]. In addition, the antioxidant activity of honey varies greatly depending on its floral origin, possibly due to differences in plant secondary metabolites and enzyme activity [118]. The influence of honey ingestion on the antioxidant capacity of plasma was studied by Al-Waili *et al.*, and it was found that phenolic antioxidants from honey are bioavailable and that they increase the antioxidant activity of plasma [119]. In addition,

Beretta *et al.* reported several positive effects of honey, such as significant suppression and prevention of cell damage and complete inhibition of cell membrane oxidation, when used on a cultured endothelial cell line subjected to oxidative stress [120]. Indeed, it is estimated that the substitution of traditional sweeteners by honey in some foods could result in an enhanced antioxidant defense system in healthy adults [119].

Honey also contains a range of compounds with antiradical and anti-inflammatory properties, such as phenolic derivates, which can play an important role, alone or in combination, in its antitumor and anti-inflammatory effects. The antitumoral activity of honey has been reported in various studies, showing its ability to prevent the spread of metastatic cells in rat models and to inhibit the growth of different bladder cancer cell lines *in vitro* and in mice models [121, 122]. Regarding the anti-inflammatory effects of honey, Al-Waili *et al.* reported that the ingestion of honey decreased several inflammation markers in human plasma, and Bilsel *et al.* reported that the ingestion of honey decreased inflammation in an experimental model of inflammatory bowel disease in rats [123, 124].

Ingestion of honey can also help treat and recover from several infections of the intestinal tract. It has been found that pure honey has bactericidal activity against many enteropathogenic organisms, including *Salmonella* and *Shigella* species, and *E. coli* [125]. In a clinical study of honey treatment in infantile gastroenteritis, honey was found to shorten the duration of diarrhoea in patients with bacterial gastroenteritis caused by these organisms [126]. Honey is also an inhibitor of *Helicobacter pylori*, the agent that causes peptic ulcers and gastritis [127], and it has been found to have prebiotics effects similar to those of fructo-oligosaccharides [128]. Consumption of honey has also been found to lower cardiovascular risk factors in healthy individuals and in patients with elevated risk factors when compared to the consumption of equal amounts of sugar [129, 130].

Finally, the benefits of honey applied topically have also been studied. It has been reported that honey reduces skin inflammation, edema and exudation, promotes wound healing, diminishes scar size and stimulates tissue regeneration [131].

2.2.6 Organic contaminants in honey and their analysis

Honey is a food of animal origin that has the particularity of being able to contain a wide range of contaminants. Because honey bees forage at distances of up to 3 km from the hive, they are not only exposed to the beekeeping-related chemicals but also to a large variety of agrochemicals and environmental pollutants, making honey a food product with the potential to contain an extensive list of contaminants. This section reviews the main organic contaminants found in honey divided into 3 families: veterinary drugs, environmental contaminants and processing contaminants. For each family, an overview of their source, incidence in honey and traditional methods of analysis are presented.

2.2.6.1 Veterinary drugs

With 35% of the global food production depending on pollinators, 90% of this pollination being managed by honey bees, the health and well-being of these insects have become a matter of importance from both a food production and environmental point of view [132, 133]. Honey bees face several threats during their lifetime, including pathogens such as bacteria, fungi and viruses, as well as parasites, so beekeepers use a wide range of products to protect their hives. In some cases, residues of these chemicals can contaminate the honey, therefore the management of the health of honey bees can have a direct impact on food safety.

The two main bacterial diseases of honey bees are American foulbrood (AFB) and European foulbrood (EFB). AFB is the most virulent brood disease known in honey bees, caused by the sporeforming bacterium *Paenibacillus larvae* [134]. Although the endospores, the infectious

form of this organism, can remain viable for more than 35 years and withstand severe weather conditions, they are infectious only to larvae; adult bees do not become infected upon ingestion of *P. larvae* spores [135, 136]. AFB results in the death of older larvae and young pupae, which are digested by enzymes secreted by the bacterium [134]. Antibiotics can only mitigate but will not eliminate the disease and therefore infected hives must be treated constantly to prevent a foulbrood outbreak. Left untreated, foulbrood destroys the hive's bee population and can annihilate an apiary. Thus, AFB is a severe problem in apiculture and causes considerable economic loss to beekeepers all over the world [137].

EFB is closely related to AFB but its causative organism, *Melissococcus plutonius*, does not form spores, and therefore the disease is considered less problematic than AFB [134]. Such as with AFB, the infection of larvae occurs when larvae ingest food contaminated with *M. plutonius*, and infected larvae die from starvation when the bacteria proliferating in their gut assimilate most of their food [138]. While AFB has been widely described, many aspects of the pathogenesis, transmission and control of EFB remain unclear.

Another critical disease for the beekeeping industry, in this case with a fungal origin, is nosemosis. This disease is the clinical outbreak of the Nosema infection caused by *Nosema apis*, which is characterized mainly by dysentery, or by *Nosema ceranae*, which is described to cause death of individuals and colonies without any previous visible symptoms [139, 140]. Unlike AFB and EFB, nosemosis affects adult bees, which become infected by ingesting Nosema spores present in faeces and in pollen, and this results in depopulation and bee colony losses [141, 142]. Indeed, this disease is suspected to be one of the agents causing Colony Collapse Disorder (CCD), a syndrome of unknown etiology that has caused massive colony losses worldwide [139, 141]. High incidences of Nosema have been directly related to stress, such as periods of long confinement or nutritional imbalance of the honey bees [144].

Among the pests that can affect honey bees, the most important ones are the mites *Varroa destructor* and *Acarapis woodi*. According to Rosenkranz *et al*, *V. destructor*, commonly referred to as *Varroa*, is still the greatest threat for apiculture as no other pathogen has had a comparable impact on both beekeeping and honey bee research in the history of apiculture [143]. This mite, which feeds on developing pupae, is responsible for the clinical symptoms of Varroosis in *A. mellifera*, including weight loss, a decreased ability to navigate and a reduced life span, among others [146, 147]. *Varroa* has been associated with CCD because of the damage it causes to the colonies and also because of being the vector of several pathogenic bee viruses [143]. The second and less problematic type of bee mite is *A. Woodi*, commonly known as tracheal mite. This parasite was first identified in 1921 in the breathing tubes of bees, where it feeds and reproduces [148]. Tracheal mites affect the overwintering capability of the colony, making bees unable to form and maintain temperatures in the winter cluster, and they are also associated with paralyzed bees displaying disjointed wings and crawling on the ground near hives [143]. Initially, tracheal mites caused devastating losses for the North American beekeepers. Nowadays, tracheal mites are still found but in far lesser amounts, because the multiple treatments used by beekeepers to try to control *Varroa* are also effective for tracheal mites [143].

The biggest problem with bee mites is that they not only damage the bee but also carry pathogenic viruses. To date, 18 different viruses have been identified from bees and many of them are vectored by mites, especially *Varroa* [149]. The three most common pathogenic viruses affecting honey bees are the acute bee paralysis virus, the deformed wing virus, and the Israeli acute paralysis virus, which has been found to be strongly correlated with CCD [138, 150]. Unfortunately, there is no treatment for bee viruses, so the only solution beekeepers have is to prevent viral vectors by maintaining the general health of the colony, with a special focus on mites [151].

There are several antibiotic classes that can be used for the treatment of AFB, EFB and nosemosis in honey bees, although many of them are only allowed in some countries and for emergency situations. Examples of these antibiotics include tetracyclines, streptomycin, sulfonamides, tylosin, erythromycin, lincomycin, chloramphenicol, nitrofurans, nitroimidazoles, fluoroquinolones and fumagillin [134]. Bee mites are generally treated with acaricides, the pyrethroids fluvalinate and flumethrin being, along with other compounds such as amitraz or coumaphos, the most commonly used ones [143]. Many of these acaricides are also used as pesticides in applications other than beekeeping. This is the reason why they are sometimes listed as pesticides in food safety reports. Alternatively, some essential oils containing thymol, eucalyptol, menthol and camphor, as well as organic acids such as formic acid, oxalic acid and lactic acid, can be used for an organic farming approach based on natural compounds [145].

In Canada, the Canadian Food Inspection Agency (CFIA) is the governmental organism in charge of the surveillance of food contaminants through the National Chemical Residue Monitoring Program (NCRMP). This screening includes several types of food from both vegetable and animal sources, domestic and imported, and comprises the analysis of a wide range of contaminants both organic and inorganic. A summary of the results of the latest three NCRMP reports for organic contaminants in honey is presented in **Table 2.3**, which contains an overview of all the veterinary drugs surveyed in honey in Canada. The veterinary drugs with high frequency of detection in both domestic and imported honey over the 3 NCRMP reports include amitraz, fumagillin, tetracyclines and macrolides. Despite the high number of positive samples detected in some cases, most of the results are below the regulatory limits, as the low number of violations shows. The range of concentrations at which the reported veterinary drugs were found was between 0.1 $ng.g^{-1}$ and 0.1 $\mu g.g^{-1}$, approximately. Among the compounds with a more notable amount of non-compliant results there is fumagillin, some sulfonamides and the macrolide tylosin. In the case of

nitrofurans, the number of positive results always corresponds to the number of violations, since these are banned compounds in Canada [152].

Traditionally, veterinary drug residues in honey have been analyzed with targeted approaches. This type of analysis usually involves an extraction step prior to quantification with LC-MS or LC-MS/MS. For example, Thompson *et al.* used SPE and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry for the determination of lincomycin and tylosin in honey [155]. In their work, honey samples were liquefied in a water bath at 60°C to remove wax and bulk debris, and then they were dissolved with a Na_2CO_3/$NaHCO_3$ buffer. The resulting solutions were loaded onto a C18 SPE cartridge. Antibiotic residues were separated from the bulk of the sample matrix, composed mainly of sugars, by using a sequence of washing steps (methanol/water 5:95 and then 30:70 (v/v) respectively). Lopez *et al.* adopted a similar approach for the multiclass determination of antibiotic residues in honey, using SPE and LC-MS/MS [156]. In another example of multiclass residue analysis, Orso *et al.* studied different extraction and cleanup methods, the optimal conditions being homogenization with McIlvaine buffer followed by extraction with acetonitrile and cleanup with Florisil® using dispersive solid phase extraction, prior to LC-MS/MS analysis [12].

Regarding non-targeted strategies, only a few authors studied the application of such approach for the analysis of veterinary drugs in honey. For example, Gómez-Pérez *et al.* investigated the non-targeted analysis of veterinary drugs in honey using direct injection to liquid chromatography coupled with TOF-MS, Orbitrap MS, and QqQ-MS [34].

Table 2.3. Summary of organic contaminants in honey reported by the NCRMP of the CFIA in their latest three reports (2012-2015).

Compound	Number of positive samples / Number of samples analyzed						Reported levels [a]
	2012-2013 [153]		2013-2014 [154]		2014-2015 [158]		
	Domestic	Imported	Domestic	Imported	Domestic	Imported	
VETERINARY DRUGS							
Amitraz	5/213 (0)[b]	11/69 (0)	29/136 (0)	32/74 (0)	20/176 (0)	22/85 (1)	0.00100-0.10100
Fluoroquinolones	0/258 (0)[c]	0/99 (0)	0/167 (0)	0/62 (0)	0/190	1/84 (0)	
Ofloxacin						1/84 (0)	0.00575
Fumagillin	26/209 (8)	1/77 (0)	48/208 (4)	1/82 (0)	26/181 (2)	1/78 (0)	0.00140-0.11360
Glycosides	0/218 (0)	0/86 (0)	0/178 (0)	0/66 (0)	0/189	0/82	
Ionophores	0/223 (0)	0/81 (0)	0/163 (0)	0/64 (0)	0/164	0/71	
Macrolides	13/251 (0)	3/85 (0)	21/209 (0)	7/82 (2)	17/190 (0)	2/74 (0)	
Desmycosin	4/251 (0)	0/85 (0)	15/209 (0)	5/82 (0)	11/190 (0)	0/74	0.00120-0.10400
Tylosin	13/251(0)	3/85 (0)	21/209 (0)	5/82 (2)	17/190 (0)	2/74 (0)	0.00038-0.18000
Nitrofurans	0/198 (0)	3/70 (1)	2/196 (2)	3/84 (3)	0/195	0/85	
Nitrofurazone		3/70 (1)	2/196 (2)	3/84 (3)			0.00020-0.01900
Nitroimidazoles	0/207 (0)	0/63 (0)	0/149 (0)	0/74 (0)	0/185	0/79	
Penicillins	0/250 (0)	3/86 (0)	0/171 (0)	0/65 (0)	0/189	0/80	
Amoxicillin		1/86 (0)					0.00013

Ampicillin		1/86 (0)					0.00010
Penicillin G		1/86 (0)					0.00012
Phenicols	0/251 (0)	0/91 (0)	0/212 (0)	0/86 (0)	0/193	0/80	
Sulfonamides	0/276 (0)	7/92 (5)	0/163 (0)	0/62 (0)	1/191 (0)	0/76	
Sulfadimethoxine		4/92 (2)			0/191		0.01280-0.19200
Sulfamethazine					1/191 (0)		0.01460
Sulfamethoxazole		3/92 (1)			0/191		0.01300-0.03500
Sulfathiazole					1/191 (0)		0.00690
Trimethoprim		2/92 (1)			0/191		0.00200-0.01200
Tetracyclines	24/231 (0)	3/66 (0)	31/214 (0)	3/108 (0)	19/184 (0)	4/72 (0)	
Epi-Oxytetracycline	1/231 (0)	0/66 (0)	1/214 (0)	0/108 (0)	3/184 (0)	0/72	0.00130-0.01200
Epi-Tetracycline	0/231 (0)	2/66 (0)	2/214 (0)	1/108 (0)	1/184 (0)	4/72 (0)	0.00160-0.01530
Oxytetracycline	23/231 (0)	1/66 (0)	29/214 (0)	1/108 (0)	17/184 (0)	1/72 (0)	0.00050-0.06400
Tetracycline	4/231 (0)	2/66 (0)	6/214 (0)	3/108 (0)	5/184 (0)	4/72 (0)	0.00050-0.01647
PESTICIDES							
Daminozide	0/281 (0)	0/94 (0)	0/105 (0)	0/52 (0)	0/84	0/26	
Carbendazim	0/221 (0)	2/79 (0)	2/156 (0)	0/72 (0)	0/174	0/80	0.01120-0.01290
Dithiocarbamate	1/209 (1)	1/88 (0)	0/209 (0)	0/82(0)	0/194	0/85	0.07800-0.22300
Ethylene diamine	3/199 (0)	5/70 (0)	0/209 (0)	6/82 (0)	16/183 (0)	2/82 (0)	0.02200-1.13000

Ethylene thiourea	0/221 (0)	0/74 (0)	0/147 (0)	0/64 (0)	0/182	0/71	
Formetanate	0/219 (0)	0/79 (0)	0/126 (0)	0/61 (0)	0/173	0/44	
Pesticides	15/274 (0)	0/114 (0)	20/158 (0)	2/64 (0)	9/151 (0)	2/72 (0)	
Bifenthrin	1/274 (0)		0/158 (0)	0/64 (0)	0/151	0/72	0.00200
Captan	2/274 (0)		1/158 (0)	0/64 (0)	0/151	0/72	0.01120-0.02820
Carbofenthion	1/274 (0)		0/158 (0)	0/64 (0)	0/151	0/72	
Coumaphos	0/274 (0)		1/158 (0)	1/64 (0)	1/151 (0)	2/72 (0)	0.00430-0.01500
Cyprodinil	3/274 (0)		0/158 (0)	0/64 (0)	0/151	0/72	0.00230-0.00920
Fludioxonil	3/274 (0)		0/158 (0)	0/64 (0)	0/151	0/72	0.00280-0.01480
Iprodione	6/274 (0)		11/158 (0)	0/64 (0)	7/151 (0)	0/72	0.00300-0.05500
p,p'-DDE	2/274 (0)		0/158 (0)	0/64 (0)	0/151	0/72	0.00130
Permethrin (total)	1/274 (0)		0/158 (0)	1/64 (0)	0/151	0/72	0.00630-0.00960
Piperonyl butoxide	0/274 (0)		7/158 (0)	0/64 (0)	1/151 (0)	0/72	0.00330-0.02900
Propoxur	1/274 (0)		0/158 (0)	0/64 (0)	0/151	0/72	0.00900
Phenol	0/137 (0)	2/47 (0)	0/11 (0)	0/7 (0)	-	-	0.01700-0.03200
Thiabendazole	0/257 (0)	0/110 (0)	0/90 (0)	0/52 (0)	0/168	0/65	
PAHs							
Acenaptthene	4/18	2/12	16/17	11/17	5/20	7/13	0.0506-13.5000
Acenapthylene	5/18	3/12	17/17	12/17	5/20	7/13	0.0313-0.3710

Anthracene	8/18	2/12	17/17	11/17	16/20	12/13	0.0300-1.2900
Benzo(a)anthracene	7/8	4/12	17/17	13/17	12/20	9/13	0.0145-0.5320
Benzo(a)pyrene	1/18	0/12	4/17	7/17	4/20	8/13	0.0216-0.1900
Benzo(b)fluoroanthene	5/18	1/12	16/17	11/17	14/20	8/13	0.0580-0.8100
Benzo(g,h,i)perylene	5/18	2/12	14/17	11/17	3/20	4/13	0.0212-4.1800
Benzo(g,h,i)fluoranthene	2/18	0/12	12/17	10/17	11/20	8/13	0.0141-0.4840
Chrysene	14/18	5/12	17/17	15/17	12/20	12/13	0.0212-0.8100
Dibenzo(a,h,)anthracene	1/18	1/12	3/17	1/17	5/20	1/13	0.0130-4.3300
Fluoranthene	18/18	12/12	17/17	17/17	18/20	13/13	0.0463-2.0000
Fluorene	14/18	10/12	17/17	17/17	18/20	13/13	0.0456-5.1400
Ideno(1,2,3-cd)pyrene	3/18	1/12	12/17	10/17	2/20	1/13	0.0268-3.5000
Naphthalene	18/18	12/12	17/17	17/17	16/20	13/13	0.6010-5.3500
Phenanthrene	18/18	12/12	17/17	17/17	20/20	13/13	0.0172-6.4000
Pyrene	18/18	12/12	17/17	17/17	17/20	13/13	0.0143-1.8600

[a] Levels are expressed in $\mu g.g^{-1}$ for veterinary drugs and pesticides, and in $ng.g^{-1}$ for PAHs. [b] In the cases where at least one sample was found positive, the number of violations (i.e. samples above the regulatory limit) is presented between parenthesis. [c] In the case of multi-residue methods for groups of compounds (i.e. fluoroquinolones, glycosides, ionophores, macrolides, nitrofurans, nitroimidazoles, penicillins, phenicols, sulfonamides, tetracyclines and pesticides), the number of positives represents the number of samples with at least one positive result for one of the compounds of the group. Details of the specific compounds that were found are presented below the multi-residue summary.

2.2.6.2 Environmental contaminants

Among the food-producing animals, honey bees have the characteristic of being excellent samplers of environmental pollution. During their foraging trips, honey bees are exposed to pesticides used in the fields they feed from, and they also accumulate environmental pollution and airborne particles on their body hair [175]. When the forager returns to the hive, she can transfer these chemicals to the home bee and they can end up contaminating the honey. For this reason, honey bees and some of their products (i.e. nectar, pollen and honey) are used as biomonitors of environmental contamination [15]. The wide range of environmental contaminants that are found in honey can be divided into two main groups: pesticides and air pollutants.

2.2.6.2.1 Pesticides

Because honey bees are great pollinators, it is very common to place bee hives strategically close to crops to benefit from both a good pollination of the crops and an extended source of nectar for the bees in one place. In many cases, bee hives can be rented by farmers who want to pollinize their crops, resulting in a win-win situation for both the beekeeper and the farmer [82]. Honey bees will be exposed during their foraging trips to all the chemicals used in the fields, reason why pesticides are included in the surveillance programs for organic contaminants in honey. The list of pesticides tested in honey by the CFIA included more than 300 compounds in the latest NCRMP report (2014-2015) between fungicides, insecticides and herbicides [158].

Among the pesticides found in honey, in recent years a particular group of compounds has been of special relevance: neonicotinoid pesticides. These compounds have been associated to CCD, so the study of their presence in honey is not only being used for food safety purposes but also to investigate their potential relationship with the massive honey bee decline worldwide [159]. Neonicotinoid pesticides include imidacloprid, thiamethoxam, thiacloprid, acetamiprid, nitenpyram, clothianidin and dinotefuran [160].

Table 2.3 contains a summary of the pesticides found in honey by the CFIA, including their frequency of detection, number of violations and range of concentrations reported. The compounds being detected more frequently in honey include cyprodinil, fludioxonil, iprodione and piperonyl butoxide. Contrary to the case of veterinary drugs, almost all pesticides surveyed in honey by the CFIA were found below the regulatory limits, with the only exception of a single violation of dithiocarbamate. The levels at which these contaminants are found in honey are between 1 $ng.g^{-1}$ and 1 $\mu g.g^{-1}$, approximately. Regarding neonicotinoid pesticides, although they are not commonly found by the CFIA screenings [153, 154, 158], the levels at which they are being reported in other parts of the world are in the order of 1-10 $ng.g^{-1}$ [161].

The analysis of pesticides in honey has many similarities with the analysis of veterinary drugs, reason why many authors develop targeted multi-residue methods for both families of compounds. Examples of methods of analysis for pesticides and veterinary drugs are presented in section 2.2.6.1.

2.2.6.2.2 Air pollutants

In addition to pesticides, during their foraging trips honey bees are also exposed to a range of air pollutants. The two families of air pollutants that are most commonly reported in honey are polychlorinated biphenyls (PCBs) and PAHs.

PCBs are a group of organic chemicals used as coolants and lubricants in transformers, capacitors and other electrical equipment [162]. Due to their persistence and adverse effects, consisting mainly of endocrine disruption and carcinogenicity, they were banned by the Stockholm convention on Persistent Organic Pollutants in 2001 [163]. However, because of uncontrolled spillage and atmosphere deposition linked to excessive use in the past, they are a ubiquitous contaminant and are found in a wide variety of food matrices [164].

PAHs are another category of hazardous organic pollutants of concern due to their environmental persistence, bioaccumulation and toxicity [165, 166]. They are emitted into the atmosphere through combustion processes and are produced as by-products of an incomplete combustion of organic matter, volcanic eruption, forest fires and vehicle emissions [167, 168]. They are also considered ubiquitous environmental pollutants, frequently observed in food [169].

Among the air pollutants that can be found in honey, the CFIA only monitors PAHs. A summary of the results of the three latest NCRMP reports regarding these contaminants in honey in Canada is presented in **Table 2.3**. All reported PAHs are found in at least one honey sample in each report, fluoranthene, fluorene, naphthalene, phenanthrene and pyrene being detected in almost all domestic and imported honey samples across all 3 reports. The levels of PAHs reported were between 0.01 and 10 $ng.g^{-1}$, approximately. Regarding PCBs, the levels found in honey by different authors were generally below the limits of detection of each study, which were in the range of 0.09 to 16.76 $ng.g^{-1}$ [14, 170].

The analysis of PCBs and PAHs in honey is usually done with GC-MS or GC-MS/MS because of the volatility of these contaminants. Saitta *et al.* developed a targeted method for the analysis of PCBs and PAHs, along with different families of pesticides, in honey samples from Italy [170]. The extraction consisted in a QuEChERS method using hexane/ethyl acetate 9:1, magnesium sulphate and sodium chloride, and samples were analyzed in a GC-MS/MS using a triple quadrupole mass spectrometer. Similarly, Al-Alam *et al.* developed a targeted method for the analysis of 90 pesticides, 16 PAHs and 22 PCBs in honey using QuEChERS multiresidue extraction followed by a preconcentration step by solid-phase microextraction (SPME) [14]. In this case, the QuEChERS method involved acetonitrile, citrate-buffered extraction salts and primary and secondary amine exchange material (PSA). The SPME fiber used for the extraction of PCBs and PAHs was coated with polydimethylsiloxane, and desorption was done directly inside the GC-MS/MS [14]. To date, no non-targeted method for the analysis of PAHs or PCBs in honey has been reported.

2.2.6.3 Processing contaminants

Once the honeycombs are collected by the beekeepers, a new group of contaminants can appear in honey as a result of its manufacture process and bottling. The two main groups of processing contaminants that can be found in honey are those which come from food-contact materials and those originating through the different heating processes.

2.2.6.3.1 Plastic-related compounds

Plastic-related compounds (PRCs) include plastic monomers, additives and non-intentionally added substances (NIAS). Plasticizers, one of the main classes of PRCs, are additives that are added to plastic materials to make them softer, more pliable and thus increase their flexibility, workability and distensibility [171]. They are often esters of polycarboxylic acids with linear or branched aliphatic alcohols with moderate chain lengths [171]. Plasticizers do not form chemical bonds with the polymer matrix, and may freely move through the matrix, spacing the polymers apart, thus significantly reducing their glass transition temperature to improve plasticization [172]. This may result in a possible migration of plasticizers into the food in contact with the plastic material. Leachables may then affect the organoleptic properties of the food or represent a food safety hazard for the consumers [173, 174]. PRCs include not only plasticizers but also compounds related to the packaging material such as surfactants, ink components and adhesives from labels, and also compounds from other sources such as environmental and food processing contaminants. Plasticizers and additives are regulated when incorporated into food contact materials. In Canada, the safety of food contact materials is controlled under Division 23 of the Food and Drugs Act and Regulations, which prohibits the sale of foods in packages that may impart any substance to the contents which might be harmful to the consumer of the food [175].

Regarding the levels of PRCs in honey in Canada, Cao *et al.* studied the presence of bis(ethylhexyl) adipate (DEHA) and 20 phthalates in honey as part of the Canadian Total Diet Study in 2015, including diisobutyl phthalate (DIBP), di-n-butyl phthalate (DBP), n-butyl benzyl phthalate (BBzP)

and di(2-ethylhexyl) phthalate (DEHP), and reported concentrations of up to 4.82 and 135 $ng.g^{1}$ for DEHA and DEHP, respectively [176]. Other studies with PRCs in honey in other countries reported bisphenols and phthalate esters in concentrations of up to 302 $ng.g^{-1}$ and 203 $\mu g.g^{-1}$, respectively [13, 177-179].

To date, the different strategies reported for the analysis of PRCs in honey are based on a targeted analysis approach based on different types of extractions, cleanups and instrumental analyses (mostly based on GC or LC). For example, Lo Turco *et al.* used SPE, with Oasis Hydrophilic-Lipophilic-Balanced glass cartridges and with water and methanol as eluents, followed by GC-MS for the study of plasticizers and bisphenol A (BPA) in Italian honeys [13]. Česen *et al.* used a similar SPE based extraction followed by derivatization using N-(tertbutyldimethylsilyl)-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane in ethyl acetate for 16 hours at 60°C so as to make the analytes more volatile, less reactive and thus to improve their chromatographic behaviour prior to GC-MS analysis for the study of bisphenols and related compounds in honey [1]. Koo *et al.* used a Solvent Terminated Dispersive Liquid-Liquid Micro Extraction method with acetonitrile as the disperser solvent and 1-hexanol as the extraction solvent, followed by high-performance liquid chromatography coupled to a diode array detector for the analysis of DBP and oleamide in stingless bee honey harvested from plastic cups [180]. To the best of our knowledge, no non-targeted methods for the analysis of PRCs in honey have been reported.

2.2.6.3.2 HMF

5-hydroxymethylfurfural (HMF) is a compound usually found in low concentrations in fresh sugar containing foods such as milk, honey, maple syrup, fruit juice and bread. HMF is a heat-induced contaminant formed as a product of the Maillard reaction occurring in many food commodities, such as bread or baked goods [181]. In honey, this compound can be formed when honey is submitted to heat treatments, such as pasteurization, or a long storage time [96]. Because this compound is found in only low concentrations in fresh honey, and its concentration raises rapidly

if honey is overheated, it is commonly used as a quality indicator in honey [100, 182]. In addition, HMF can be formed in food commodities even at low temperatures in acidic conditions, such as the case of honey [183].

HMF can cause a range of adverse effects including mutagenicity, carcinogenicity, genotoxicity and cytotoxicity [181, 184]. For this reason, its concentration in foods is regulated. According to the Codex Alimentarius, HMF should not exceed 40 $\mu g.g^{-1}$ in honey with the exception of honeys from tropical areas, in which case the maximum is risen to 80 $\mu g.g^{-1}$ [76].

The International Honey Commission suggests three methods for the determination of HMF content in honey: the White and Winkler methods, both based on spectrophotometry, and a direct injection-HPLC-UV method [184]. Alternatively, some authors have developed LC-MS methods for the detection of HMF along with other contaminants. For example, Tomasini *et al.* developed a method for the targeted analysis of HMF and pesticides in honey based on extraction with QuEChERS using acetonitrile and anhydrous magnesium sulfate followed by analysis with atmospheric-pressure chemical ionization LC-MS/MS [185]. To date, no non-targeted methods have been reported for the analysis of HMF in honey.

2.3 CONCLUSIONS

In a world where the list of contaminants reported in food is continuously increasing, the classical targeted analysis design has become obsolete, the new trends being the detection and identification of currently unknown or unexpected contaminants through the use of foodomics and non-targeted analysis. While some non-targeted analysis methods have already been developed for the analysis of food contaminants, the need to further develop non-targeted methods to better characterize human exposure to chemicals and to identify potential risk compounds in food matrices has been detected. Further work is needed in order to optimize the different steps of the non-targeted workflow, especially in terms of data filtering, as well as to explore the wide range of data treatment possibilities and their applicability in the analysis of trace contaminants in food.

Among the infinite variety of food matrices that can be used as a model for the study of non-targeted approaches, honey offers the advantage of being able to contain an extended range of contaminants, both agricultural and environmental, at trace levels (in the order of $\mu g.g^{-1}$ to $ng.g^{-1}$). Being a food produced and consumed worldwide, the results from the analysis of organic contaminants in honey can provide answers not only from a food safety perspective but also for environmental monitoring. Non-targeted approaches, which have only been reported for organic contaminants in honey by a very few authors, open the door to an infinite number of new studies that were not possible before with the use of targeted analysis. These include the screening of new contaminants and study of their evolution through the re-examination of previously acquired data, the determination of the fate of trace contaminants during food processing or the comparison of samples based on their contaminant load, among others. In conclusion, non-targeted approaches in food, specially in honey, need to be further explored and developed, as they can hold the key to a new era in food safety risk assessment.

CONNECTING PARAGRAPH

Chapter 2 provided a summary of the use of non-targeted approaches for the analysis of trace organic contaminants, as well as an overview of the world of honey and its main contaminants. After identifying the multiple knowledge gaps in this area, Chapter 3 presents the development of a method for the targeted and non-targeted analysis of contaminants in honey, focusing on the first two steps of the non-targeted workflow: sample preparation and instrumental analysis. Chapter 3 has been accepted for publication in the Journal of Food and Drug Analysis*:* A. von Eyken, D. Furlong, S. Arooni, F. Butterworth, J. F. Roy, J. Zweigenbaum, S. Bayen; *Direct injection high-performance liquid chromatography coupled to data independent acquisition mass spectrometry for the screening of antibiotics in honey.*

CHAPTER 3: DIRECT INJECTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO DATA INDEPENDENT ACQUISITION MASS SPECTROMETRY FOR THE SCREENING OF ANTIBIOTICS IN HONEY

3.1 ABSTRACT

The targeted analysis of veterinary drug residues in honey traditionally involves a series of extraction and purification steps prior to quantification with high-performance liquid chromatography coupled to high resolution or tandem mass spectrometry. These steps, designed to separate the target analytes from interferences, are generally time-consuming and costly. In addition, traditional cleanup steps are likely to eliminate other compounds whose analysis could prove decisive in current or future assessment of the honey sample. Alternatively, direct injection without complex sample preparation steps has been introduced for the fast analysis of trace compounds in environmental and food matrices. The aim of this study was to develop a rapid method for the targeted analysis of 7 key veterinary drug residues in honey based on direct injection high-performance liquid chromatography coupled to quadrupole time-of-flight, while simultaneously recording data-independent MS/MS (e.g. All Ions MS/MS data) for future re-examination of the data for other purposes. The new method allowed for the detection of the target residues at levels approximately 20 to 100 times lower than current regulatory limits, for a total analysis time of about 45 min. The recoveries (103-119%), the linearity ($R \geq 0.996$) and the repeatability (RSD ≤ 7%) were satisfactory. The method was then applied to 35 honey samples from the Canadian market. Residues of tylosin A, tylosin B, sulfamethazine and sulfadimethoxine were detected in 6, 9, 6 and 23% of the samples respectively, at levels below the regulatory limits in Canada. The possibility of adding a hydrolysis step to study sulfonamides in honey was tested, which provided good results for this family of compounds but led to degradation of some of the other analytes. Finally, the non-targeted identification of several compounds was demonstrated as a proof of concept of future re-examination of All Ions MS/MS data. This paper illustrates the capacity of this novel method to combine targeted and non-targeted screening of chemical residues in honey.

3.2 INTRODUCTION

The health and the well-being of honey bees is critical for both the natural environment and human food production systems. Indeed, 35% of the global food production is dependent on pollinators [1]. For some crops, up to 90% of the pollination is provided by honey bees [2]. In beekeeping, bees are exposed to three major diseases, namely the American foulbrood, the European foulbrood and nosemosis. In case of such threats, bee hives can be protected using antibiotics. Compounds such as tetracyclines, streptomycin, sulfonamides, tylosin, erythromycin, lincomycin, chloramphenicol, nitrofurans, nitroimidazoles, fluoroquinolones and fumagillin have been reported for bee protection [3]. In Canada, Maximum Residue Limits (MRLs) have been defined for the residues of oxytetracycline, tylosin and fumagillin in honey [4]. Health Canada has also defined and recommended some safe Working Residue Levels (WRLs) for a number of veterinary drugs approved for use in other species that may be detected in domestic or imported honey [5]. **Table 3.1** describes the current MRLs and recommended WRLs for veterinary drug residues in honey in Canada.

The targeted analysis of veterinary drug residues in honey traditionally involves an extraction step prior to quantification with liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS). For example, Thompson *et al.* developed a method for the determination of lincomycin and tylosin in honey, based on solid-phase extraction (SPE) and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry [6]. In their work, honey samples, previously liquefied in a water bath at 60°C to remove wax and bulk debris, were dissolved with a Na_2CO_3/$NaHCO_3$ buffer. The resulting samples were loaded onto a C18 SPE cartridge. Using a sequence of washing steps (methanol/water 5:95 and then 30:70 (v/v) respectively), antibiotic residues were separated from the bulk of the sample matrix, notably sugars. A similar approach was adopted by Lopez *et al.* for the multiclass determination of antibiotic residues in honey, using SPE extraction and LC-MS/MS [7]. Recently, Orso *et al.* studied different extraction and cleanup methods for the determination of multiclass pesticides and

antibiotics in honey samples, and the optimal conditions were determined to be homogenization with McIlvaine buffer followed by extraction with acetonitrile and cleanup with Florisil® using dispersive solid phase extraction, prior to LC-MS/MS analysis [8].

Table 3.1. MRLs and recommended WRLs for veterinary drug residues in honey in Canada [4, 5]

Compound	**Regulated concentration ($\mu g.g^{-1}$)**
Oxytetracycline	0.3 (MRL)
Tylosin (as tylosin A+B)	0.2 (MRL)
Fumagillin	0.025 (MRL)
Chlortetracycline	0.03 (WRL)
Erythromycin	0.03 (WRL)
Lincomycin	0.03 (WRL)
Streptomycin	0.0375 (WRL)
Sulfonamide drugs*	0.03 (WRL)
Tetracycline	0.075 (WRL)
Chloramphenicol	No MRL/WRL (Banned substance)
5-Nitrofuran compounds	No MRL/WRL (Banned substance)

*Only refers to the sulfonamide drugs listed in the "Table of Approved Administrative Maximum Residue Limits and Maximum Residue Limits" posted on Health Canada's website, which includes sulfacetamide, sulfabenzamide, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfaethoxypyridazine, sulfaguanidine, sulfamerazine, sulfamethazine, sulfanilamide, sulfanitran, sulfapyridine, sulfaquinoxaline and sulfathiazole.

The above purification steps, designed to separate the target analytes from interferences, are generally time-consuming and costly. In addition, traditional cleanup steps are likely to eliminate other compounds whose analysis could prove decisive in current or future assessment of the honey sample (e.g. presence of other contaminants, chemical tracers, metabolites, etc.). Alternatively, direct injection without complex sample preparation steps has been introduced for

the fast analysis of trace compounds in environmental and food matrices. For example, Bayen *et al.* reported a direct injection approach for the study of veterinary antibiotics in surface freshwater and seawater using liquid chromatography – electrospray ionization mass spectrometry (LC-ESI-MS) [9]. According to the authors, the recoveries obtained for the spiked compounds had an average of 95 ± 14% and 96 ± 28% for freshwater and seawater, respectively; linearity and limits of detection were acceptable for ecological risk assessment applications. Direct injection of seawater, which contains high concentrations of salts that could damage the instrument, was made possible by a post-column switch on the system that diverted the salt-containing solutions flushed out of the column to the waste. Similarly, Ciofi *et al.* recently investigated the applicability of direct injection of waste, surface, ground and drinking water samples into a LC-MS/MS system for the determination of perfluoro-alkyl acids [10]. Their method, based on the direct injection of the centrifuged water sample without any other treatment, was reported to have better sensitivity and repeatability than those achieved with other extraction methods, such as on-line SPE-LC-MS/MS. In the field of food analysis, Bayen *et al.* applied direct injection LC-ESI-MS/MS for the analysis of pharmaceuticals and endocrine-disrupting chemicals in mussels and clams [11]. This “cleanup-free” approach, which relied on a simple solvent extraction with acetonitrile/methanol (50:50), was made possible using isotopically labeled surrogates to correct for matrix effects. As in the case of seawater analysis, a post-column switch on the LC-MS/MS system was used to remove potential interferences. Olmo-García *et al.* developed a method for metabolic profiling of phenolic compounds in olive oil using direct injection LC-ESI-MS [12]. The sample preparation was reduced to the dilution of olive oil in acetone, and the method was successfully validated and applied to the quantification of 21 phenolic compounds without any other step. In the context of honey analysis, direct injection LC-MS was successfully applied for the screening of various syrup adulterants and the presence of 10% sugar syrup in honey could be detected in less than 30 min [13]. "Dilute and shoot" approaches for honey have also been reported for the targeted determination of pesticides, veterinary drugs and other trace contaminants in honey prior to liquid chromatography coupled with time-of-flight MS, Orbitrap MS, and triple quadrupole MS [14-16].

The list of contaminants and toxins reported in food such as honey is continuously increasing, including new agrochemicals, emerging environmental pollutants and food contact material residues [17-19]. In this context, it appears essential to develop tools for the detection of currently unknown or unexpected contaminants. The need to further develop non-targeted methods has been highlighted by chemical risk assessment community to better characterize human exposure to chemicals [20], and to identify potential risk compounds in food matrices [21]. Among others, liquid chromatography coupled with high resolution mass spectrometry (HRMS) has emerged as a promising tool for the non-targeted analysis of food [21, 22]. HRMS systems may be operated in full-scan mode or when using data-independent acquisitions for example, to obtain structural information about virtually all ionized compounds. In All Ions MS/MS, ions are fragmented in the collision cell without the selection of any specific precursor ion (data independent acquisition). Thus, unlike data-dependent acquisition modes, All Ions MS/MS provides fragmentation patterns for all the precursors. Perez-Ortega *et al.* applied All Ions MS/MS to the screening of over 625 multiclass organic food contaminants using high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS), and found that this acquisition mode was more appropriate for the large-scale screening than the classic product ion scans because it provided excellent fragmentation information for confirmatory purposes for a theoretically unlimited number of compounds [23]. When comparing the performance of HRMS and All Ions MS/MS, the latter was found to preserve full-scan acquisition flexibility and to have the benefits of acquiring all the information all the time without the time window boundaries of targeted MS/MS mode and without the potential loss of sensitivity of non-targeted MS/MS. Therefore, All Ions MS/MS mode could be seen as a combination of the mass resolution capabilities of HRMS and the identification capabilities of MS/MS spectra obtained without compromising the detection of trace compounds in complex matrices. As direct injection allows for a minimal modification of the sample, its coupling with high resolution mass spectrometry is expected to provide a broad screening of samples' composition. To date, direct injection in combination with HRMS in the All Ions MS/MS mode for the screening of food contaminants has not been reported.

The aim of this study was to develop (i) a rapid method for the targeted analysis of seven veterinary drug residues in honey based on direct injection HPLC-Q-TOF-MS, while (ii) simultaneously recording non-targeted information (fast high-resolution MS scans combined with All Ions MS/MS) for future re-examination of the data (e.g. for exposure assessment). Seven target compounds were selected to explore the performances of the approach for different families of veterinary drugs related to beekeeping (i.e. macrolides, lincosamides, nitrofurans and sulfonamides) [3]. They were all reported to be of concern due to their toxicity, probability of antibiotic resistance, frequency of dosing or evidence of detectable residues [24]. It should be noted that sulfonamides are known to bind to sugars in honey, and acid hydrolysis is commonly required in order to liberate them and to study the total amount in honey (free+bound) [25]. Therefore, the presented approach was tested with and without an acid hydrolysis step to study sulfonamides in honey samples. The data acquired with the method developed in the present study could be used in the future to re-examine for the presence of currently unknown contaminants, or to identify some shift in the quality of honey over time. The novelty of this study is the use of direct injection combined with HRMS in the All Ions MS/MS mode for the targeted and non-targeted screening of food contaminants.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals and reagents

Analytical standards (tylosin A (CAS Number 1404-69-0), lincomycin (CAS Number 859-18-7), furazolidone (CAS Number 67-45-8), sulfamethoxazole (CAS Number 723-46-6), sulfadimethoxine (CAS Number 122-11-2) and sulfamethazine (CAS Number 57-68-1)) were purchased from Sigma-Aldrich (St Louis, MO, USA). Tylosin B (CAS Number 11032-98-7) was purchased from Toku-E (Bellingham, WA, USA). Labelled internal standards, D3-diphenhydramine (CAS Number 170082-18-5) and D3-6-acetylmorphine (CAS Number 152477-90-2), were purchased from Cerilliant (Round Rock, TX, USA). All standards were of analytical

grade. HPLC grade solvents (water, methanol, acetonitrile, acetone and 2-propanol), as well as LC/MS grade formic acid were all purchased at Fisher Chemical (Pittsburgh, PA, USA). Hydrochloric acid (37%) and D-(+)-glucose (≥ 99.5%) were purchased from Sigma-Aldrich.

3.3.2 Honey Samples

Twenty-six honey samples (H1-H26) were purchased from different stores and farmers' markets in the Montreal and Calgary regions (Canada) in May 2016. Details from the samples are presented in **Table S3.1**. They were all unpasteurized and of various prices and types (i.e. non-organic, organic farming, different colors and different floral origins). Nine additional selected honey samples (H27-H35) were obtained from the Canadian Food Inspection Agency (CFIA) in December 2016. These nine samples had been earlier analyzed by the CFIA Calgary Laboratory using class-specific multi-residue methods developed and validated in-house, and were used to test the performance of the present new method. All samples were transferred from their original container to 40 mL amber glass vials and kept in the freezer at -18°C until analysis.

3.3.3 Sample Preparation

<u>Method A: Sample preparation without acid hydrolysis</u>

Sample preparation was adapted from Du *et al.* [13]. Approximately 0.2 g of honey was weighed in a glass conic tube and 2 mL of a mixture of acetonitrile and water (1:1) was added. Samples were vortexed for about 2 min, or until the honey was completely dissolved, and then filtered through a 0.22 µm PTFE filter from Chrom4 (Thüringen, Germany). Before injection into the HPLC, the extract was further diluted with water to a final concentration corresponding to 1% of honey, and 50 µL of a 0.4 $\mu g.mL^{-1}$ mixture of the two internal standards was added. These internal standards were not used for quantification in this study but were spiked to provide a reference for sensitivity and retention time, necessary for the future non-targeted data treatment.

Method B: Sample preparation with acid hydrolysis

Approximately 0.2 g of honey was weighed in a glass conic tube and 1 mL of HCl 2M was added. Samples were vortexed for about 5 min, and then they were allowed to sit at room temperature for 1 h. Samples were then filtered, diluted and spiked with internal standards as above in Method A.

3.3.4 Method validation

In a preliminary study, three honey samples without any detectable amounts of the target antibiotics were selected as matrix blanks (H7, H18 and H26). They were from different floral and geographical origins, as well as different colours and farming methods, representing the variability of matrices within the study. To establish the absolute recoveries and the linearity of both Methods A and B, these samples were spiked with 50 µL of standard of the target antibiotic analytes in methanol at 7 levels in the 0.004 to 2 $\mu g.g^{-1}$ range before sample dilution. For Method B, samples were allowed to sit overnight at room temperature after spiking in order for the sulfonamides to react with sugars [26]. To study the matrix effect, the native antibiotic standards were spiked directly on the 1% honey sample ready for LC-QTOF analysis, at 7 levels in the 0.04 to 20 $ng.mL^{-1}$ range in the injected sample, corresponding to 0.004 to 2 $\mu g.g^{-1}$ in honey. Procedural blanks (n=10) were analyzed and used to derive the limits of detection (3σ). For repeatability studies, 5 replicates of three spiked honey samples were analyzed.

To further investigate the effect of acid hydrolysis on sugar-sulfonamide conjugates, additional honey samples were spiked with the 3 sulfonamides (0.2 $\mu g.g^{-1}$) and left to sit overnight for the sulfonamides to bind with sugars [27]. Samples were then prepared using both Method A and Method B (n=3 each). Signals corresponding to glucose-sulfonamide conjugates were identified in the chromatograms through the comparison with three standard mixtures rich in glucose-sulfonamide conjugates. These mixtures were then prepared by mechanochemical mixing of an equimolar mixture of glucose and individual sulfonamide in a Retsch Mixer Mill (MM 400

Newtown, PA, US) at room temperature using two stainless steel balls and a frequency of 30Hz for 30 minutes. The reaction mixture for each sulfonamide was then suspended in water and filtered, and the residue was analyzed after dilution in water/methanol 95:5.

3.3.5 Instrument analysis

Samples were analyzed using a 1290 series LC system from Agilent Technologies (Santa Clara, CA, USA) equipped with an InfinityLab Poroshell 120 Phenyl Hexyl (3.0 × 100 mm, 2.7 µm) column fitted with an InfinityLab Poroshell 120 EC-C18 (3.0 × 5 mm, 2.7 µm) guard column, both from Agilent Technologies. The mobile phase consisted in a mixture of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B), at a flow rate of 0.2 mL/min. The mobile phase gradient profile was as follows: 1 min 5% B, from 1 to 15 min gradient to 100% B, from 15 to 20 min 100% B, from 20 to 20.10 min gradient to 5% B and from 20.10 to 25 min 5% B. The injection volume was 20 µL and the column temperature was set to 20°C. The LC system was coupled to a 6545 series Q-TOF from Agilent Technologies (Santa Clara, CA, USA) equipped with a Dual AJS ESI ion source operating in positive ionization mode. Drying gas temperature was 325°C with a flow of 5 L/min, sheath gas temperature was 275°C with a flow of 12 L/min, the pressure on the nebulizer was 20 psi, the capillary voltage was 4000 V, the fragmentor voltage was 175 V, the skimmer voltage was 65 V and the nozzle voltage was 2000 V. All Ions MS/MS data was collected as MS scans between *m/z* 100 and 1700 at a scan rate of 3 spectra/s for four different collision energies (0, 10, 20 and 40 V). A diode-array detector (DAD; scan range: 190 to 640 nm with a 2 nm step and a slit of 4 nm) coupled to the HPLC-Q-TOF-MS system was also used for the study of the diversion of elution to waste. Samples were kept at 4°C in the multisampler compartment.

3.3.6 Data treatment

Instrument response linearity was calculated for each compound as the relative standard deviation (RSD) of the response factors (RF) of the seven calibration curve standards (ranging

0.08 to 40 ng.mL^{-1}). Antibiotic concentrations were calculated using the Agilent Mass Hunter Workstation Software – Quantitative Analysis B.07.01, for three different *m/z* extraction window values (± 5, ± 10 and ± 20 ppm). Extraction window values were selected to represent the range of values used by other authors in similar targeted and non-targeted studies [28-30]. Quantification was done based on external calibration, and peaks with a signal-to-noise ratio below 10 were considered below the limit of quantification. The following mass-to-charge ratios were used for quantification: 916.5270 for tylosin A, 772.4483 for tylosin B, 407.2216 for lincomycin, 226.0464 for furazolidone, 254.0599 for sulfamethoxazole, 279.0916 for sulfamethazine and 311.0814 for sulfadimethoxine. Matrix effect, recovery, instrument linearity, method linearity, repeatability, instrument detection limit (IDL), method detection limit (MDL) and limit of quantification (LOQ) were calculated for each compound for each of the three *m/z* extraction window values. Matrix effect (ME) and recovery (RE) were calculated according to the equations proposed by Matuszewski by comparing the response of each compound in samples spiked before dilution (RE), after dilution (ME) and in solvent [31]. The overall method linearity was assessed from the Pearson coefficient of the linear correlation between the experimental and theoretical spiked concentrations. Repeatability was assessed from the RSD obtained for five replicates of three spiked honey samples. IDL was calculated as the concentration leading to a signal-to-noise ratio (S/N) of 3, derived from the S/N of the lowest standard of the calibration curve. MDL was calculated as 3σ of the signals of 10 procedural blanks around the retention time of each compound. LOQ was calculated as 3.3 times the MDL.

Two-way analysis of variance (ANOVA) tests were performed using SigmaPlot v13.0 (Systat Software Inc) to compare the performances of the method (matrix effect, recovery, repeatability, method linearity and MDL) obtained for the different *m/z* extraction window values.

After confirmation of the linearity of the method with three matrix matched curves, antibiotics were quantified in all the 35 honey samples based on the standard addition method with one single level of spiking corresponding to a concentration of 0.2 µg.g^{-1} in honey. This standard

addition, which was within the range of linearity, was done to the already diluted extract to compensate for the matrix effect of each honey sample [32].

For the non-targeted applicability of the method, the honey samples were screened for the 7 veterinary drugs and other compounds using Agilent Mass Hunter Workstation Software – Qualitative Analysis B.07.00. The chromatogram was explored using the algorithm Find By Formula, using All Ions MS/MS and a customized database of honey-related compounds created with Personal Compound Database and Library software (PCDL) for LC/TOF. For the library screening, match tolerance was set to ± 5 ppm and expansion values for chromatogram extraction at ± 10 ppm.

3.4 RESULTS AND DISCUSSION

3.4.1 Diversion of elution to waste

Introducing a high amount of sugars, the main components of honey, into the ion source could increase needs for cleaning and maintenance. To circumvent this issue, a post-column switch was used to divert the fraction containing the sugars directly to waste. This diversion of elution to waste to avoid the introduction of high amounts of matrix-related highly-polar compounds into the ionization source was successfully applied by other authors during the analysis of contaminants in seawater and seafood using direct injection [9, 11]. **Figure S3.1** shows the total wavelength chromatogram obtained for a honey sample spiked with the mixture of the seven veterinary drug residues. The first peak at around 2.5 min corresponded to the sugars, which are expected to elute early in reversed-phase chromatography. From a targeted point of view, the first compound of interest (lincomycin, Log K_{OW} = 0.86 [33]) did not elute until 9 min, so the first 0.6 mL (3 min) eluting out of the column were directly sent to waste after which the post-column switch position changed to the ion source. From a non-targeted point of view, since most contaminants are expected to be less polar than the first-eluting matrix-related salts and sugars,

it was decided to compromise the possible loss of a minority of molecules of interest for the lifetime of the ion source and the throughput capabilities of the method.

3.4.2 Method performance (Method A – sample preparation without acid hydrolysis) and *m/z* extraction window selection

Instrument linearity, IDL, matrix effects, MDL, LOQ, recoveries, method linearity and repeatability are presented in **Table 3.2** for each of the seven analytes and each *m/z* extraction window. RSD values of the RF of the calibration curve standards were used to assess instrument linearity. RSDs were generally below 30% with the only exception of tylosin A at ± 5 ppm, which presents an RSD of 44%. In general, as the *m/z* extraction window decreased, so did the signal intensities. This may result in a decreased precision of the RF, especially for the lowest concentrations, and poorer instrument linearity. IDLs were in the range of 0.002 and 0.017 $ng.mL^{-1}$ for the three *m/z* extraction windows tested.

Matrix effects obtained for a ± 5 ppm *m/z* extraction window were significantly different than those at ± 10 and ± 20 ppm (ANOVA, P=0.014). According to the equation used, an absence of matrix effect corresponds to a value of 100%. Values below 100% correspond to matrix suppression, while values above 100% correspond to matrix enhancement. Generally, matrix effects are considered to be mild when the values are between 80 and 120%, medium between 50 and 80% or 120 and 150%, and strong for values below 50% or above 150% [34]. In this study, even though the results obtained for a ± 5 ppm *m/z* extraction window were statistically different than those at ± 10 and ± 20 ppm, most of the matrix effect values were mild and only two cases of medium matrix effects were noted, for furazolidone at ± 5 ppm (ME=63%) and sulfamethazine at ± 20 ppm (ME=123%). Matrix effects in this study were overall lower than those reported by Orso *et al.* for veterinary drugs in honey using a traditional extraction approach with SPE. In their study, strong matrix suppression was reported for tylosin A (ME=23.4%) and strong matrix enhancement was observed for sulfamethoxazole and sulfadimethoxine (ME=181.0% and ME=183.8% respectively) [8]. In the present study, signal suppression was noted for tylosin A,

lincomycin, furazolidone and sulfamethoxazole at all *m/z* extraction window values. However, Orso *et al.* reported signal suppression for tylosin A only, while mild to strong signal enhancements were recorded for lincomycin, furazolidone, sulfamethoxazole, sulfamethazine and sulfadimethoxine [8]. On the other hand, Lopez *et al.* reported signal suppression for lincomycin in honey using SPE extraction [7]. In the present study, matrix effects varied among honey samples, as illustrated by the standard deviation on the ME values. This suggests that matrix effects are strongly dependent on the characteristics of individual honey samples. The application of an average matrix effect may therefore inaccurately correct for the matrix effects, and we would recommend the assessment of the matrix effects for each honey sample.

Table 3.2. Method performance for the seven targeted veterinary drug residues for *m/z* extraction windows of ± 5, ± 10 and ± 20 ppm.

Parameter	Extraction window	Tylosin A *m/z* 916.5270 RT[b] 14.64 min	Tylosin B *m/z* 772.4483 RT 13.91 min	Lincomycin *m/z* 407.2216 RT 9.17 min	Furazolidone *m/z* 226.0464 RT 11.69 min	Sulfamethoxazole *m/z* 254.0599 RT 11.57 min	Sulfamethazine *m/z* 279.0916 RT 10.73 min	Sulfadimethoxine *m/z* 311.0814 RT 12.96 min
Instrument	± 5 ppm	44	17	9	24	13	25	15
linearity	± 10 ppm	8	12	9	25	12	10	13
(RSD % of RF)	± 20 ppm	6	10	9	26	14	9	14
IDL	± 5 ppm	0.007	0.009	0.002	0.003	0.012	0.002	0.002
($ng.mL^{-1}$)	± 10 ppm	0.012	0.007	0.001	0.003	0.010	0.002	0.002
	± 20 ppm	0.011	0.009	0.002	0.003	0.017	0.002	0.002
Matrix effect	± 5 ppm	97 ± 37	108 ± 14	82 ± 10	63 ± 26	86 ± 17	97 ± 21	84 ± 23
(%)[a]	± 10 ppm	98 ± 8	111 ± 9	82 ± 10	98 ± 31	88 ± 17	115 ± 23	102 ± 17
	± 20 ppm	98 ± 8	111 ± 10	85 ± 12	92 ± 24	94 ± 18	123 ± 42	102 ± 21
MDL	± 5 ppm	0.0025	0.0015	0.0008	0.0016	0.0022	0.0009	0.0005
($\mu g.g^{-1}$ honey)	± 10 ppm	0.0023	0.0015	0.0003	0.0014	0.0018	0.0007	0.0005
	± 20 ppm	0.0017	0.0017	0.0008	0.0020	0.0023	0.0009	0.0008

LOQ	± 5 ppm	0.0084	0.0048	0.0027	0.0054	0.0072	0.0029	0.0016
($\mu g.g^{-1}$ honey)	± 10 ppm	0.0076	0.0050	0.0011	0.0047	0.0060	0.0025	0.0018
	± 20 ppm	0.0055	0.0056	0.0026	0.0067	0.0077	0.0030	0.0025
Recovery (%)[a]	± 5 ppm	107 ± 39	112 ± 21	117 ± 17	126 ± 36	110 ± 30	111 ± 33	102 ± 47
	± 10 ppm	109 ± 10	115 ± 12	119 ± 13	103 ± 16	115 ± 23	118 ± 21	108 ± 12
	± 20 ppm	112 ± 13	115 ± 12	120 ± 10	114 ± 24	113 ± 18	123 ± 44	113 ± 20
Method	± 5 ppm	0.9736	0.9983	0.9995	0.9993	0.9987	0.9990	0.9975
linearity (R)	± 10 ppm	0.9980	0.9988	0.9996	0.9963	0.9987	0.9990	0.9987
	± 20 ppm	0.9979	0.9984	0.9996	0.9960	0.9986	0.9990	0.9987
Repeatability	± 5 ppm	37	5	6	24	6	11	20
(RSD %)	± 10 ppm	4	4	5	5	6	7	7
	± 20 ppm	4	4	5	5	5	7	7

[a] Matrix effects and recovery values are presented as mean of all concentration levels ± standard deviation (n= 21). [b] RT = Retention Time

MDLs were not statistically different using ± 5, ± 10 or ± 20 ppm of *m/z* extraction window (ANOVA, P=0.166). With values in the range of 0.0003 to 0.0025 $\mu g.g^{-1}$ in honey, these MDLs correspond to the detection of 0.6 to 5 pg of antibiotic injected. These MDLs are in the same range as those reported by other authors for organic contaminants using the direct injection approach in other matrices [10]. These MDLs are also in the same range as those reported for veterinary drugs and pesticides in honey using conventional extraction approaches [8, 35]. Most importantly, our MDLs are around 20 to 100 times lower than their respective regulatory limits in Canada, the MRLs or WRLs for these substances in honey (**Table 3.1**). No MRLs have been set up for these antibiotics in honey in other countries such as Australia, the European Union or the United States [36-38]. LOQ were in the range of 0.0011 to 0.0084 $\mu g.g^{-1}$ in honey, which is around 25 times lower than the regulatory limits.

All the recoveries were within the 80-120% acceptable range, with the only exception of furazolidone at ± 5 ppm and sulfamethazine at ± 20 ppm with values of 126 and 123%, respectively. These values were in the same order as those reported by other authors for pesticides and veterinary drugs in honey, where the recoveries generally ranged 80-120% with a few exceptions above or below this range [8, 15]. There was no statistical difference (ANOVA, P=0.591) amongst the recoveries obtained for different *m/z* extraction windows. However, since all the recoveries for the ± 10 ppm *m/z* extraction window were systematically <120%, this value was selected for the rest of this study.

Regarding method linearity, results showed no significant difference for any of the compounds at all three *m/z* extraction window values (ANOVA, P=0.462). Pearson coefficients were between 0.9960 and 0.9996 with the only exception of tylosin A, which presented a slightly lower R value of 0.9736 at ± 5 ppm. These high Pearson coefficients were similar or higher than those reported for honey or for other matrices using the direct injection approach [8, 12]. For this reason, the method is considered linear.

With regards to repeatability, there was a clear difference between the results at ± 5 ppm and at ± 10 and ± 20 ppm, and RSD values of up to 37% for tylosin A were recorded at ± 5 ppm. Repeatability for ± 10 and ± 20 ppm were all below 10%. This difference was confirmed by the statistical tests (ANOVA, P=0.014). As commented before with the instrument linearity, this can be due to the fact that the signals are generally smaller at ± 5 ppm in comparison with ± 10 and ± 20 ppm, and this may affect repeatability. At ± 10 and ±20 ppm, the RSD values obtained in this study were in the same order or lower than those reported by other authors [7, 12].

In conclusion, a *m/z* extraction window of ± 10 ppm was selected for the treatment of the honey sample data as satisfactory performances were obtained at that value. This value has also been used by other authors doing similar studies on organic contaminants and metabolites in food with HPLC-HRMS [23, 39].

Figure 3.1. shows the overlapped extracted ion chromatograms of the 7 analytes spiked in one of the validation samples at a concentration of 0.2 $\mu g.g^{-1}$, corresponding to the MRL of tylosin in honey, extracted using a *m/z* extraction window of ± 10 ppm. Chromatographic peaks for all 7 compounds can be clearly identified with minimal background interferences with the present method. Altogether, satisfactory performances were obtained for 7 key veterinary compounds with the present method, with the added benefits of (i) much shorter analysis times compared to current methods, and (ii) recording non-targeted information for future re-assessment of the data. In addition, this direct injection approach would satisfy some of the requirements of green analytical chemistry, since it is a direct analytical technique that avoids sample treatment, it has minimal sample size and reduced reagent consumption in comparison with the traditional methods of honey analysis, derivatization is avoided, and it is a multi-analyte method [40].

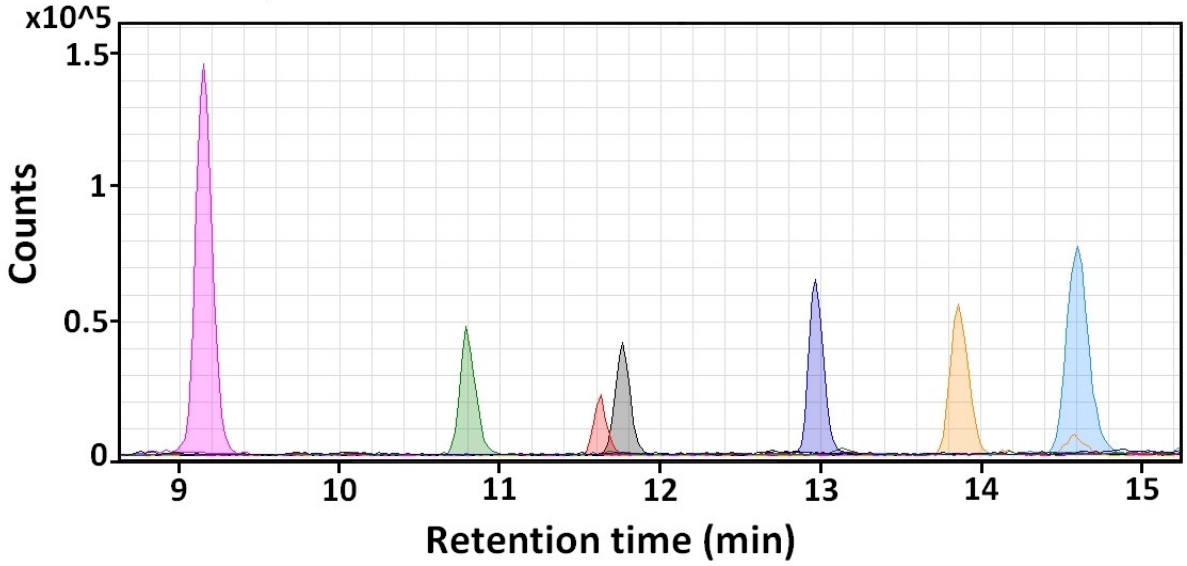

Figure 3.1. Overlapped extracted ion chromatograms for the 7 antibiotics in sample H7 spiked at a concentration corresponding to 0.2 µg.g^{-1} in honey (Sample preparation Method A). Order of elution: lincomycin, sulfamethazine, sulfamethoxazole, furazolidone, sulfadimethoxine, tylosin B and tylosin A.

3.4.3 Application to honey samples

The above optimized method was applied to 35 honey samples collected from the Canadian market. Most of the results were below the MDL, and only tylosin A, tylosin B, sulfamethazine and sulfadimethoxine were detected in some samples. The concentrations of these four antibiotics in the samples they were detected are shown in **Table 3.3**. As mentioned earlier, in absence of hydrolysis, these concentrations correspond to the free species of the compounds in honey. The highest values were obtained for tylosin B, with concentrations up to 0.0703 µg.g^{-1}. None of the samples contained residues of tylosin (A+B) above the MRL of 0.2 µg.g^{-1} set in Canada. Similarly, the levels of free sulfamethazine and sulfadimethoxine were below their respective recommended WRL in Canada (0.03 µg.g^{-1}).

Table 3.3. Concentration ($\mu g.g^{-1}$) of tylosin A, tylosin B, sulfamethazine and sulfadimethoxine in the honey samples they were detected, at a *m/z* extraction window of ± 10 ppm.

Sample	Tylosin A	Tylosin B	Sulfamethazine[c]	Sulfadimethoxine[c]
1	ND[a]	0.0021	ND	ND
6	ND	ND	ND	0.0045
7	ND	ND	ND	0.0039
8	ND	ND	ND	0.0042
11	ND	ND	ND	0.0017
14	ND	ND	<0.0023[b]	0.0035
15	ND	ND	ND	0.0074
18	ND	ND	ND	0.0022
29	ND	ND	ND	<0.0017[b]
30	ND	ND	<0.0023[b]	ND
32	<0.0076[b]	0.0221	ND	ND
35	0.0176	0.0703	ND	ND

[a] ND = non-detected. [b] Compounds detected at concentrations below the LOQ.

[c] Concentration of sulfamethazine and sulfadimethoxine refers to the free form of these compounds in honey.

In their most recent National Chemical Residue Monitoring Program (NCRMP, 2013-2014 report), the CFIA reported the occurrence of tylosin A in 10.05% of the domestic honey samples and in 6.10% of the imported ones. The frequency of detection for tylosin B was 19.74 and 14.71% for domestic and imported honey, respectively [41]. In this study, 6% of the samples were positive for tylosin A and 9% for tylosin B, so the rates of detection of these compounds are similar to those found by the CFIA. No sulfonamide antibiotics were reported by the CFIA in their 2013-2014 report, but sulfadimethoxine and sulfamethoxazole had been detected in 4.35 and 3.26% of the imported honeys, respectively, in the 2012-2013 NCRMP CFIA Report [42]. In the present

study, free sulfadimethoxine was detected in 23% of the honeys, so the present rate of detection of this compound was higher than that reported by the CFIA in 2012-2013.

Tylosin A is the main component of the commercial tylosin formulation applied to the honeybees [43]. The ratio of mass concentrations of tylosin A to tylosin B in the present study was measured to be 0.16 and 0.25 for samples 32 and 35 respectively. These values are lower than the overall average reported by Thompson *et al.* of 1.2 ± 0.2 in honey in Canada [44]. Tylosin A degrades into tylosin B in honey, suggesting that the ratio of their concentrations can decrease over time [43, 45]. Bohm *et al.* studied tylosin A and tylosin B in honey following the application of tylosin A tartrate on honeybees, and their ratio decreased from 4.31 after 3 days of application to 0.73 after 52 days [46]. As a consequence, the World Health Organization Expert Committee on Food Additives (JECFA) listed tylosin B as a major end product of tylosin A in honey, and recommended to take into account both tylosin A and B when considering food safety [47].

3.4.4 Comparison with a standard method

The nine samples provided by CFIA had been previously tested for their content of tylosin A using the official CFIA method ACC-066. These results were used as a blind comparison to test the performance of the present new method. As shown in **Table 3.4**, the results of this study matched the positive and negative results reported by the CFIA. Moreover, the concentrations of tylosin A measured with the present method were comparable to those obtained with the official methods for the two positive samples. In conclusion, this method is expected to perform well for the monitoring of veterinary drugs in honey, with low chances of misclassification of samples (false negatives or false positives).

Table 3.4. Concentration of tylosin A in the CFIA honey samples according to the reference method and in the present method, expressed as $\mu g.g^{-1}$.

Sample	Reference method (CFIA ACC-066)	Present method (MDL=0.0023 $\mu g.g^{-1}$)
27	ND	ND[a]
28	ND	ND
29	ND	ND
30	ND	ND
31	ND	ND
32	0.0060	<0.0076[b]
33	ND	ND
34	ND	ND
35	0.0136	0.0176

[a] ND = non-detected. [b] Detected at a concentration below the LOQ.

3.4.5 Performances of the method including an acid hydrolysis step (Method B)

The addition of an acid hydrolysis step (Method B) was tested to assess the total content of sulfadimethoxine, sulfamethazine and sulfamethoxazole in honey. Method performances are presented in **Table S3.2**. The direct injection method following an acid hydrolysis gave overall satisfactory results for the three sulfonamides. Matrix effects were however greater for sulfadimethoxine and sulfamethoxazole, and precision was slightly poorer (19-23%) for sulfonamides with respect to the initial direct injection method. It is important to highlight that tylosin A and furazolidone were detected in honey samples treated by acid hydrolysis. Low recoveries for tylosin A were expected for Method B, since tylosin A has been reported to degrade under acidic conditions [48].

In order to confirm the effect of acid hydrolysis on sugar-sulfonamide conjugates, additional spiked honey samples (H7, H18 and HX26) were equilibrated overnight and prepared using both Method A and Method B. **Figure 3.2 A-C** show the peaks of sulfamethoxazole, sulfamethazine and sulfadimethoxine spiked in sample H18 and extracted with both methods. In all three samples, the amount of sulfonamides detected following acid hydrolysis (Method B) was greater than without hydrolysis (Method A), confirming the release of conjugated sulfonamides under acidic conditions. In parallel, the mass spectra and the retention time of the glucose-sulfonamide conjugates were determined in the HPLC-QTOF-MS chromatograms obtained for the three sulfonamide-glucose standard mixtures (**Figure S3.2**). This information was then used to interpret the chromatograms obtained for the three spiked honey samples equilibrated overnight and extracted with and without acid hydrolysis. As observed in **Figure 3.2 D-F**, the glucose-sulfonamide conjugates were detected in honey samples injected in the HPLC-QTOF-MS without hydrolysis, but not after acid hydrolysis. The attribution of this signal to a glucose-sulfonamide conjugate was confirmed by comparison of the retention time (**Figure S3.2**) and the All Ions MS/MS spectra of the synthesized conjugate. Indeed, the $[M+Na]^+$ ion (*m/z* 438.0947 for glucose-sulfamethoxazole conjugate, *m/z* 463.1263 for glucose-sulfamethazine conjugate and *m/z* 495.1162 for glucose-sulfadimethoxine conjugate) and one characteristic fragment (*m/z* 254.0594 for glucose-sulfamethoxazole conjugate, *m/z* 186.0330 for glucose-sulfamethazine conjugate and *m/z* 156.0764 for glucose-sulfadimethoxine conjugate) were observed for each conjugate in the honey samples and in the standard mixture with similar relative abundances. To the best of our knowledge, this is the first time these glucose-sulfonamide conjugates have been reported in honey, and this was made possible through the coupling of direct injection and HRMS and the interpretation of All Ions MS/MS data. The detection of these conjugates in food samples has very promising applications, as it opens the doors to including sulfonamides in multi-residue and non-targeted methods without compromising the stability of other analytes (e.g. tylosin A) with an extra acid hydrolysis step. Further studies are required to fully validate the quantification of these conjugates.

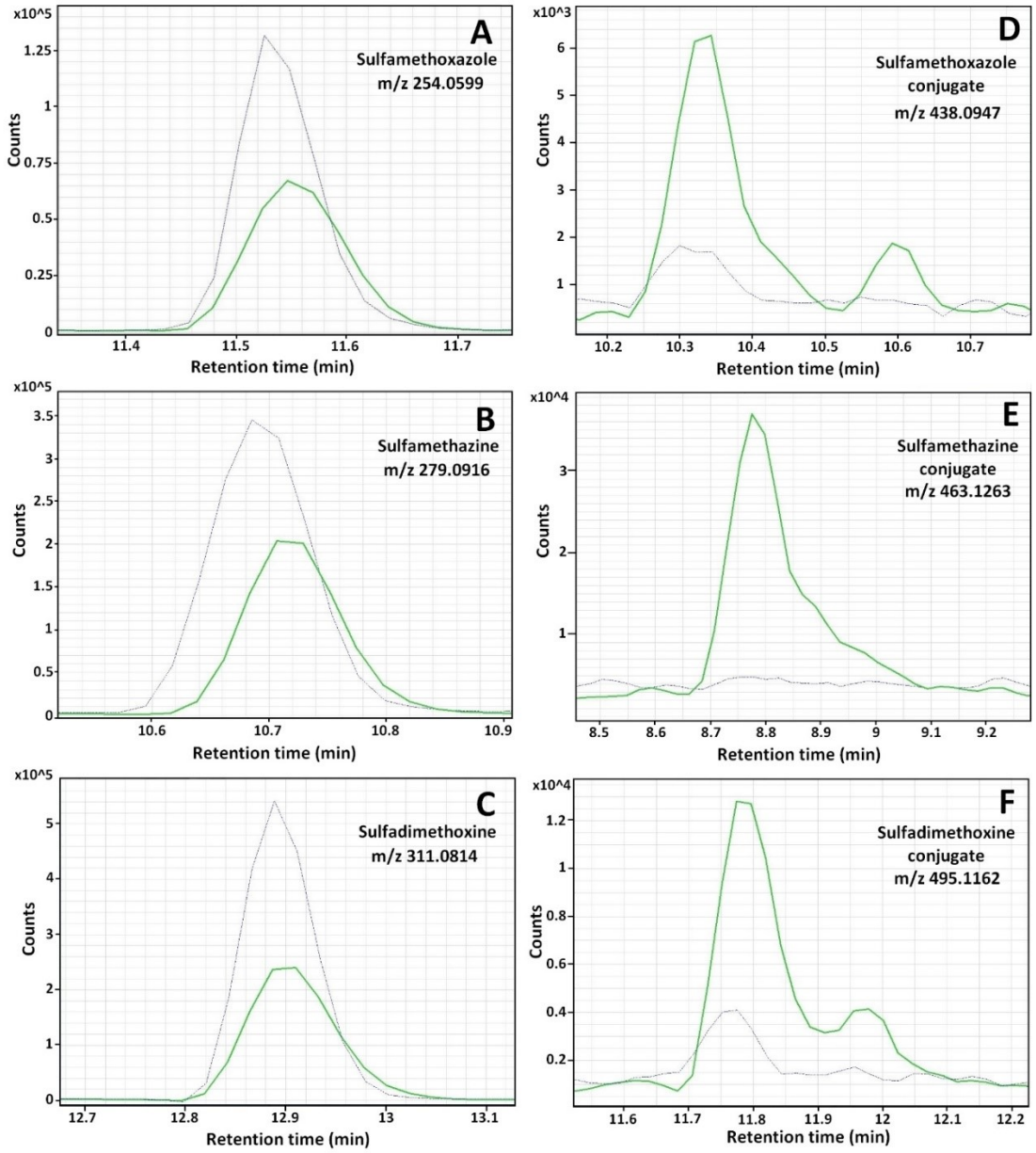

Figure 3.2. Extracted ion chromatograms of sulfamethazine (A), sulfamethoxazole (B), sulfadimethoxine (C), glucose-sulfamethazine conjugate (D), glucose-sulfamethoxazole conjugate (E) and glucose-sulfadimethoxine (F) in sample H18 spiked with all 7 target veterinary drugs at a level corresponding to 0.2 $\mu g.g^{-1}$ in honey, which was extracted with hydrolysis (blue dotted line) and without hydrolysis (green line). The extracted ions in A-C and D-F were $[M+H]^+$ and $[M+Na]^+$, respectively.

3.4.6 Application of the method for the non-targeted identification of contaminants in honey

The ultimate application of this method is to provide non-targeted information for future re-examination of the data for other purposes (e.g. in the context of exposure assessment).

To first illustrate the capacity of this approach in identifying unknown compounds in honey based on All Ions MS/MS, the data obtained for one of the matrix blanks spiked with the seven target analytes at a concentration of 2 $ng.g^{-1}$ were treated using Agilent Mass Hunter Workstation Software – Qualitative Analysis B.07.00. The chromatogram was explored using the algorithm Find By Formula, using All Ions MS/MS data and a customized database of honey-related compounds created with Personal Compound Database and Library software (PCDL) for LC/TOF. This algorithm was applied by other authors for identification of suspects using HPLC-QTOF-MS [49]. All 7 analytes were successfully identified in this sample with total scores above 70%, confirming the non-targeted capability of the approach. The total score reflects the probability that a feature being correctly identified as a specific compound, with a score of 100% representing a perfect fit [22]. As an example, **Figure 3.3** shows the All Ions MS/MS spectra of tylosin B in sample H35 and in the standard. The characteristic $[M+H]^+$ ion of tylosin B can be observed in the honey sample at 0, 10 and 20 V with an *m/z* of 772.4459, 772.4454 and 772.4456 respectively (exact mass: 772.4483). Its main fragment $[C_8H_{16}NO_3]^+$, commonly reported by others [50], can be observed at a CE of 20 and 40 V with an *m/z* of 174.1123 and 174.1122 respectively (exact mass: 174.1130). Thus, comparison of the characteristic fragments of this compound between sample and standard to confirm its identification was possible using All Ions MS/MS mode.

To further demonstrate the non-targeted applicability of the method beyond the veterinary drugs of interest, the 35 honey samples were screened for other compounds related to beekeeping using the same Find By Formula Algorithm and different databases of honey-related compounds created with PCDL LC/TOF. Nine out of the 35 honey samples were found to contain hydroxymethylfurfural (HMF), a heat-induced contaminant commonly found in honey samples

that had been submitted to heat treatments or a long storage time [51]. Once the exact mass of this compound was identified as HMF by the Find By Formula Algorithm with a score above 70%, the confirmation of its identity was carried out in the same way as for tylosin B by comparing the All Ions MS/MS spectra with a standard. The $[M+H]^+$ ion of HMF (exact mass: 127.0395) and one of its characteristic fragments (exact mass: 109.0289) were observed with similar relative intensity in the honey samples and in the standard, thus confirming the identity of this compound.

In conclusion, the non-targeted identification of the 7 veterinary drugs in honey as well as another compound beyond the list of spiked compounds was possible using All Ions MS/MS mode, showing the promising non-targeted applications of this method. Further studies are required to explore and optimize the characteristics of the non-targeted identification workflow using such type of data acquisition.

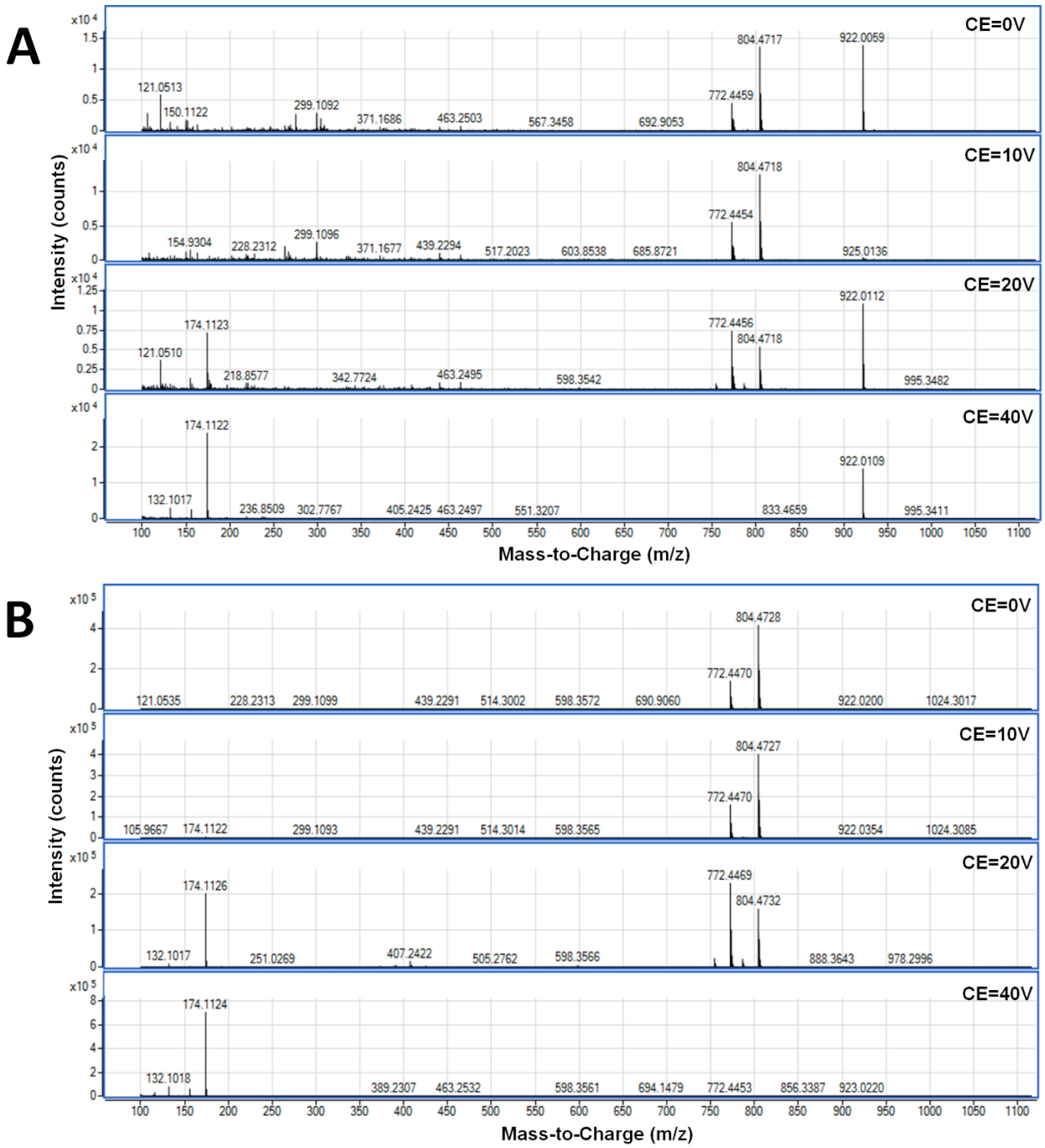

Figure 3.3. All Ions MS/MS spectra of tylosin B for different collision energies (CE). A: in honey sample H35. B: in an analytical standard (20 ng.mL^{-1} in methanol/water).

3.5 CONCLUSIONS

A fast screening and quantification method was successfully developed and validated for the targeted analysis of 7 veterinary drug residues in honey, using direct injection HPLC-QTOF-MS. This method allows for the detection of the selected veterinary drug residues at levels approximately 20 to 100 times lower than the actual regulatory limits, with acceptable recoveries, linearity and repeatability. The total analysis time is only 45 min per sample (sample preparation + analysis in ESI+ mode). Negative ionization will be added in the future to allow for the analysis of other residues (e.g. chloramphenicol). The method was successfully applied to 35 honey samples from the Canadian market. Tylosin A, tylosin B, sulfamethazine and sulfadimethoxine were detected in some samples at levels below the regulatory limits for honey in Canada. All Ions MS/MS data was recorded at four different voltages, allowing for the confirmation of the identity of the target analyte. The continuous recording of accurate mass and All Ions MS/MS data could also allow for non-targeted screenings of other compounds (e.g. pesticides), and this approach will be studied in future work.

3.6 ACKNOWLEDGMENTS

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3.8 SUPPLEMENTARY INFORMATION

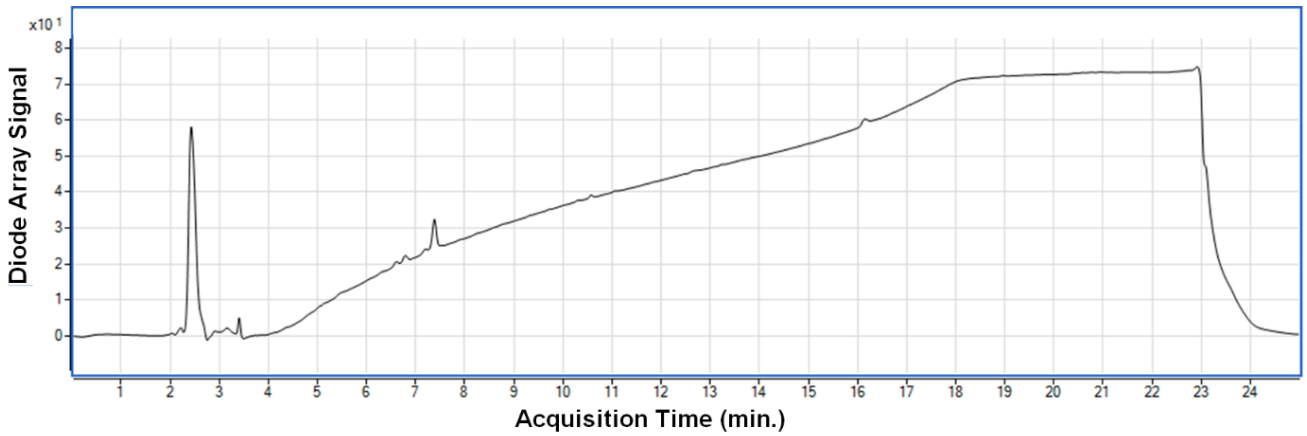

Figure S3.1. Total wavelength chromatogram of a honey sample spiked with the seven veterinary drug residues using HPLC-DAD (scan range: 190 to 640 nm). The first large peak (RT~2.5 min.) corresponds to a potential interference which was diverted to waste.

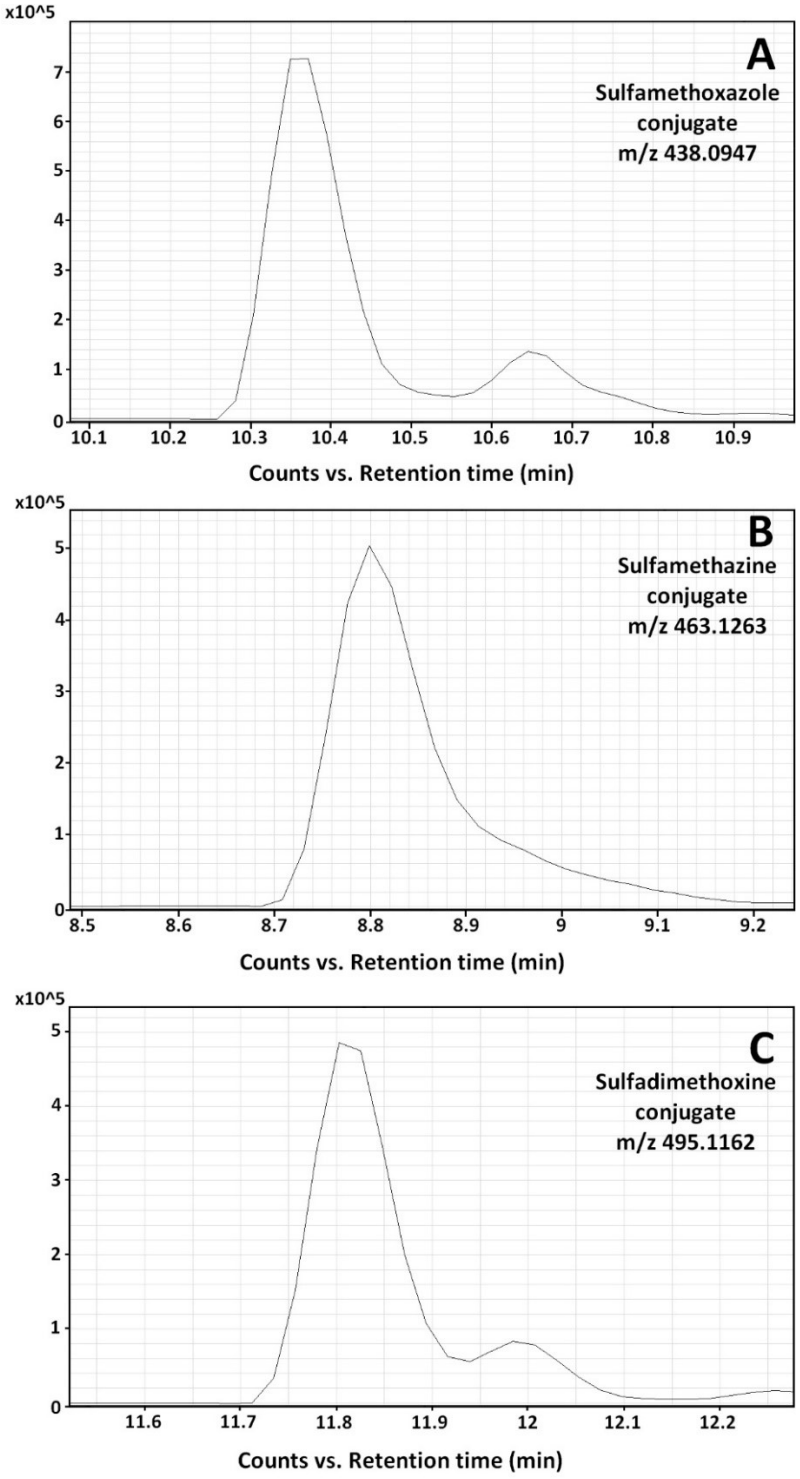

Figure S3.2. Extracted ion chromatograms, corresponding to the $[M+Na]^+$ of the three glucose-sulfonamide conjugates in water/methanol (95:5).

Table S3.1. List of 26 commercial honey samples and detailed information as obtained from their labels.

Sample	Colour[a]	Type of farming	Botanical origin	Geographical origin
H1	Amber	Non-organic	Wildflowers	USA
H2	White	Non-organic	Clover	Canada
H3	White	Non-organic	Acacia	France
H4	White	Non-organic	Clover	Canada
H5	Golden	Non-organic	Meadow and wildflowers (vetch, sweet clover, clover, milkweed)	Canada
H6	Dark	Non-organic	Unknown	Canada
H7	Golden	Organic	Unknown	Canada
H8	Golden	Non-organic	Clover	Canada
H9	Golden	Non-organic	Forest honey	Canada
H10	Golden	Organic	Summer flowers (white clover, melilot, raspberry bush, alfalfa, linden, Wildflowers)	Canada
H11	White	Non-organic	Unknown	Canada
H12	White	Non-organic	Wildflowers	Canada
H13	White	Non-organic	Clover	Canada
H14	White	Organic	Wildflowers	Canada
H15	Golden	Non-organic	Wildflowers	Bulgaria, Guatemala, Mexico, Spain, Thailand and Ukraine

H16	Golden	Non-organic	Orange blossom	Spain
H17	Golden	Non-organic	Unknown	Canada
H18	Dark	Non-organic	Buckwheat	Canada
H19	White	Non-organic	Meadow flowers (spring flowers)	Canada
H20	White	Non-organic	Unknown	Canada
H21	Amber	Non-organic	Wildflowers (fall flowers)	Canada
H22	Amber	Non-organic	Wildflowers	Canada
H23	White	Non-organic	Clover	Canada
H24	White	Non-organic	Clover	Canada
H25	White	Non-organic	Clover, alfalfa, and Wildflowers (summer flowers)	Canada
H26	White	Non-organic	Clover	Canada

[a] In the cases where the colour of the honey was not specified in the label, this was determined by visual comparison with other samples of similar colour whose colour was specified by the manufacturer in the label.

Table S3.2. Comparison of the method performances for the seven targeted veterinary drug residues with and without an acid hydrolysis step (*m/z* extraction window of ± 10 ppm).

Parameter	**Extraction window**	**Tylosin A** ***m/z* 916.5270** **RT[b] 14.64 min**	**Tylosin B** ***m/z* 772.4483** **RT 13.91 min**	**Lincomycin** ***m/z* 407.2216** **RT 9.17 min**	**Furazolidone** ***m/z* 226.0464** **RT 11.75 min**	**Sulfamethoxazole** ***m/z* 254.0599** **RT 10.60 min**	**Sulfamethazine** ***m/z* 279.0916** **RT 10.76 min**	**Sulfadimethoxine** ***m/z* 311.0814** **RT 12.96 min**
Matrix effect (%)[a]	Hydrolysis	ND[d]	255 ± 30	94 ± 24	ND	168 ± 53	100 ± 28	147 ± 32
	No-hydrolysis	98 ± 8	111 ± 9	82 ± 10	98 ± 31	88 ± 17	115 ± 23	102 ± 17
MDL ($\mu g.g^{-1}$ honey)	Hydrolysis	0.0005	0.0508	0.0282	0.0004	0.0047	0.0002	0.0023
	No-hydrolysis	0.0023	0.0015	0.0003	0.0014	0.0018	0.0007	0.0005
LOQ ($\mu g.g^{-1}$ honey)	Hydrolysis	0.0017	0.1677	0.0931	0.0013	0.0155	0.0006	0.0076
	No-hydrolysis	0.0076	0.0050	0.0011	0.0047	0.0060	0.0025	0.0018
Recovery (%)[a,c]	Hydrolysis	ND	83 ± 7	127 ± 41	ND	127 ± 35	137 ± 10	117 ± 23.
	No-hydrolysis	109 ± 10	115 ± 12	119 ± 13	103 ± 16	115 ± 23	118 ± 21	108 ± 12
Method linearity (R)	Hydrolysis	ND	0.9985	0.9948	ND	0.9971	0.9979	0.9926
	No-hydrolysis	0.9980	0.9988	0.9996	0.9963	0.9987	0.9990	0.9987
Repeatability	Hydrolysis	ND	20	22	ND	19	21	23

(RSD %)	No-hydrolysis	4	4	5	5	6	7	7

[a] Matrix effects and recovery values are presented as mean of all concentration levels ± standard deviation (n= 21). [b] RT = Retention Time. [c] For sulfamethoxazole, sulfamethazine and sulfadimethoxine, recovery of the non-hydrolysis method represents that of the compounds in their free form. [d] ND = All or most of the validation samples were below the limit of detection, so the parameter could not be calculated.

CONNECTING PARAGRAPH

The method developed in Chapter 3 showed promising results in non-targeted approaches for the analysis of trace organic contaminants in honey. After developing the two first steps of the analysis workflow (i.e. sample preparation and instrumental analysis), Chapter 4 focuses on the third step: data pre-treatment. Chapter 4 has been accepted for publication in the Journal of the American Society for Mass Spectrometry: A. von Eyken, S. Bayen; *Optimization of the data treatment steps of a non-targeted LC-MS based workflow for the identification of trace chemical residues in honey*.

CHAPTER 4: OPTIMIZATION OF THE DATA TREATMENT STEPS OF A NON-TARGETED LC-MS BASED WORKFLOW FOR THE IDENTIFICATION OF TRACE CHEMICAL RESIDUES IN HONEY

4.1 ABSTRACT

Non-targeted screening (e.g. suspected-target) is emerging as an attractive tool to investigate the occurrence of contaminants in food. The sample preparation and instrument analysis steps are known to influence the identification of analytes with non-targeted workflows, especially for complex matrices. However, for methods based on mass spectrometry, the impact of the post-analysis data treatment (e.g. feature extraction) on the capacity to correctly identify a contaminant at trace level is currently not well understood. The aim of the study was to investigate the influence of seven post-analysis data treatment parameters on the non-targeted identification of trace contaminants in honey using high-performance liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS). Seven compounds reported as veterinary drugs for honey bees were applied as model compounds. Among the parameters studied, the expansion window for chromatogram extraction and the average scans included in the spectra influenced significantly the identification process results. The optimized data treatment was applied to the non-targeted screening of veterinary drugs, pesticides and other contaminants in 55 honey samples as a proof-of-concept. Among the 43 compounds included in a library of honey-related compounds that was used for screening, 8 compounds were tentatively identified in at least one honey sample. The tentative identity of two of these compounds, tylosin A and hydroxymethylfurfural, was further confirmed with analytical standards.

4.2 INTRODUCTION

There has been an increasing demand for analytical techniques that can detect and identify unexpected or unknown contaminants in food matrices without any prior knowledge on their occurrence. As a result, non-targeted strategies, as opposed to the traditional targeted analysis in which methods are designed based on the availability of analytical standards, need to be

developed. The interest for novel non-targeted tools is not limited to food analysis but is also emerging in numerous fields such as environmental analysis or forensics [1-3].

There are generally four components in a non-targeted workflow: sample preparation, instrument analysis, post-analysis data treatment and data interpretation. The non-targeted analysis of trace contaminants in food, where the low concentration of the analytes poses an added challenge, can be achieved using state-of-the-art instruments, e.g. high-performance liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS) [4, 5]. This approach results in relatively large datasets and requires advanced data treatment in order to extract and identify the relatively small signals of the contaminants [6]. The non-targeted identification of compounds based on liquid or gas chromatography coupled to mass spectrometry (LC/GC-MS) data is a multistep process. First, the whole chromatogram needs to be examined in order to create a compound list of all peaks that represent real molecules [7]. During this process, detected ions are assigned a monoisotopic peak with a corresponding *m/z* value, and an isotopic distribution analysis is performed to identify isotopic clusters that might be characteristic of certain elemental compositions. Next, the presence of adducts that may be associated with the eluting compounds is assessed based on a list of potential adducts specified beforehand [8]. At this point, if the mass accuracy is sufficient and there is a minimal isotopic distribution error, the correct molecular formula can be generated for the compounds of interest [9, 10]. Finally, if a compound database is available, mass spectral similarities between an experimental mass spectrum and each mass spectrum in a reference library are assessed [11]. Often, several compounds from a specific database can be associated with a single molecular formula. In that case, acquisition methods using data-dependent or data-independent MS/MS can provide some information (exact mass, isotopic patterns) for both the parent ions and their fragments that can be used to further confirm the correct identification of the target compounds [12].

The correct detection and identification of contaminants in food is critical to ensure the safety of the consumers. Contaminated food samples wrongly assigned as a “non-detect” result (false negative) could lead to an unacceptable exposure of the consumers to potential toxicants. On the other hand, food products wrongly identified as contaminated (false positive) could potentially result into unnecessary food recalls and economic losses. Since the unbiased identification of chemical residues in food products is a matter of concern for public health, regulations such as the 2002/657/EC European Commission Decision have established a system of identification points (IPs) to confirm the identification of organic residues and contaminants using mass spectrometric techniques [13]. The main advantage of this system is to provide a standardized approach to confirm the identity of chemical contaminants [14]. So far, most non-targeted studies have focused on improving the accuracy of compound identification, but little attention has been paid to reducing the false discovery rate [15].

Analytical steps such as sample preparation or instrument analysis are known to influence the list of molecular features obtained in a non-targeted metabolomics [16]. In the case of complex matrices, such as food samples, matrix effects are also known to affect the automatic identification of compounds such as pesticides [12]. More specifically, interferences from the matrix (e.g. signal suppression, co-elution with isobaric compounds) can induce a loss of mass accuracy and lead to erroneous identification of unknowns [7]. This underlines the need for a thorough study and optimization of the data treatment steps to ensure the correct identification of compounds in food matrices. Some researchers have reported the influence of the “exact mass tolerance” parameter used to compare the measured *m/z* (experimental) with that in the database (theoretical) on the non-targeted identification of contaminants in food matrices (i.e. fruits and vegetables) [7, 17, 18]. In metabolomics, the need for identifying the parameters that have the main impact on number and quality of reported metabolites has been recognized for a while [19]. Recently, Tian *et al*. demonstrated the influence of post-acquisition data processing for the non-targeted screening of trace leachable residues from reusable plastic bottles [20]. However, to the best of our knowledge, there has been no systematic assessment of the

influence of any other parameters of post-analysis data treatment other than the exact mass tolerance on the identification of trace contaminants in food.

Among the infinite variety of food matrices that can be used as a model for the study of the influence of non-targeted identification parameters, honey offers the advantage of being able to contain an extended range of contaminants, both agricultural and environmental, at trace levels (in the order of mg kg^{-1} to µg kg^{-1}) [21-23]. Honey bees fly up to 4 km in all directions from their hive and thus have access to an area of 50 km^2, which allow them to be exposed to a wide variety of contaminants in a large area [24]. In addition, a method for the determination of seven veterinary drugs in honey based on a simple dilute-and-shoot procedure followed by direct injection into HPLC-QTOF-MS/MS was recently developed [25]. Although this was initially a targeted analysis method, the use of a data-independent MS/MS acquisition such as the All Ions mode showed promising applications in non-targeted analysis of contaminants in honey.

The aim of the study was to investigate the influence of the post-analysis data treatment parameters on the non-targeted identification of trace contaminants (suspected-target) in honey, using seven veterinary drugs as model compounds. More specifically, an assessment was made to study the impact of the following: match mass tolerance, the mass extraction window, the isotope abundance score, the peak filter absolute height, the average of spectra, the exclusion of TOF spectra and the post-processing peak filters. To date, there has no comprehensive study of the impact of these parameters in the non-targeted identification of contaminants in honey. Optimized data treatment conditions were then applied to the non-targeted screening of veterinary drugs, pesticides and other contaminants in honey collected in Canada.

4.3 EXPERIMENTAL

4.3.1 Chemicals and reagents

Analytical standards (tylosin A (≥ 94.2%), lincomycin (≥ 95.0%), furazolidone (≥ 99%), sulfamethoxazole (≥ 99%), sulfadimethoxine (≥ 98.5%), sulfamethazine (≥ 99%) and 5-hydroxymethyl-2-furaldehyde (≥ 99%)) were purchased from Sigma-Aldrich (St Louis, MO, USA). Tylosin B (≥ 99%) was purchased from Toku-E (Bellingham, WA, USA). Deuterated internal standards, D_3-diphenylhydramine (≥ 99.4%) and D_3-6-acetylmorphine (≥ 99.5%), were purchased from Cerilliant (Round Rock, TX, USA). HPLC grade solvents (water, methanol, acetonitrile, acetone and 2-propanol), as well as LC/MS grade formic acid were all obtained from Fisher Chemical (Pittsburgh, PA, USA).

4.3.2 Honey Samples

Fifty-five honey samples were purchased from different stores and farmers' markets in the Montreal and Calgary regions (Canada) in May 2016 and May 2017. They were all labelled as "unpasteurized" and were of various prices and quality (i.e. different colours and different floral and geographical origins, some labelled as organic as defined in Canada) [26]. Subsamples of each honey were transferred from their original container to 40 mL amber glass vials and kept in the freezer at -18˚C until analysis. In an earlier study [25], these samples were analyzed and fifteen of them showed no detectable residues of any of the 7 tested antimicrobials, so they were considered as blank matrices for the present study.

4.3.3 Sample Preparation

The sample preparation follows a method described in an earlier paper [25]. In Short, approximately 0.2 g of honey was weighed in a glass conic tube and 2 mL of acetonitrile:water mixture (1:1) were added. Samples were vortexed until the honey was completely dissolved, and

then filtered through a 0.22 µm PTFE filter from Chrom4 (Thüringen, Germany). Before injection into the HPLC system, the extract was further diluted with water to a final concentration corresponding to 1% of honey (w/v), and 50 µL of a 0.4 µg mL^{-1} mixture of the two deuterated internal standards were added. These internal standards were not added for quantification purposes, but serve as a reference for retention time and sensitivity [27].

For the optimization of the identification parameters, the fifteen matrix blanks were spiked before dilution with 50 µL of a mixture of the seven antimicrobials at a concentration corresponding to 0.2 µg g^{-1} of honey. This concentration corresponds to the maximum regulatory limit (MRL) for tylosin A + B in honey in Canada [28].

4.3.4 Instrument analysis

Samples were analyzed using a 1290 series LC system from Agilent Technologies (Santa Clara, CA, USA) equipped with an InfinityLab Poroshell 120 Phenyl Hexyl (3.0 x 100 mm, 2.7 µm) column fitted with an InfinityLab Poroshell 120 EC-C18 (3.0 x 5 mm, 2.7 µm) guard column, both from Agilent Technologies. This column, which has improved selectivity for aromatic compounds, was used in a previous study and was found to successfully separate the seven veterinary drugs used in the present study [25, 29]. The mobile phase composition was prepared as reported in the literature on non-targeted analysis using reverse-phase liquid chromatography. In that case, the mobile phase commonly consists of a mixture of water and methanol or acetonitrile, often with the addition of a small quantity of mobile phase modifiers (e.g. ammonium formate, ammonium acetate, formic acid or acetic acid) to improve the separation and detection of compounds [30, 31]. This mobile phase is generally used starting with a low proportion of organic phase (i.e. 5-10%), held in isocratic mode for a few minutes. A gradient is then generally applied until 100% of organic phase. Finally, the mobile phase composition went back to the initial conditions to re-equilibrate the system for a few minutes [31]. In the present study, the mobile phase consisted of water (solvent A) and methanol (solvent B), both with 0.1% formic acid, at a flow rate of 0.2 mL min^{-1}. The mobile phase gradient profile was as follows: 1 min 5% B, from 1 to 15 min gradient

to 100% B, from 15 to 20 min 100 % B, from 20 to 20.10 min gradient to 5% B and from 20.10 to 25 min 5% B. The injection volume was 20 µL and the column temperature was set to 20°C.

The LC system was coupled to a 6545 series Q-TOF from Agilent Technologies equipped with a Dual AJS ESI ion source operating in positive ionization mode. Drying gas temperature was 325°C with a flow of 5 L min^{-1}, sheath gas temperature was 275°C with a flow of 12 L.min^{-1}, the pressure on the nebulizer was 20 psi, the capillary voltage was 4000 V, the fragmentor voltage was 175 V, the skimmer voltage was 65 V and the nozzle voltage was 2000 V. All Ions MS/MS data was collected (scans between *m/z* 70 and 1700 at a scan rate of 3 spectra/s for four different collision energies: 0, 10, 20 and 40 V). This scan speed corresponded to about 14 spectra per peak for each collision energy. This data-independent MS/MS acquisition mode has been reported to be more appropriate for large-scale screening than the classic product ion scans because it provided excellent fragmentation information for confirmatory purposes for an unlimited number of compounds [32]. The first 3 min of elution were diverted to waste. Samples were kept at 4°C in the multisampler compartment.

4.3.5 Data treatment

The datasets were processed with MassHunter Profinder B.08.00 software (Agilent Technologies), using "*Batch Targeted Feature Extraction*". This algorithm extracts features from the acquired data, combining the "Find Compounds by Formula" algorithm with a database containing molecular formulas, mass, and/or retention time information [33]. *Batch Targeted Feature Extraction* was run under different conditions to determine which parameter(s) influence the rates of detection and identification of veterinary drugs present in honey at trace level. The parameters were selected based on what has been reported by others, and other additional parameters representative of the different steps of the feature extraction algorithm were also added [7, 8, 18, 34, 35]. The 7 parameters tested included: *match tolerance mass, expansion values for chromatogram extraction, isotope abundance score, peak filter absolute height, spectra to include average scans, exclude TOF spectra if above, and post-processing filter absolute*

height. A detailed explanation of the selected parameters and the values tested for each of them is presented in the Supplementary Information. For each experiment, all parameters were fixed at an initial value and only one of them was altered at a time. Compound identification in the non-targeted mode was completed using a reduced library dataset that contained only the 7 antimicrobials, which was created from previously existing libraries using the software Mass Hunter PCDL Manager for Metabolomics B.07.00 from Agilent Technologies. The library for the first part of this study was reduced to 7 compounds with the purpose of speeding up the experiment, but the size of the library does not influence the identification results from the *Batch Targeted Feature Extraction* algorithm. Retention time was not considered when searching in the database, as it is specific to the HPLC conditions used to acquire the data. The positive ions and adducts considered were H^+, Na^+ and K^+. Finally, only compounds with a minimum total score of 70% were considered, which is what is commonly reported for this type of analysis [7, 11].

4.3.6 Experimental design

According to the literature, veterinary drug residues may be detected at a low frequency in actual honey samples. In 2013-2014, the Canadian Food Inspection Agency carried out tests for veterinary drug residues on 280 samples of domestic honey and 108 samples of imported honey in Canada, and the percentage of samples with detected residues was 26.07% and 12.04% for domestic and imported honeys, respectively [36]. As a result, we studied the influence of the number of samples above the LOD in a batch, on the correct non-targeted identification of trace compounds. Different groups of datasets were produced using different ratios of matrix blanks, spiked and unspiked samples, to mimic a range of contaminant frequencies of the veterinary drug residues in honey samples (**Table 4.1**). Each batch of data files contained 15 honey sample extracts and 5 procedural blanks, so a total of 20 data files per batch were used. Group A, B and C corresponded to 75%, 25% and 5% of the samples in the batch containing detectable amounts of veterinary drug residues, respectively. The spiked samples in the 3 batches of group B and C

were different, and were selected to represent a range of colour, floral origin, packaging and organic/non-organic farming matrices.

Two-way analyses of variance (ANOVA) were performed using IBM SPSS Statistics 23 (NY, USA) to determine whether the different parameters tested produced statistically significant differences.

Table 4.1. Composition of the groups of samples used to optimize the data pre-treatment parameters.

Group	Batch	Spiked samples	Matrix blanks	Procedural blanks	Samples per batch	Samples per group	Expected positives	Expected negatives
A	A	15	0	5	20	20	15	5
B	B1	5	10	5	20	60	15	45
	B2	5	10	5	20			
	B3	5	10	5	20			
C	C1	1	14	5	20	60	3	57
	C2	1	14	5	20			
	C3	1	14	5	20			

4.3.7 Application of the optimized data pre-treatment to the non-targeted analysis of actual honey samples

As a proof-of-concept, the optimized data treatment was used to screen 55 honey samples from the Canadian market for veterinary drugs, pesticides and other contaminants. Since no commercial library dataset was found containing only compounds related to honey, a subset

from METLIN Metabolome Database was created based on 42 chemicals reported in the production of honey or previously detected in honey. Hydroxymethylfurfural (HMF) was not part of the METLIN Metabolome Database, so it was manually added using the experimental exact mass and spectral data with LC-MS recorded from an analytical standard. Details on the compounds included in the library are provided as Supplementary Information (**Table S4.1**). Five procedural blanks were run together with the honey samples and were included in the data treatment. In this study, only compounds whose signal was greater than the average + 3 σ of the blank signals were considered.

4.3.8 Confirmation of the non-targeted identification of compounds in honey samples

According to the level system proposed by Schymanski *et al*. [37], the level of confidence of the identification for the compounds obtained using the *Batch Targeted Feature Extraction* algorithm is 4, since it is based on the exact mass and the isotopic signature. In order to increase the identification confidence level of these compounds, MS/MS data should be compared with that of a compound database to provide a probable structure. Ultimately, the structure should be confirmed with a reference standard to achieve level 1 of confidence, which represents the highest level. In the present study, the identity of the compounds identified by the *Batch Targeted Feature Extraction* was further confirmed using the parent ion and most abundant daughter ion found with the All Ions MS/MS data with those reported in the database or in the literature. For each compound, both the presence of these ions in the MS/MS spectra as well as their intensity ratio were studied.

When the MS/MS spectra of the sample matched those reported elsewhere, a final confirmation was performed with analytical standards to achieve a confidence level of 1. This identification was done following the IPs system established by the 2002/657/EC European Commission Decision [13]. For the confirmation of the substances listed in group B of Annex I of Directive 96/23/EC, which include veterinary drugs and other contaminants (i.e. organochlorine compounds including PCBs, organophosphorus compounds, chemical elements, mycotoxins,

dyes and others), a minimum of 3 IPs is required [38]. In addition to having 3 IPs, the 2002/657/EC European Commission Decision establishes that a minimum of one ion ratio should be measured, that all measured ion ratios need to meet maximum permitted tolerances for relative ion intensities, and that a maximum of three separate techniques can be combined to achieve the minimum number of IPs [13]. For LC or GC with mass-spectrometric detection, IPs are assigned depending on the type of ion measured (i.e. parent or fragment ions) and the resolving power of the mass analyzer. However, some difficulties appear when trying to classify mass analyzers as low- or high-resolution because modern low-resolution mass analyzers can now reach resolutions comparable to the traditional HRMS instruments [39]. Alternatively, Hernández *et al.* proposed a criterion for assignment of IPs based on mass measurement accuracy instead of resolution, which was used in the present study [40]. For the calculation of the IPs, two ions were selected for each compound: MS1 (parent ion) and MS2 (most abundant fragment). The abundance of each ion was read at the collision energy (0, 10, 20 or 40 V) that produced the highest peak. In addition to the IPs, the retention time difference between the compound in honey and the analytical standard was calculated, and only differences below 0.3 min were considered a match [17].

4.4 RESULTS AND DISCUSSION

4.4.1 Optimization of the identification workflow

The aim of this study was to describe and optimize the influence of the post-analysis data treatment parameters on the non-targeted identification of trace contaminants in honey using seven veterinary drugs as model compounds. The number of erroneous identifications (i.e. false positives and false negatives) for each parameter for test groups A, B and C are presented in **Table 4.2**, **Table 4.3** and **Table 4.4**, respectively.

The maximum mass error between the measured ion (experimental) and the database (theoretical) to consider a match did not influence the correct identification of compounds (ANOVA, p=0.084). In general, an increase in mass tolerance makes the search in the compound database less restrictive, and this could lead to an increase in the number of false positives. For this reason, when screening for pesticide residues in food, Mezcua *et al.* recommended the use of a mass tolerance of ± 10 mDa for screening purposes, and to lower the tolerance to ± 1 mDa and/or 5 ppm when confirming the identity of compounds [18]. Similar mass tolerances have been reported for the non-targeted analysis of trace contaminants using a library database. Indeed, a mass tolerance of ± 5 ppm was reported in environmental monitoring studies, including the screening of environmental contaminants in honey bees and pollen, and the occurrence of xenobiotics in blood of sea turtles [11, 41]. Herrera-Lopez *et al*. reported a mass tolerance of ± 2.5 and ± 5 ppm for MS and MS/MS data, respectively, for the screening of organic contaminants in water samples and Sjerps *et al*. used a mass tolerance of ± 5 ppm [12, 34]. Regarding food matrices, Malato *et al*. performed a screening for pesticides in fruits and vegetables with three mass tolerances (0.6 mDa, 1 mDa and 5 ppm) and determined that the best option according to their results was to use a mass tolerance ≤ 5 ppm when confirming the identity [7]. Malato *et al*. discussed the difference between the use of relative (ppm) or absolute (mDa) mass tolerance values and concluded that a relative mass tolerance value should be applied in order to avoid dependence on the search criteria with each compound mass (especially when dealing with a wide range of compounds) [7]. Another factor to consider when determining which mass match tolerance to use is the mass accuracy of the instrument. Using a match mass tolerance value far lower than the error of the acquired mass could result in an increase of false negatives, as the measured mass would not match those reported in the library. The average mass accuracies obtained in the present study were -1.8 ± 0.3, -1.8 ± 0.3, -1.4 ± 0.4, -2.4 ± 0.5, -1.9 ± 0.5, -2.2 ± 0.3, and -2.5 ± 0.6 ppm for the $[M+H]^+$ fragments of tylosin A, tylosin B, lincomycin, furazolidone, sulfadimethoxine, sulfamethazine, and sulfamethoxazole, respectively. Therefore, a match mass tolerance of ± 5 ppm was selected as it led to acceptable results in this study, in line with what has been reported by others.

Next, the influence of the expansion value for chromatogram extraction was tested. The rate of erroneous identifications of the compounds increased significantly with the expansion value (ANOVA, $p<0.001$). Indeed, up to 11 identifications were incorrect when the expansion window was increased to ± 500 ppm (**Table 4.2**, **Table 4.3** and **Table 4.4**). An increase of false positives was expected when increasing the extraction window, because this criterion would become less restrictive and more features may be matched with a specific formula. However, this was not observed in the present study. Instead, the number of false negatives increased, notably for tylosin B and sulfadimethoxine when applying an expansion value of ± 500 ppm. To further explore this, the signal-to-noise ratio (S/N) was assessed for each compound in their respective chromatograms extracted using ± 10 and ± 500 ppm (**Figure 4.1**). S/N significantly decreased with an increasing mass extraction window for tylosin A, lincomycin, furazolidone, sulfamethoxazole, sulfamethazine, and sulfadimethoxine (ANOVA, $p<0.001$ in all cases). Dasenaki et al. have demonstrated that unsatisfactory fragmentation or insufficient sensitivity of fragment ions can hinder the identification of veterinary residues in fish and milk using HPLC-QTOF-MS [42]. Using the present method, S/N were systematically above 10 for the 7 trace contaminants (spike level 0.2 $\mu g.g^{-1}$; see **Figure 4.1**) and molecular features corresponding to the compounds were detected. The chemical noise or the presence of interferences could be at the origin of the false identifications, for example for tylosin B and sulfadimethoxine. There was no significant difference in the number of erroneous identifications between ± 10 and ± 50 ppm (ANOVA, $p=0.844$), so ± 10 ppm was selected as expansion value for chromatogram extraction. This value has been reported by other authors [35, 41].

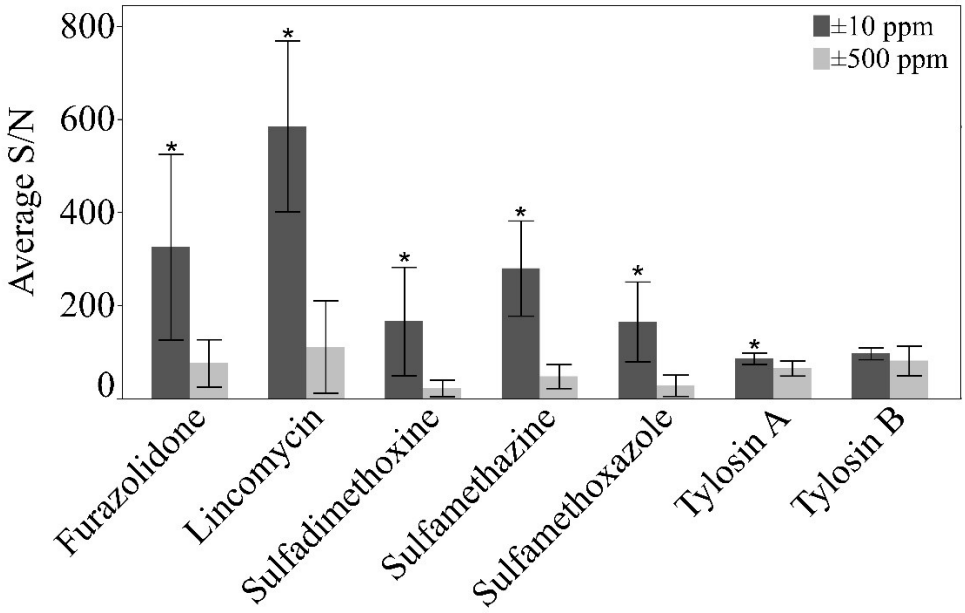

Figure 4.1. Comparison of the mean S/N obtained from chromatograms extracted with a mass extraction window of ± 10 and ± 500 ppm (*m/z* for each compound are described in **Table 4.2**).
* Indicates significant difference between S/N at ± 10 and ± 500 ppm.

The next parameter, the isotope abundance score, had no significant impact on the identification of trace contaminants in honey (ANOVA, p=0.334), as the same rate of correct identification was obtained for 60% and 100% of isotope abundance score (**Table 4.2**, **Table 4.3** and **Table 4.4**). This parameter is rarely reported in similar studies. Sjerps *et al*. used an isotopic pattern similarity of above 90% for the analysis of different organic contaminants in water, but no information on its optimization had been reported [34]. Instead, the literature generally focusses on the total score, which is a combination of mass match score, isotope abundance score, isotope spacing score and retention time score. In this study, only the identifications with a total score ≥ 70% were considered, as suggested by other authors in similar studies [7, 11]. Alternatively, Knolhoff *et al*. used an overall quality score of ≥ 50% for the non-targeted analysis of organic compounds in orange juice and milk [8]. However, lowering the total score makes the identification less

restrictive, and this could potentially lead to an increase of the rate of erroneous identifications. Since altering the isotope abundance score did not affect the identification, the default value of 60% given by the software was chosen for the final method.

Three absolute height filters were tested and there was no statistical difference among the false identification rates (ANOVA, p=0.556). An increase in false negatives could be expected when increasing the absolute height filter, especially for trace contaminants as their peaks may be relatively small. However, increasing this threshold from 100 to 1000 counts did not affect the rate of correct identification of the veterinary drug residues in the present study (**Table 4.2**, **Table 4.3** and **Table 4.4**). This study was performed at a residue concentration of 0.2 mg kg^{-1}, and this resulted in peaks whose height was in the order of 10^5 to 10^6 counts, explaining the lack of impact of the absolute height filter. This threshold could however become of great importance when screening for compounds in concentrations in the low µg kg^{-1} to ng kg^{-1} range. Knolhoff *et al.* used a minimum ion peak height of 500 counts for the non-targeted analysis of organic compounds in orange juice and milk, while Mezcua *et al.* and Malato *et al.* used a peak filter of 100 for the analysis of pesticides in fruits and vegetables [7, 8, 18]. In the present study an intermediate value of 200 counts was finally selected.

Regarding the two parameters affecting the spectra to be included in the identification (s*pectra to include average scans* and *exclude TOF spectra if above)*, the different average of scans produced significantly different results, while the possible exclusion of the TOF spectra did not affect them (ANOVA, spectra to include average scans p<0.001, exclude TOF spectra p=0.556). When examining closely the multiple comparisons between pairs of values, it was observed that taking the spectra at the apex of the peak or at above 30% of the peak height lead to the exact same results (ANOVA, p=1.00), which correspond to the lowest rate of erroneous identifications (**Table 4.2**, **Table 4.3** and **Table 4.4**). For the rest of the study, spectra at the apex of the peaks were used in order to avoid possible interferences from coeluting compounds on trace contaminants. Consequently, the option of excluding part of the TOF spectra was deselected.

Finally, the different post-processing peak filters were tested and did not significantly affect the correct identification of the veterinary drug residues (ANOVA, p=0.556). As in the case of peak filters, selecting a high value for the post processing peak filters could lead to the omission of relevant peaks, and thus increase the rate of false negatives for trace contaminants. The range of post processing peak filters used by other authors in similar studies is in the same order of those tested in the present study, going from a compound filter of only 400 counts for the analysis of pesticides in fruits and vegetables by Malato *et al*., to up to 5000 counts for the analysis of organic compounds in orange juice and milk by Knolhoff *et al* [7, 8]. In some studies, this parameter has been expressed as a relative abundance instead of an absolute height. Indeed, Mezcua *et al*. used a compound filter of 0.01% of the most abundant peak [18]. However, the use of a relative abundance filter for the identification of trace contaminants, especially in a dilute-and-shoot approach such as the one used in the present study where the final extract is a very complex matrix, could lead to a significant elimination of peaks corresponding to trace-level compounds. An intermediate value of 1000 counts was selected in the present study.

The number of spiked samples in the sample batch was found to influence the rate of correct identification of the seven veterinary drugs for all tested parameters (ANOVA, match mass tolerance $p<0.001$, expansion values for chromatogram extraction $p<0.001$, isotope abundance score $p<0.001$, peak filter absolute height $p<0.001$, spectra to include average scans $p<0.001$, exclude TOF spectra $p<0.001$, post processing filters $p<0.001$). As shown in **Table 4.5**, the lowest amount of erroneous identifications (0.5% of false negatives and no false positives) was obtained for group C, which corresponded to a frequency of true positives of 5% (1 spiked sample per batch of 20 samples). In their two most recent National Chemical Residue Monitoring Program (NCRMP, 2013-2014 and 2012-2013 reports), the CFIA reported the occurrence of tylosin A, tylosin B, sulfadimethoxine and sulfamethoxazole at rates of detection of 6.10-10.05%, 14.71-19.74%, 4.35% and 3.26%, respectively [36, 43]. Thus, the frequency of true positives of group C would correspond to the frequency of detection of these compounds in a real-case scenario. Therefore, based on these results, the optimized identification workflow developed in the

present study should be applied to the screening of veterinary drugs in honey in order to expect the lowest rate of erroneous results.

Table 4.2. Number of false positives (+) and false negatives (-) for each compound in samples from group A.

		Tylosin A		**Tylosin B**		**Lincomycin**		**Furazolidone**		**Sulfadimethoxine**		**Sulfamethazine**		**Sulfamethoxazole**	
		***m/z* 916.5270**		***m/z* 772.4483**		***m/z* 407.2216**		***m/z* 226.0464**		***m/z* 311.0814**		***m/z* 279.0916**		***m/z* 254.0599**	
		+	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
Match mass tolerance	± 1 ppm	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	± 10 ppm[a]	0	1	0	0	0	0	0	0	0	0	0	1	0	0
	± 50 ppm	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Expansion values for	± 10 ppm[a]	0	1	0	0	0	0	0	0	0	0	0	1	0	0
chromatogram	± 50 ppm	0	0	0	0	0	0	0	0	0	2	0	0	0	0
extraction	± 500 ppm	0	1	0	4	0	0	0	0	0	2	0	0	0	4
Isotope abundance	60%[a]	0	1	0	0	0	0	0	0	0	0	0	1	0	0
score	100%	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Peak filter absolute	100 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0
high	200 counts[a]	0	1	0	0	0	0	0	0	0	0	0	1	0	0
	1000 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Spectra to include	> 1% of peak height	0	1	0	0	0	0	0	0	0	0	0	1	0	0
average scans	> 10% of peak height[a]	0	1	0	0	0	0	0	0	0	0	0	1	0	0

	> 30% of peak height	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	At apex of peak	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exclude TOF spectra	> 20% saturation[a]	0	1	0	0	0	0	0	0	0	0	0	1	0	0
	> 40% saturation	0	1	0	0	0	0	0	0	0	0	0	1	0	0
	Unselected	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Post-processing filter	≥ 200 counts[a]	0	1	0	0	0	0	0	0	0	0	0	1	0	0
absolute height	≥ 1000 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0
	≥ 2500 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0

[a]Initial conditions of the test.

Table 4.3. Number of false positives (+) and false negatives (-) with scores above 70% for each compound in samples from group B.

		Tylosin A		**Tylosin B**		**Lincomycin**		**Furazolidone**		**Sulfadimethoxine**		**Sulfamethazine**		**Sulfamethoxazole**	
		***m/z* 916.5270**		***m/z* 772.4483**		***m/z* 407.2216**		***m/z* 226.0464**		***m/z* 311.0814**		***m/z* 279.0916**		***m/z* 254.0599**	
		+	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
Match mass	± 1 ppm	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tolerance	± 10 ppm[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	± 50 ppm	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Expansion values	± 10 ppm[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
for chromatogram	± 50 ppm	0	0	0	0	0	0	0	0	0	2	0	0	0	0
extraction	± 500 ppm	0	1	0	4	0	0	0	0	0	0	0	0	0	4
Isotope abundance	60%[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
score	100%	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Peak filter absolute	100 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0
high	200 counts[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1000 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Spectra to include	> 1% of peak height	0	1	0	0	0	0	0	0	0	0	0	1	0	0
average scans	> 10% of peak height[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	> 30% of peak height	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	At apex of peak	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exclude TOF	> 20% saturation[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
spectra	> 40% saturation	0	1	0	0	0	0	0	0	0	0	0	1	0	0
	Unselected	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Post-processing	≥ 200 counts[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
filter absolute	≥ 1000 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0
height	≥ 2500 counts	0	1	0	0	0	0	0	0	0	0	0	1	0	0

[a]Initial conditions of the test.

Table 4.4. Number of false positives (+) and false negatives (-) with scores above 70% for each compound in samples from group C.

		Tylosin A		**Tylosin B**		**Lincomycin**		**Furazolidone**		**Sulfadimethoxine**		**Sulfamethazine**		**Sulfamethoxazole**	
		***m/z* 916.5270**		***m/z* 772.4483**		***m/z* 407.2216**		***m/z* 226.0464**		***m/z* 311.0814**		***m/z* 279.0916**		***m/z* 254.0599**	
		+	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
Match mass	± 1 ppm	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tolerance	± 10 ppm[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	± 50 ppm	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Expansion values	± 10 ppm[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
for chromatogram	± 50 ppm	0	0	0	0	0	0	0	0	0	1	0	0	0	0
extraction	± 500 ppm	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Isotope abundance	60%[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
score	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peak filter absolute	100 counts	0	0	0	0	0	0	0	0	0	0	0	0	0	0
high	200 counts[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1000 counts	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spectra to include	> 1% of peak height	0	0	0	0	0	0	0	0	0	0	0	0	0	0
average scans	> 10% of peak height[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	> 30% of peak height	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	At apex of peak	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exclude TOF	> 20% saturation[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
spectra	> 40% saturation	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Unselected	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Post-processing	≥ 200 counts[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
filter absolute	≥ 1000 counts	0	0	0	0	0	0	0	0	0	0	0	0	0	0
height	≥ 2500 counts	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]Initial conditions of the test.

Table 4.5. Summary of the rate (%) of false positives and false negatives compared to the total number of identifications for all the compounds and all the samples for each test group.

Test group	Samples per group	Total identifications	% of all identifications being false positives	% of all identifications being false negatives
A	20	140	0	32.1
B	60	420	0	6.9
C	60	420	0	0.5

4.4.2 Screening of veterinary drugs and other contaminants in actual honey samples

Forty-three compounds were included in the library of honey-related compounds to screen 55 honey samples collected in Canada. Eight compounds were tentatively identified in at least one sample with a score above 70% and with an intensity above the mean + 3σ of the blank signals (**Table 4.6**). All Ions MS/MS data were compared to MS/MS fragmentation information from the literature or from database to investigate the identity of these 8 compounds. Among the compounds with spectral information in the database (i. e. carvone, metolcarb, thiacloprid and tylosin A), only tylosin A presented MS/MS spectra similar to those in the library. With regards to the remaining four compounds (i.e. HMF, nitenpyram, piperonyl butoxide and rolitetracycline), only HMF was found to match the spectra reported in the literature [44-47].

Table 4.6. List of compounds from the library of honey-related products identified by the non-targeted workflow in at least one honey sample collected in Canada. Information is provided for each compound on the number of samples in which the compound was detected, the average score for each compound and whether the compounds had similar MS/MS to those reported in the literature or in the database.

Compound	Samples	Score	Similar MS/MS
Carvone	3	99	No
HMF	21	100	Yes
Metolcarb	41	99.9	No
Nitenpyram	1	82.4	No
Piperonyl butoxide	9	94.1	No
Rolitetracycline	2	96.3	No
Thiacloprid	1	76	No
Tylosin A	1	92.2	Yes

The identity of tylosin A and HMF was further confirmed using analytical standards according to the IPs system established by the 2002/657/EC European Commission Decision [13]. Details on the identification of both compounds are presented in **Table 4.7**. For tylosin A, a total of 4 IPs was earned from MS1 and MS2 and the error of the measured MS2/MS1 was 1%, confirming the identity of this compound with the maximum level of confidence. Regarding HMF, a total of 4 IPs was earned from MS1 and MS2 and the error of the measure MS2/MS1 was 10 ± 8%, confirming the identity of this compound with the maximum level of confidence. Additionally, in both cases the difference in retention time between the compound in honey and the analytical standard was below 0.3 min. Visual comparisons of the retention time and MS/MS spectra between the honey samples and standards for tylosin A and HMF are presented in **Figures 4.2** and **4.3**.

Table 4.7. Identification of tylosin A and HMF following the IPs system established by the 2002/657/EC European Commission Decision.

	Tylosin A (n=1) **MS1 (0 V): *m/z* 916.5270** **MS2 (40 V): *m/z* 772.4483**	**HMF (n=21)** **MS1 (0 V): *m/z* 127.0395** **MS2 (10 V): *m/z* 109.0289**
Mass error of MS1	< 2 mDa	< 2 mDa
IPs earned for MS1	2	2
Mass error of MS2	< 2 mDa	< 2 mDa
IPs earned for MS2	2	2
Total IPs earned	**4**	**4**
MS2/MS1 sample	0.411	1.099 ± 0.122
MS2/MS1 standard	0.406	1.015
Error in the measured ratio	**1%**	**10 ± 8%**
Maximum permitted tolerance	± 25%	± 20%
CONFIRMED?	**YES**	**YES**

Tylosin A and HMF are both commonly detected in honey. Tylosin A is a macrolide antibiotic approved for example by the US Food and Drug Administration for emergency use in the control of American foulbrood (AFB) of honey bees, the most virulent disease known to affect these animals [48]. The CFIA, in their 2013-2014 National Chemical Residue Monitoring Program report, detected tylosin in up to 10.05% of the tested domestic honeys and 6.10% of honeys imported to Canada [36]. HMF is a heat-induced contaminant formed as a product of the Maillard reaction occurring in many food commodities, such as bread or baked goods [49]. In honey, this compound can be formed when honey is submitted to heat treatments, such as pasteurization, or a long storage time [50]. For this reason, HMF is commonly used as a quality indicator in honey [51]. In addition, HMF can be formed in food commodities even at low temperatures in acidic conditions, such as the case of honey [52].

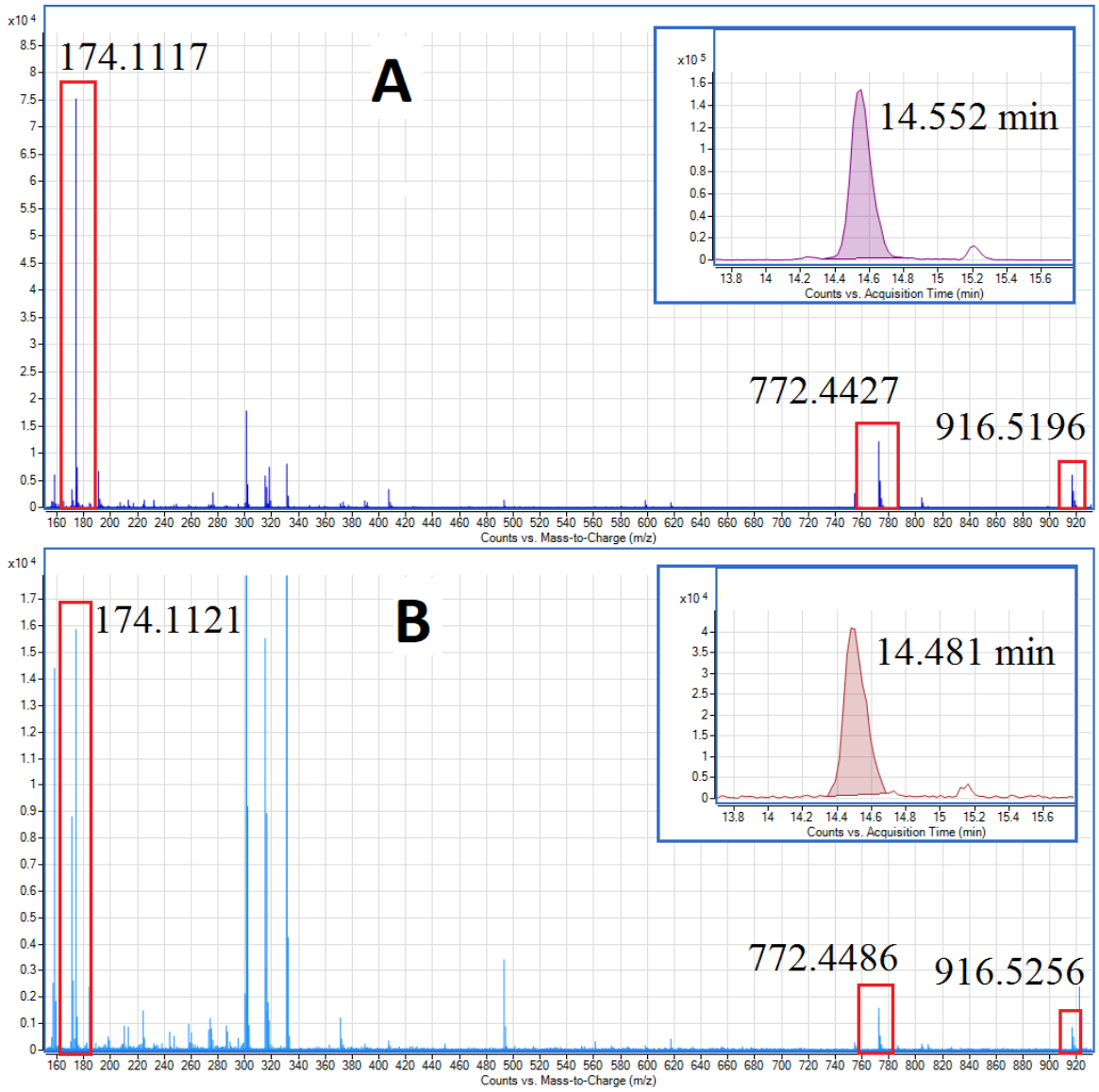

Figure 4.2. Extracted chromatograms and MS spectra (collision energy=40 V) of tylosin A obtained through All-Ions MS/MS in a 20 ng mL-1 standard solution in methanol (A) and in the honey sample where it was identified (B).

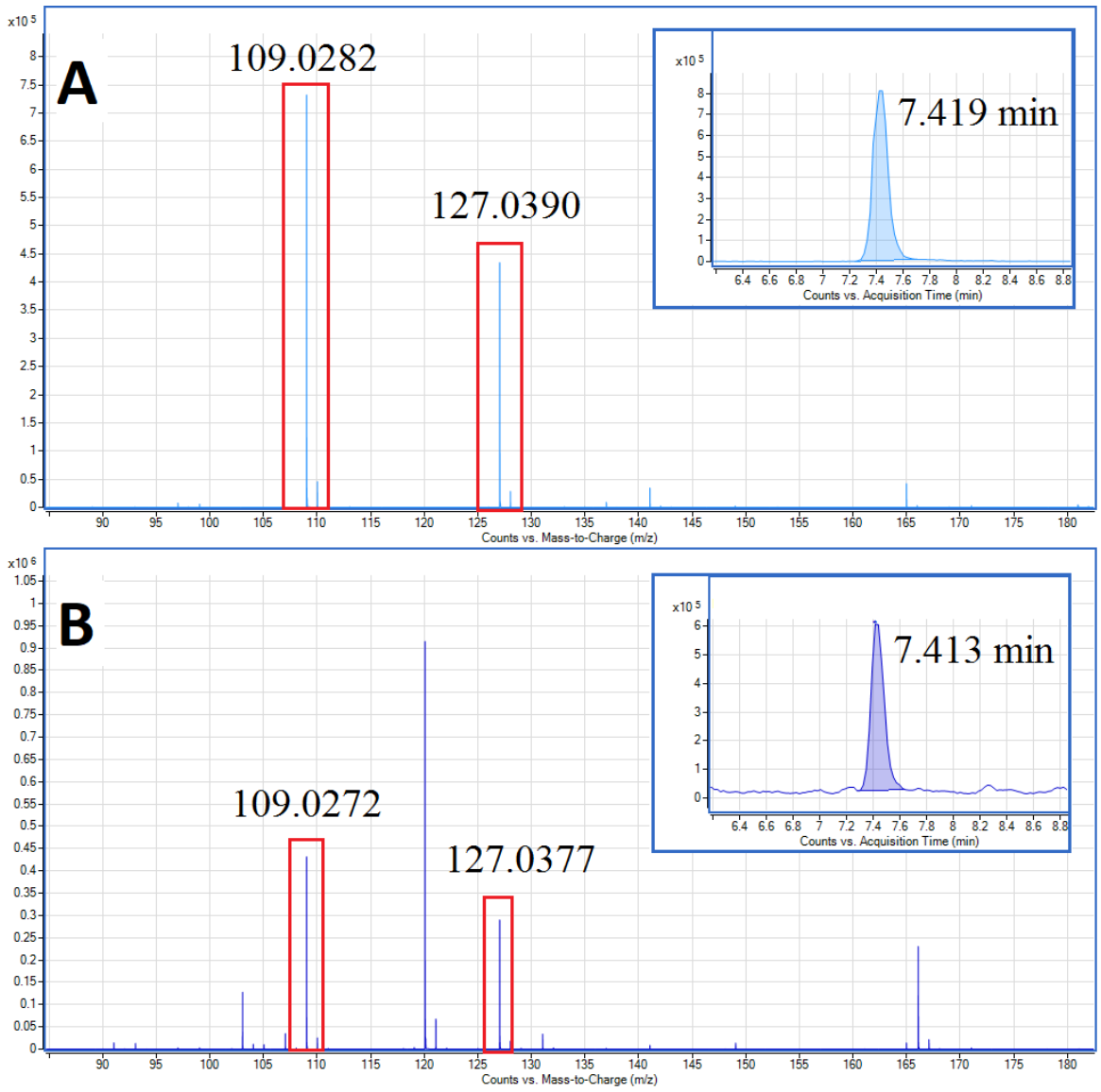

Figure 4.3. Extracted chromatograms and MS spectra (collision energy=0 V) of HMF obtained through All-Ions MS/MS in a 10 µg mL-1 standard solution in water (A) and in one of the honey samples where it was identified (B).

4.5 CONCLUSIONS

The impact of the match mass tolerance, the mass extraction window, the isotope abundance score, the peak filter absolute height, the average of spectra included, the exclusion of TOF spectra and the post-processing peak filters on the correct identification of seven veterinary drug residues (model trace residues) in honey was assessed. Among these 7 parameters, the expansion window for chromatogram extraction and the average scans included in the spectra influenced significantly the identification results. Based on the present comprehensive study of the influence of each parameter and a review of values reported in the literature, the following optimized conditions were selected: match mass tolerance of ±5 ppm, an expansion value for chromatogram extraction of ±10 ppm, an isotope abundance score of 60%, a peak filter absolute height of 200 counts, the spectra being recorded only at the apex of the peak and a post processing peak filter absolute height of 1000 counts.

This optimized method was used to screen 55 honey samples from the Canadian market using a library of 43 honey-related compounds, including veterinary drugs, pesticides and other contaminants. Eight compounds were tentatively identified in at least one sample with a score above 70% and with an intensity significantly above the blanks. Among these compounds, tylosin A and hydroxymethylfurfural were further identified using analytical standards. The results of this study show that post-acquisition data treatment parameters can affect the identification of trace contaminants in food such as honey. Since the presence of chemical residues in food products is a matter of concern for public health, a systematic assessment of post-acquisition data treatment parameters should be conducted before applying non-targeted workflows to the identification of trace contaminants in food matrices.

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4.8 SUPPLEMENTARY INFORMATION

List of parameters

- *Match tolerance mass*: this parameter represents the maximum mass error between the measured *m/z* and the *m/z* of the putative match in the database. It is based on an abundance weighted average of the mass errors for each of the ions recorded [1]. The initial value was set to ±10 ppm, and two additional tolerances of ±1 and ±50 ppm were tested.
- *Expansion values for chromatogram extraction*: this parameter corresponds to the *m/z* extraction window used during the feature extraction. The initial extraction window was set to ± 50 ppm (symmetric), therefore in the same order of what has been reported by other authors [2]. In a previous study using a targeted approach with the same compounds in honey, an extraction window of ± 10 ppm was found to yield the best results, so this extraction window was also tested in the present study [3]. An extreme extraction window of ± 500 ppm was also evaluated in order to assess the impact of this parameter when using an extreme case.
- *Isotope abundance score*: this parameter represents the weight given to the isotope abundance score on the total score calculation. The total score reflects the probability that a feature being correctly identified as a specific compound, being a score of 100% a perfect fit [4]. It is calculated as the average of four components: the mass match score, the isotope abundance score, the isotope spacing score and the retention time score [1]. Because isotopic pattern matching generally plays an important role on the identification of compounds, the effect of the isotope abundance score was evaluated in the present study [5]. To do so, a value of 60% was initially selected by default by the software, and a value of 100% was also tested.
- *Peak filter absolute height*: this parameter limits the number of peaks extracted based on their absolute height. The specification of a minimum height will reduce the likelihood of

small peaks or noise being detected and incorrectly assigned to a compound. This filter is typically set to 200 counts just as a threshold for the background or chemical noise [6]. Thus, the initial value was set to 200 counts, and two additional filters were tested: 100 and 1000 counts.

- *Spectra to include average scans*: this parameter defines which spectra are extracted for each peak in the chromatogram. It can either be the average of all spectra acquired within a specified percentage of the total height of the peak, or only the single spectrum recorded at the apex of the peak. When the latter option is selected, then the parameters corresponding to the TOF spectra are not available. At the beginning of the experiment, this parameter was set by default to extract the average of scans above 10% of the peak height. Subsequently, the extractions at above 1 and 30% of the peak height as well as the extraction of spectrum at the apex of the peak were tested.
- *Exclude TOF spectra if above*: during the extraction of spectra for each peak, this parameter excludes from the calculation of the average those peaks whose intensity is above a given percentage of the saturation of the detector. High ion counts, causing saturation of the detector, can result in a higher mass measurement error [7]. An option is offered in the MassHunter software to exclude TOF spectra above a given percentage of the saturation of the detector in order to minimize these effects [4]. This value was initially set by default at “20% of saturation”, and a value of “40% of saturation” was also tested. The omission of this option, which would result in no exclusion of any TOF spectra and, thus, the inclusion of all TOF spectra for the identification, was also tested.
- *Post-processing filter absolute height*: as part of the filters that can be used in processing the compound groups, this filter sets the requirement of a minimum absolute peak height to include the compound as found. The initial value was set to ≥ 200 counts, and two additional values of ≥ 1000 and ≥ 2500 counts were tested.

Table S4.1. List of compounds included in the library along with their description and relation with honey.

Compound	Description
Acetamiprid	Neonicotinoid insecticide [8]. Found in Austrian honeys at concentrations between < 0.6 and 15.2 µg kg-1 [8].
Amitraz	Pesticide used in Canada for the control of parasitic varroa mites for honey bees [9]. Found in Canada in 21.32% of domestic honeys and 43.24% of imported honeys, at concentrations between 0.00200 and 0.06280 µg g-1 [9].
Ampicillin	Veterinary drug used for the control of American foulbrood (AFB) [10]. Found in Canada in 1 imported honey in 2013 at a concentration of 0.00010 µg g-1 [11].
Bacitracin	Veterinary drug used for the control of AFB [12].
Bifenthrin	Pyrethroid insecticide found in wax, pollen and bee bodies in North American hives [13]. In honey, it was found in Canada in 2013 at a concentration of 0.00200 µg g-1 [11].
Carbendazim	Fungicide extensively used to control various leaf and fruit pests [14]. Found in Canada in less than 5% of domestic and imported honeys at concentrations between 0.01220 and 0.01290 µg g-1 [9]
Carvone	Natural component of essential oils used to treat AFB [15].
Chloramphenicol	Veterinary drug used in the past for the control of AFB [16]. Banned in Canada for use in any food producing animal [17]. Found in honey samples in Ukraine at concentrations between 0.2 and 2.2 µg kg-1 [18].
Chlortetracycline	Broad-spectrum veterinary drug used in honey bees [19].
4-epi-chlortetracycline	Broad-spectrum veterinary drug used in honey bees [19].
Ciprofloxacin	Veterinary drug used as a prophylaxis for bee diseases [19]. Found in Canada in 7 imported honeys in 2014 at concentrations between 0.00267 and 0.00110 µg g-1 [9].

Clothianidin	Neonicotinoid insecticide found in Egypt in pollen and bees at concentrations of 4.53 and 0.06-10.38 ng g-1, respectively [20].
Coumaphos	Acaricide used to control varroatosis ascospherosis in hives [21]. Found in Canada in less than 5% of domestic and imported honeys in 2014 at concentrations between 0.00430 and 0.01500 μg g-1 [9].
Cyprodinil	Fungicide found in Canada in 3 domestic honeys in 2013 at concentrations between 0.00230 and 0.00920 μg g-1 [11]
Diethyltoluamide (DEET)	Widely used insect repellent found in honeybee wax combs in Belgium [22].
Dimetridazole	Used to prevent and control Nosema apis in honey bees [23]. Banned in Canada for use in any food producing animal [17]. It has been reported that it can migrate from the wax to the honey [24].
Erythromycin A	Veterinary drug used for the treatment of AFB [12]. Found in honey in China at a concentration of up to 14.9 ng g-1 [25].
Fludioxonil	Pesticide found in Canada in 3 domestic honeys in 2013 at concentrations between 0.00280 and 0.01480 μg g-1 [11].
Fumagillin	Veterinary drug used to prevent and control nosemosis [19]. Found in Canada in 24.37% of domestic honeys and 1.22% of imported honeys in 2014 at concentrations between 0.00110 and 34.00000 μg g-1 [9].
Furazolidone	Veterinary drug used to treat several bacterial diseases in bees [26].
Hydroxymethylfurfural (HMF)	Heat-induced contaminant formed as a product of the Maillard Reaction occurring in many food commodities, such as bread or baked goods [27]. Used as indicator of quality of honey [28].
Imidacloprid	Neonicotinoid insecticide [8]. Found in honey in France at concentrations between <0.3 and 21.8 μg kg-1 [29]
Iprodione	Pesticide found in Canada in 6.95% of domestic honeys in 2014 at concentrations between 0.00630 and 0.05500 μg g-1 [9].
Lincomycin	Veterinary drug used to control AFB [19].

Metolcarb	Insecticide that has been extensively used to control rice leafhoppers, plant hoppers and fruit flies in agricultural production [30]. It has not been reported in honey, but in other food matrices such as fruits and vegetables [31].
Metronidazole	Used to prevent and control Nosema apis in honey bees [23]. Banned in Canada for use in any food producing animal [17]. Found in honeys in China at concentrations between <0.64 and 66.95 μg kg-1 [32].
Nitenpyram	Neonicotinoid insecticide found to be <5 μg kg-1 in 41 Austrian honey samples [8].
Norfloxacin	Veterinary drug used as a prophylaxis for bee diseases [19].
Oxytetracycline	Veterinary drug used for the control of AFB and European foulbrood (EFB) [33, 34]. Found in Canada in 13.55% of domestic honeys in 2014 at concentrations between 0.00050 and 0.02930 μg g-1 [9].
trans/cis-Permethrin	Pesticide found in Canada in less than 5% of domestic honeys in 2014 at concentrations of 0.00420 and 0.00540 μg g-1 for cis- and trans-permethrin, respectively [9].
Piperonyl butoxide	Pesticide found in Canada in less than 5% of imported honeys in 2014 at concentrations between 0.00330 and 0.02900 μg g-1 [9].
Propoxur	Pesticide found in Canada in 1 domestic honey in 2013 at a concentration of 0.00900 μg g-1 [11].
Rolitetracycline	Veterinary drug used for the control of AFB and European foulbrood (EFB) [33, 34].
Ronidazole	Used to prevent and control Nosema apis in honey bees [23]. Banned in Canada for use in any food producing animal [17]. It has been reported that it can migrate from the wax to the honey [24].
Streptomycin	Veterinary drug used to protect bees against a variety of brood diseases [19].
Sulfadimethoxine	Veterinary drug used for the control of different bacterial and protozoal diseases in bees [19]. Found in Canada in 4.35% of imported honeys in 2013 at concentrations between 0.01280 and 0.19200 μg g-1 [11]

Sulfamethazine	Veterinary drug used for the control of different bacterial and protozoal diseases in bees [19]. Found in Canada in 1 imported honey in 2013 at a concentration of 0.44800 μg g-1 [11]
Sulfamethoxazole	Veterinary drug used for the control of different bacterial and protozoal diseases in bees [19]. Found in Canada in 1 imported honey sample in 2014 at a concentration of 0.66500 μg g-1 [9].
Thiacloprid	Neonicotinoid insecticide found in Austrian honeys at concentrations between <0.6 and 27.4 μg kg-1 [8].
Thiamethoxam	Neonicotinoid insecticide found in Austrian honeys at concentrations <2 μg kg-1 [8].
Tinidazole	Used to prevent and control Nosema apis in honey bees [23]. Banned in Canada for use in any food producing animal [17].
Tylosin A	Veterinary drug used for the control of AFB [35]. Found in Canada in 10.05% of domestic honeys and 6.10% of imported honeys in 2014 at concentrations between 0.00106 and 0.18000 μg g-1 [9].
Tylosin B	Main degradation compound of tylosin A [36]. Found in Canada in 19.74% of domestic honeys and 14.71% of imported honeys in 2014 at concentrations between 0.00120 and 0.10400 μg g-1 [9].

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CONNECTING PARAGRAPH

A method for the non-targeted analysis of trace organic contaminants in honey was developed and validated in Chapters 3 and 4. This included the main three steps of the analysis workflow: sample preparation, instrumental analysis and data filtering. Chapter 5 continues the non-targeted workflow development with the discussion of different data treatment approaches for the non-targeted study of plastic-related contaminants in honey and comparison of honey samples sold in glass and plastic jars. This chapter will be submitted for publication in the Journal of Agricultural and Food Chemistry: A. von Eyken, S. Ramachandran, S. Bayen; *Suspected-target screening for the assessment of plastic-related chemicals in honey.*

CHAPTER 5: SUSPECTED-TARGET SCREENING FOR THE ASSESSMENT OF PLASTIC-RELATED CHEMICALS IN HONEY

5.1 ABSTRACT

Plastic-related compounds (PRCs) are commonly reported in a variety of foods as a result of the interaction between the food and the packaging material. PRCs include plastic monomers, additives and non-intentionally added substances (NIAS). These contaminants may affect the organoleptic properties of the food or represent a food safety hazard for the consumers. For this reason, the incorporation of such compounds into food contact materials is regulated in many countries. In the case of honey, a few studies have reported trace amounts of these compounds. However, a non-targeted approach capable of screening for PRCs in honey without being limited by the availability of standards has not been reported to date. The aim of this study was to apply a previously developed non-targeted method to screen PRCs in honey and investigate the differences among honey products sold in glass and plastic jars. A total of 662 putative compounds were detected and the identification of two of these compounds, namely 2-ethyhexyladipate (DEHA) and tris (2-butoxyethyl) phosphate (TBOEP), was further confirmed with standards as proof-of-concept for the approach. Their concentrations and estimated daily intakes were calculated, resulting in a total of 0.001 and 0.006 $\mu g.kg^{-1}bw.day^{-1}$ for DEHA and TBOEP, respectively. This led to the conclusion that the levels of these two compounds in the honey samples from the present study were below the tolerable daily intake. With regards to the comparison of the chemical burden in honey samples sold in either glass or plastic jars, various data treatment approaches were used which resulted in different lists of relevant contaminants. As expected based on the low frequency of detection of the 662 PRCs found in honey, unique entity analysis with 100% detection and volcano plot with $p<0.05$ found none and 2 compounds, respectively. When applying a data treatment approach based on the differential frequency of detection, however, the number of relevant compounds increased to 13 in glass and 40 in plastic, among which 6 were unique to honey samples sold in plastic jars and 3 were unique to honey samples sold in glass jars. The different results obtained with the various data treatment approaches suggest that the relatively low frequency of contaminants in food needs to be accounted for when selecting the appropriate data treatment tool to compare groups of samples.

5.2 INTRODUCTION

Honey is a natural food containing mostly sugars but also other constituents such as enzymes, organic acids, amino acids, vitamins and minerals [1]. It is a natural sweetener that has a distinctive colour, aroma and flavour depending on the flowers, geographical regions, climate and honeybee species involved in the production [2, 3]. Honey, as any other food products, can be contaminated with a range of chemicals. Veterinary drugs have been detected in honey as residues of their use to protect the queen, workers, drones or the larvae from fungi, bacteria or viruses [4]. Acaricides, fungicides and insecticides are another class of residues commonly detected in honey for they are applied to control and protect the beehives from diseases such as verroatosis and ascospheriosis [5-7]. Indirect contamination of honey by pesticides can also happen because of extensive use and distribution of pesticides in the environment [8, 9].

Among the many classes of contaminants that have been tested in honey, plastic-related compounds (PRCs) are amidst the least reported so far. PRCs include plastic monomers, additives and non-intentionally added substances (NIAS). Plasticizers are additives that are added to plastic materials to make them softer, more pliable and thus increase their flexibility, workability and distensibility [10]. They are often esters of polycarboxylic acids with linear or branched aliphatic alcohols with moderate chain lengths [10]. Plasticizers do not form chemical bonds with the polymer matrix, and may freely move through the matrix, spacing the polymers apart, thus significantly reducing their glass transition temperature to improve plasticization [11]. This may result in a possible migration of plasticizers into the food in contact with the plastic material. Leachables may then affect the organoleptic properties of the food or represent a food safety hazard for the consumers [12, 13]. Other types of PRCs include compounds related to the packaging material such as surfactants, ink components and adhesives from labels, and also compounds from other sources such as environmental and food processing contaminants.

Plasticizers and additives are regulated in particular when incorporated into food contact materials. In the European Union (EU) for example, food contact materials are regulated by the EU Commission Directive 1935/2004, 2023/2006/EC and 10/2011/UE (modified by EU Regulation 1282/2011) [14, 15]. This directive requires that the manufacturers of all food contact materials provide assurance that the product does not transfer its constituents in quantities that endanger human health [15]. In Canada, the safety of food contact materials is controlled under Division 23 of the Food and Drugs Act and Regulations, which prohibits the sale of foods in packages that may impart any substance to the contents which might be harmful to the consumer of the food [16]. In addition, the Phthalates Regulation under the Canada Consumer Product Safety Act establishes that the concentrations of six majorly used phthalates, which constitute one of the main families of plasticizers, in child care articles and toys that can be placed in the mouth of a child under four years of age have to be below 0.1% by weight [17].

PRCs are commonly reported in a variety of foods (including tea, vegetable oils and fruit jellies, among others), as a result of the interaction between the food and the packaging material [18-21]. In the case of honey, Silva *et al.* studied the migration of diphenyl butadiene, an optical brightener, in various food matrices and found it to be negligible for honey [22]. However, a few other studies have reported trace amounts of various PRCs in honey. A summary of the literature on the occurrence of PRCs in commercial honey samples is presented in **Table 5.1**. Bisphenol A (BPA) and bisphenol F (BPF) were studied in honey (n=107) by Inoue *et al.*, and BPA was detected in a few samples at concentrations up to 33.3 $ng.g^{-1}$, while BPF was not detected [23]. Zhou *et al.* studied 22 phthalate esters in honey (n=10) and found the rates of positive results for dimethyl phthalate (DMP), diethyl phthalate (DEP), diisopropyl phthalate (DIPP), diisobutyl phthalate (DIBP), di-*n*-butyl phthalate (DBP), dicyclohexyl phthalate (DCHP) and di(2-ethylhexyl) phthalate (DEHP) to be 50, 20, 60, 70, 80, 60 and 90%, respectively [24]. Concentrations of these contaminants in honey ranged from non-detected (ND) to 483.1 $ng.g^{-1}$ [24]. Lo Turco *et al.* analyzed 26 plasticizers and BPA in Sicilian and Calabrian honey (n=39) and DEHP was the most abundant plasticizer found with a concentration of up to 202.7 ± 153.1 $\mu g.kg^{-1}$, followed by DBP

with a concentration of 40.3 ± 9.3 µg.kg^{-1} [25]. Česen *et al.* studied bisphenols and related compounds in honey (n=36) and found BPA, bisphenol AF (BPAF), bisphenol E (BPE), BPF, bisphenol S (BPS), and bisphenol Z (BPZ) in amounts up to 107, 53.5, 12.8, 31.6, 302, and 28.4 ng.g^{-1}, respectively [26]. Cao *et al.* studied the presence of 2-ethyhexyladipate (DEHA) and 20 phthalates in honey as part of the Canadian Total Diet Study in 2015, including DIBP, DBP, n-butyl benzyl phthalate (BBzP), DEHA and DEHP, and reported concentrations of up to 4.82 and 135 ng.g^{1} for DEHA and DEHP, respectively. [27].

Table 5.1. Summary of studies reporting the presence of PRCs in commercial honey samples.

PRC	**Level (ng.g^{-1})**	**Type of jar**	**Frequency of detection**	**Geographical origin of the honey**	**Suspected origin of PRC**	**Ref.**
BPA	ND – 33.3 ND – 11.4	Glass (n = 51) Plastic (n = 56)	15 % (n = 51) 16 % (n = 56)	Various countries	Manufacture or transport of honey	[23]
DMP	ND – 41.5	Not specified	50 % (n = 10)	Not specified	Not discussed	[24]
DEP	ND – 25.2		20 % (n = 10)			
DIPP	ND – 82.4		60 % (n = 10)			
DIBP	ND – 79.7		70 % (n = 10)			
DBP	ND – 58.4		80 % (n = 10)			
DCHP	ND – 45.8		60 % (n = 10)			
DEHP	ND – 483.1		90 % (n = 10)			
DMP	ND – 41.5	Collected directly from beekeeper, stored in glass	59 % (n = 39)	Italy	Honey production, environmental pollution, agricultural practices	[25]
DEP	ND – 25.2		26 % (n = 39)			
DIBP	ND – 68.2		61 % (n = 39)			
DBP	ND – 58.4		100 % (n = 39)			
DEHP	ND – 483.1		100 % (n = 39)			

BPA	ND – 97.7	Glass (n = 10)	75 % (n = 36)	Various	Packaging and	[26]
	ND – 107	Plastic (n = 26)		countries	other additional	
BPAF	ND – 47.2	Glass (n = 10)	64 % (n = 36)		sources of	
	ND – 53.5	Plastic (n = 26)			contamination	
BPE	ND – 12.8	Glass (n = 10)	17 % (n = 36)			
	ND – 8.18	Plastic (n = 26)				
BPF	ND	Glass (n = 10)	17 % (n = 36)			
	ND – 31.6	Plastic (n = 26)				
BPS	ND	Glass (n = 10)	3 % (n = 36)			
	ND – 302	Plastic (n = 26)				
BPZ	ND	Glass (n = 10)	14 % (n = 36)			
	ND – 28.4	Plastic (n = 26)				
DIBP	< 16.5	Not specified	Not specified	Not specified	Not discussed	[27]
DBP	< 68.2					
BBzP	< 3.49					
DEHA	4.82					
DEHP	135					

To date, the different strategies reported for the analysis of PRCs are based on a targeted analysis approach based on different types of extractions, cleanups and instrumental analyses (mostly based on gas or liquid chromatography). For example, Lo Turco *et al.* used solid-phase extraction (SPE), using Oasis Hydrophilic-Lipophilic-Balanced glass cartridges and with water and methanol as eluents, followed by gas chromatography coupled to mass spectrometry (GC-MS) [25]. Česen *et al.* used a similar SPE based extraction followed by derivatization using N-(tertbutyldimethylsilyl)-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane in

ethyl acetate for 16 hours at 60°C to make the analytes more volatile, less reactive and thus to improve their chromatographic behaviour prior to GC-MS analysis [26]. Koo *et al.* used a solvent terminated dispersive liquid-liquid microextraction method with acetonitrile as the disperser solvent and 1-hexanol as the extraction solvent, followed by high-performance liquid chromatography (HPLC) coupled to a diode array detector [28].

Despite providing satisfactory results in the aforementioned studies, targeted methods are limited by the availability of analytical standards [29]. As an alternative, non-targeted analysis approaches are currently being developed to identify and semi-quantify a broader range of chemical compounds, particularly in the absence of standards [30, 31]. A non-targeted method for the analysis of contaminants in honey based on direct injection and high-performance liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry (HPLC-QTOF-MS/MS) using data-independent MS/MS acquisition was recently developed [32]. This method was successfully applied for the analysis of free-form veterinary drugs, pesticides and other trace organic contaminants in honey, showing promising results for its potential application to the non-targeted analysis of other contaminants such as PRCs in honey [33].

The aim of the study was to apply the above method to screen PRCs in honey and investigate the differences among honey products sold in glass and plastic jars. More specifically, the goals were to (i) apply a non-targeted workflow to detect and identify PRCs in 104 honey samples collected in Canada, (ii) compare the chemical burden in honey samples sold in either glass or plastic jars, and (iii) use this example to provide recommendation on the application of a non-targeted workflow to the study of trace chemicals in food matrices.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals and reagents

Labelled internal standards, D_3-diphenylhydramine (≥ 99.4%) and D_3-6-acetylmorphine (≥ 99.5%), were purchased from Cerilliant (Round Rock, TX, USA). 1-Ethinyl-1-cyclohexanol (≥ 99%), N-vinylcaprolactam (98%), 2-amino-5-methylbenzoic acid (99%) and tris (2-butoxyethyl) phosphate (TBOEP) (95%) standards were purchased from Sigma-Aldrich (Darmstadt, Germany). DEHA (98%) standard was purchased from Toronto Research Chemicals (Toronto, Canada). HPLC grade solvents (water, methanol, acetonitrile, acetone and 2-propanol) and LC/MS grade formic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA).

5.3.2 Honey Samples

One hundred and four honey samples were obtained from different stores and farmers' markets in the Montreal and Calgary regions (Canada) between May 2016 and May 2018. They were all labelled as unpasteurized and were of various prices and quality (i.e. different types of farming, different colours and different floral and geographical origins). Out of the 104 samples, 31 were sold in a plastic jar and 73 were sold in a glass jar. More information on each sample can be found in the **Table S5.1**. Samples were transferred from their original container to 40 mL amber glass vials and kept in the freezer at -18^{o}C until analysis.

5.3.3 Sample Preparation

Samples were prepared according to a method developed previously [32]. In short, approximately 0.2 g of honey were weighed in a glass conic tube and 2 mL of a mixture of acetonitrile and water 1:1 was added. Samples were vortexed for about 2 min, or until the honey was completely dissolved, and then filtered through a 0.22 µm PTFE filter from Chrom4 (Thüringen, Germany). The objective of the present study was to screen for free chemicals in

honey, so no hydrolysis was performed prior to the injection into the HPLC system [34]. The extract was further diluted with water to a final concentration corresponding to 1% of honey (w/v), and 50 µL of a 0.4 $\mu g.mL^{-1}$ mixture of the two deuterated internal standards was added. These internal standards were added to have a reference for retention time, sensitivity and mass accuracy. Five procedural blanks were prepared following the same method. Additionally, five Quality Control (QC) samples were prepared by mixing equal volumes of all the extracted samples. In the absence of targeted compounds that could be monitored using standards, such QC samples are used in non-targeted analysis as indicators of the quality of the analysis and to detect any possible instrumental artifacts. Post analysis, the results of the QC samples can be examined visually, generally through an unsupervised method such as Principal Component Analysis (PCA), to give an indication of the reproducibility of the analysis [35].

5.3.4 Instrument analysis

Samples were analyzed using a 1290 series LC system from Agilent Technologies (Santa Clara, CA, USA) equipped with an InfinityLab Poroshell 120 Phenyl Hexyl (3.0 x 100 mm, 2.7 µm) column fitted with an InfinityLab Poroshell 120 Ec-C18 (3.0 x 5 mm, 2.7 µm) guard column, both from Agilent Technologies. The mobile phase consisted in water (solvent A) and methanol (solvent B), both with 0.1% formic acid, at a flow rate of 0.2 mL/min. The mobile phase gradient profile was as follows: 1 min 5% B, from 1 to 15 min gradient to 100% B, from 15 to 20 min 100 % B, from 20 to 20.10 min gradient to 5% B and from 20.10 to 25 min 5% B. The injection volume was 20 µL and the column temperature was set to 20°C.

The LC system was coupled to a 6545 series Q-TOF from Agilent Technologies, equipped with a Dual AJS ESI ion source operating in positive ionization mode. Drying gas temperature was 325°C with a flow of 5 $L.min^{-1}$, sheath gas temperature was 275°C with a flow of 12 $L.min^{-1}$, the pressure on the nebulizer was 20 psi, the capillary voltage was 4000 V, the fragmentor voltage was 175 V, the skimmer voltage was 65 V and the nozzle voltage was 2000 V. All Ions MS/MS data was collected as MS scans between *m/z* 70 and 1700 at a scan rate of 3 spectra/s for four different

collision energies (0, 10, 20 and 40 V). This scan speed corresponded to about 14 spectra per peak for each collision energy. The first 3 min of elution were diverted to waste. Samples were kept at 4°C in the multisampler compartment. Samples were analyzed randomly to ensure there was no trend in the results created by an instrumental drift.

In a later stage of this work, MS/MS spectra of two compounds were acquired using the same method using *Targeted MS/MS* mode (instead of All Ions MS/MS mode) to confirm the structure of the suspect compounds in honey. The targeted masses were *m/z* 125.0950 at 12.45 ± 0.15 min and *m/z* 140.1070 at 4.05 ± 0.15 min. The spectra were recorded at three collision energies (10, 20 and 40 V).

5.3.5 Data treatment

5.3.5.1 Feature extraction

The extraction of the molecular features and the alignment of the datasets were performed using the software MassHunter Profinder B.08.00 from Agilent Technologies. The molecular feature extraction parameters have been optimized in a previous study [33]. Peak filter height was set at 200 counts, expansion window for chromatogram extraction was ± 10 ppm, retention time tolerance was 0.30 min and the post-processing peak filter height was 1000 counts. Since the present study focuses on trace contaminants, it is important to set the threshold for the filtration by abundance to a low value to make sure they are properly detected. The isotope model selected was common organic molecules, and the ion and adducts considered were H^+, Na^+ and K^+.

5.3.5.2 Suspect screening

After peak alignment, multivariate analyses were performed using the software Mass Profiler Professional B.14.8 from Agilent Technologies. In this software, all data was normalized with 75.0

percentile shift. First, a PCA based on data for all samples was performed to investigate the overall performances of the workflow. The grouping of the QC samples in the PCA plot was used as an indication of the good reproducibility of the analysis, and thus its quality [35]. Then, compounds detected in honey samples were screened against the library Agilent Extractable & Leachable LC/QTOF PCDL. Based on a non-targeted identification method developed previously, the mass tolerance for the library match was set to ± 5 ppm and the minimum total score for the identification to be considered was 70% [33]. The total score reflects the probability that a feature is a real compound, a score of 100% being a perfect fit [36]. It is calculated as the average of four components: a mass match score, an isotope abundance score, an isotope spacing score and a retention time score [37]. Retention time was not considered when searching in the database, as it is specific to the HPLC conditions used to acquire the data. The positive ions and adducts considered were H^+, Na^+ and K^+. In addition, a requirement that there must be at least 2 ions present for any compound was applied. Among the tentatively identified compounds, only those whose peak area was above the "*limit of detection*" (i.e. mean + 3σ of the peak area of the procedural blanks) were considered for further data treatment.

5.3.5.3 Verification of the putative identities with standards

Confirmation of the identity of compounds with standards was performed following the EU identification points (IPs) system established in the 2002/657/EC European Commission Decision [38]. This system was developed to confirm the identification of organic residues and contaminants in live animals and animal products. For the confirmation of substances listed in group B of Annex I of Directive 96/23/EC, which include veterinary drugs, environmental contaminants, and other substances, a minimum of 3 IPs is required [39]. In addition to having 3 IPs, the 2002/657/EC European Commission Decision establishes that a minimum of one ion ratio should be measured, and that all measured ion ratios need to meet maximum permitted tolerances for relative ion intensities presented in **Table 5.2** [38]. In the present study, the assignment of IPs was performed following the criterion proposed by Hernández *et al.* based on

the measurement of the mass accuracy of each ion [40]. A total of 2 IPs per ion was attributed when mass error was below 2 mDa, 1.5 IPs per ion for mass errors between 2 and 10 mDa, and 1 IP per ion for mass errors greater than 10 mDa. After achieving a minimum of 3 IPs, the identity of each compound was only confirmed when the difference between the ratio of parent/fragment in the standard solutions and in samples was compliant with the limits presented in **Table 5.2**. For the calculation of the IPs and the ratio parent/fragment, two ions were selected for each compound using the acquired All Ions MS/MS data: MS1 (parent ion) and MS2 (most abundant fragment). The peak height of each ion was assessed with Agilent Mass Hunter Workstation Software – Qualitative Analysis B.07.00, taken at the collision energy (either 0, 10, 20 or 40 V) that produced the highest signal. In addition, the retention time of the compound in the standard solution and in the honey sample had to match within a tolerance of ± 0.15 min.

Table 5.2. Maximum permitted tolerances for relative ion intensities according to 2002/657/EC European Commission Decision [38].

Relative intensity (% of base peak)	Maximum permitted tolerance
> 50%	±20%
>20% to 50%	±25%
>10% to 20%	±30%
≤10%	±50%

5.3.5.4 Estimation of the dietary exposure to the identified compounds from honey

Two confirmed suspects (DEHA and TBOEP) were quantified based on 5-point calibration curves in the range of 0.01 to 1 ng.mL $^{-1}$ for DEHA and 0.1 to 10 ng.mL $^{-1}$ for TBOEP (external calibration). Concentrations were calculated using the Agilent Mass Hunter Workstation Software – Quantitative Analysis B.07.01. A mass extraction window of ± 10 ppm was applied based on a previous report [32]. Final concentrations in honey included a correction based on matrix effects

for each honey sample, which was calculated based on a single-point standard addition of 1 and 5 ng.mL^{-1} for DEHA and TBOEP, respectively. Method limit of detection (MDL) was calculated as 3σ of the equivalent signals of 10 procedural blanks. Peaks with a signal-to-noise ratio below 10 were also considered below the limit of quantification. Finally, estimated daily intakes of DEHA and TBOEP from honey were calculated from the concentrations of the compounds multiplied by the average daily consumption of honey in Canada (2.5 g.day^{-1}) and divided by a body weight of 70 kg [41].

5.3.5.5 Discrimination of honey samples through PCA

PCA was performed using the software Mass Profiler Professional to investigate the possible correlation between the presence of the PRCs found in the honey samples and the material of the honey jars. PCA was first used to find trends in the honey samples based on the material of the jar (plastic or glass), and then any possible trends according to both the material and the sampling year were also explored. The loadings of the principal components were investigated to determine the main compounds responsible for the trends observed in the PCAs.

5.3.5.6 Comparison of the PRC profiles among the honey samples commercially sold in plastic or glass jars

A unique entity analysis was performed with MS Excel using the peak areas for the 662 PRCs obtained from Mass Profiler Professional. Samples were divided into two groups based on the material of their jars (glass or plastic) and a unique entity analysis was applied to search for compounds present in 100% of the samples of one group and absent in 100% of the samples of the other group.

Volcano plots were created using the software Mass Profiler Professional to compare honey samples in glass and plastic jars based on the peak area for each of the 662 features identified as

PRCs. To do so, a moderated Student t-test was performed, followed by a Benjamini-Hochberg test. The p-value threshold was set at 0.05.

Pearson's Chi-squared test was applied using the statistical software JMP (SAS Institute Inc., Cary, NC, USA) in order to compare the frequency of detection of each of the 662 PRCs in the honey samples from plastic and glass jars. The p-value threshold was set at 0.05.

5.3.5.7 Structure elucidation of selected compounds

The Agilent MassHunter Molecular Structure Correlator B.07.00 software was used to create a list of potential candidate structures from the MS/MS spectra of some selected compounds whose MS/MS information was not available in the compound library, or whose identification was not conclusive (i.e. compounds G1 and P1). In order to do so, since this software only recognizes MS/MS spectra obtained through targeted or auto MS/MS (but not All-Ions MS/MS data), targeted MS/MS spectra were acquired for each of the compounds. The search for MS/MS spectra was done using the ChemSpider and Metlin libraries.

5.4 RESULTS AND DISCUSSION

5.4.1 QA/QC

Quality assurance and quality control (QA/QC) in a non-targeted workflow in the absence of standards for the target analytes is critical [35]. Several validation steps were used prior to the data treatment. First, the performance of the method in terms of precision of the measured retention time (RT), sensitivity and exact mass measurement was assessed from the results of the two labelled internal standards (IS). The precision of the retention time was calculated with the relative standard deviation (RSD) of the retention time of the IS across all the 114 analyzed samples (104 honey samples, 5 procedural blanks and 5 QCs). The results showed high precision, with an RSD of 0.2 % for D_3-diphenylhydramine (RT = 13.483 min) and 1.0% for D_3-6-

acetylmorphine (RT = 9.515 min). Next, any changes on the sensitivity of the instrument were monitored by calculating the RSD of the signal intensity for both IS across all the 114 analyzed samples. The results, with an RSD of 8.3% for D_3-diphenylhydramine and 8.5% for D_3-6-acetylmorphine, showed an acceptable repeatability of the instrument. Then, the accuracy of the exact mass measurement of the instrument was evaluated throughout the run by calculating the mass error of the mass measured for each IS across all samples. The average mass errors obtained were acceptable, with an error of 10 ± 2 ppm for D_3-diphenylhydramine ($[M+H]^+$ *m/z* 259.1914) and 8 ± 1 ppm for D_3-6-acetylmorphine ($[M+H]^+$ *m/z* 331.1762). After seeing the results for the precision and accuracy of different parameters obtained for the two IS, the overall performance of the instrument during the run was considered acceptable.

Finally, to ensure the quality of the data and verify that there was no instrumental artifact during the analysis, a PCA was performed with all the samples. The objective of this PCA was to verify the position of the QC samples, as they are used in non-targeted studies to monitor the quality of the run. As shown in **Figure 5.1**, QC samples were grouped together in the PCA plot, indicating that the analysis was reproducible and there were no time related trends [35]. Therefore, the recorded samples could be used for the next steps of the data treatment.

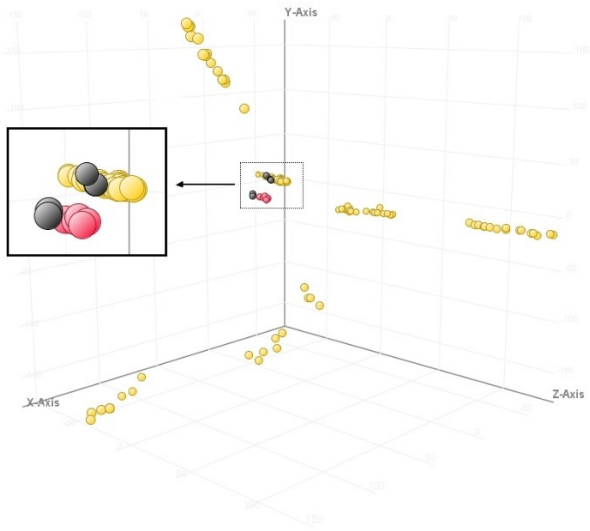

Figure 5.1. PCA plot of all samples (PC1-PC3), including the 104 honey samples (yellow), 5 procedural blanks (red) and 5 QCs (black). PC1, PC2 and PC3 explained 5.21, 4.54 and 3.47% of the variation of the data, respectively.

5.4.2 Data reduction and screening of plastic-related compounds

In total, 104,051 molecular features were obtained from the data (ESI+) for the 104 honey samples. After confirmation of the quality of the acquired data, the molecular features were screened against a commercial compound database of PRCs. In total, 763 were tentatively identified in the Agilent Extractable & Leachable LC/QTOF PCDL database based on their exact mass measurements and isotopic signature, which would correspond to a level of confidence of 4 according to the level system proposed by Schymanski *et al.* [42]. Out of the 763 compounds, the signal for 101 of them in real samples were found to be below the average + 3σ of the blanks, so the list of compounds tentatively identified in honey with a confidence level of 4 and a concentration significantly higher than that of the blank was reduced to 662 (**Figure 5.2-A**).

In the system proposed by Schymanski *et al.*, a confidence level of 4 corresponds only to the assignation of a molecular formula based on spectral information (e.g. adduct, isotope, and/or fragment information) [42]. An identification at level 4 is, thus, only tentative. In order to increase

the identification confidence level of these compounds, MS/MS data should be compared with that of a compound database to provide a probable structure and, ultimately, the structure should be confirmed with a reference standard to achieve level 1 of confidence [42]. All Ions MS/MS data is currently not recognized by the used software as such, and so automatic screening using All Ions MS/MS data is not possible. However, this data can be screened manually to identify characteristic ion fragments and increase the confidence level to 2. Since the manual identification of 662 compounds in 104 honey samples would be extremely time-consuming, it was decided to further confirm the identity of only some selected compounds of interest.

Figure 5.2-B depicts the frequency of the above 662 compounds tentatively identified as PRCs among honey samples. Most of these compounds were detected at a low frequency. Indeed, 63% of the compounds were detected in only 10% of the samples or less. As shown in **Table 5.1**, there is a wide range of reported frequencies for the detection of PRCs in honeys, ranging from 3% for BPS in Lo Turco *et al.* to 100% for DBP and DEHP in Česen *et al.* [25, 26]. Other food contaminants such as veterinary drugs and pesticides are also often detected at a low frequency in honey in market studies [43].

The number of compounds per honey sample ranged from 38 to 143 in the present study (out of the above 662 tentatively identified as PRCs) and is presented in **Figure 5.2-C**. The average number of compounds in honey samples sold in glass or plastic jars was very similar (88 ± 21 compounds in glass jars and 90 ± 28 compounds in plastic jars). This finding further supports the need for non-targeted methods of analysis with a multi-contaminant capability for honey. The 5 most abundant chemicals (in terms of average peak area across all 104 samples) tentatively identified regardless of frequency of occurrence were N-lauryldiethanolamine, tetraethyleneglycol dimethacrylate, 5-hydroxyquinoline, palmitamide, and bis (3,3-dimethyl-dibenzylidene sorbitol), with a total score of 99.41, 85.39, 99.76, 89.56, and 98.81%, respectively.

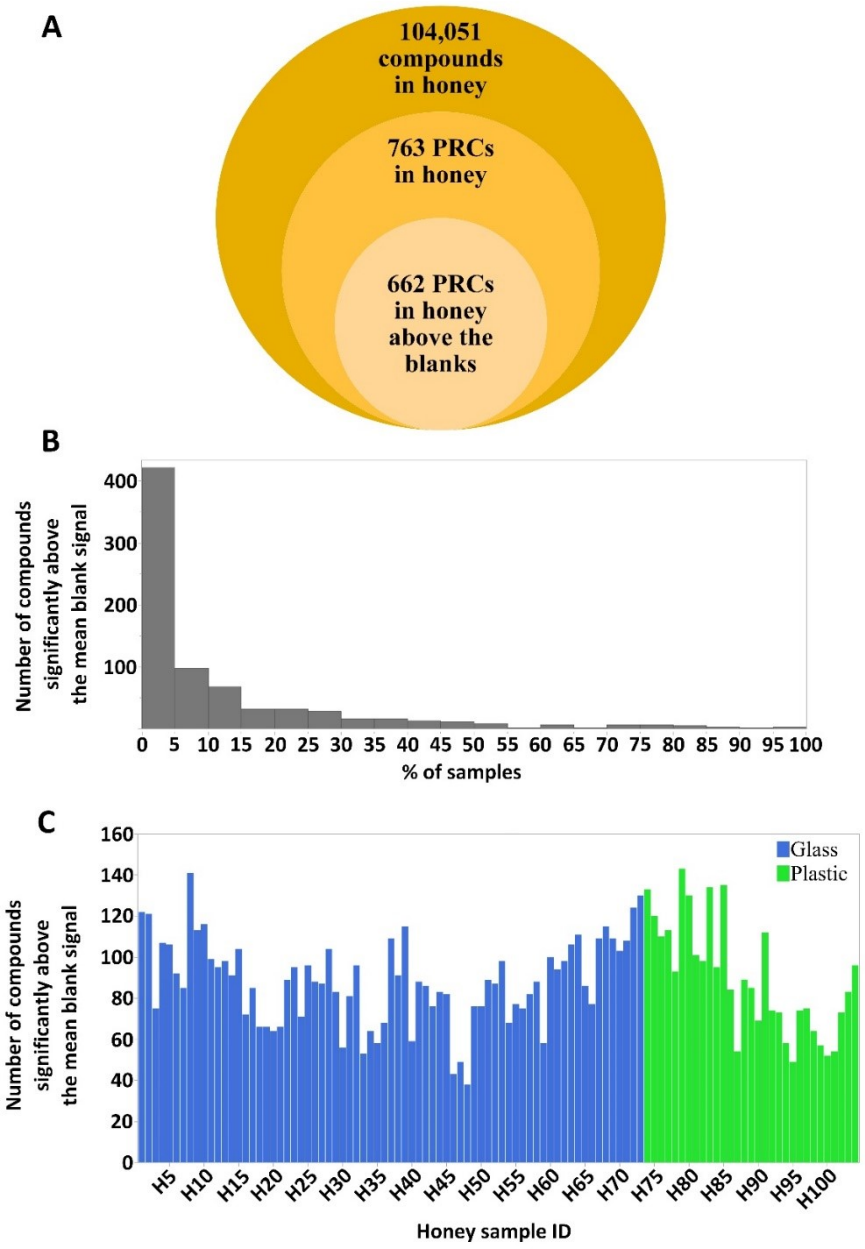

Figure 5.2. A: Graphical representation of the data filtering steps from the initial pool of all entities in all samples to the reduced group of tentatively identified PRCs. B: Frequency plot of the 662 PRCs tentatively identified in all 104 honey samples. C: Number of detected PRCs in each of the 104 honey samples.

Two compounds, C1 and C2, were selected to illustrate two different frequencies of detection of PRCs in the honey samples from the present study: C1 to represent high frequency (74% detection) and C2 for low frequency (7% detection). The confirmation of the identity of these 2 compounds is presented in detail in the supplementary information. Briefly, C1 was initially identified with a confidence level of 4 as DEHA ($C_{22}H_{42}O_4$, *m/z* 370.3083, total score of 74.09%), and C2 was initially identified with a confidence level of 4 as TBOEP ($C_{18}H_{39}O_7P$, *m/z* 398.2433, total score of 95.26%). The chromatograms of the 2 compounds were acquired from analytical standards to validate their identity after finding similarities between the recorded All Ions MS/MS spectra for these compounds in the honey matrices and the literature. Since the retention time difference between the standards and the honey samples was within ± 0.15 min, the identity of DEHA and TBOEP was further assessed according to the IPs system established in the 2002/657/EC European Commission Decision [38]. The results of this identification are presented in **Table 5.3**. Using two ions, the parent ion (MS1) and one fragment (MS2), 4 IPs were achieved for both DEHA and TBOEP, with an error in the measured MS2/MS1 ratio below the maximum tolerance limits in all cases. Therefore, C1 and C2 were successfully identified as DEHA and TBOEP, respectively, with a confidence level of 1, which is the highest level in the identification system proposed by Schymanski *et al.* [42]. The chromatographic peaks of both compounds in standard solution and in one honey sample, as well as their MS/MS spectra used for identification, are presented in **Figure S5.1** and **Figure S5.2** as an example.

Table 5.3. Calculation of IPs and parent/fragment ion ratio for the confirmation of DEHA and TBOEP based on the All Ions MS/MS data acquired for the standards and the honey samples in which these compounds were detected.

	DEHA (n= 77)	**TBOEP (n= 9)**
	MS1 (0 V): *m/z* 371.3156	**MS1 (0 V): *m/z* 399.2506**
	MS2 (20 V): *m/z* 129.0546	**MS2 (10 V): *m/z* 299.1618**
Mass error of MS1	< 2 mDa	< 2 mDa
IPs earned for MS1	2	2
Mass error of MS2	< 2 mDa	< 2 mDa
IPs earned for MS2	2	2
Total IPs earned	**4**	**4**
MS2/MS1 sample	0.575 ± 0.033	0.369 ± 0.012
MS2/MS1 standard	0.634	0.434
Error in the measured MS2/MS1	**9 ± 5%**	**15 ± 3%**
Maximum permitted tolerance	± 20%	± 25%
CONFIRMED?	**YES**	**YES**

DEHA is a common plasticizer applied in food contact materials which has been reported in polyvinyl chloride (PVC) food wrapping film and in polyethylene terephthalate (PET) bottles [44, 45]. Wrapping food in PVC films has been suggested as the source of high levels of DEHA in different types of food in Canada [27]. TBOEP is used in a variety of applications as a plasticizer, lubricant and flame retardant, among others [46]. In conclusion, the occurrence of the above 2 contaminants in honey could be related to their use in food packaging materials. However, the packaging material is probably not the only possible source of these compounds in honey. Other food products such as coffee and tea have been found to be contaminated with PRCs during their

production steps as result of being in contact with plastic parts of the production machinery [18, 47]. In the case of honey, contamination with PRCs during processing could happen through contact with plastic components of the equipment for honey production, such as the honey extractors [25]. Moreover, some of these contaminants such as DEHP could also come from agricultural practices (e.g. using plastic mulch film) and/or environmental contamination that would contaminate the nectar used to produce the honey [27]. Because DEHA and TBOEP may have multiple sources other than the food packaging materials, their distribution among honey samples cannot be explained unequivocally based on the material of the jar.

5.4.3 Implications for food safety

DEHA and TBOEP are of increasing concern to humans and have been identified as priority chemicals for assessment of risk under the Government of Canada's Chemicals Management Plan due to the finding of adverse effects from toxicology studied in animals [27]. DEHA and TBOEP obtained in this study were quantified through external calibration with standards and considering matrix effect for each honey sample (**Table 5.4**). Both compounds were successfully determined, with an MDL of 1.19 and 16.99 $\mu g.kg^{-1}$ for DEHA and TBOEP, respectively. The maximum concentrations of DEHA measured in the present study were in the same order of what had been previously reported in the literature. Cao *et al.* studied DEHA as part of the Canadian Total Diet Study in 2015, and found a concentration in honey of 4.82 $\mu g.kg^{-1}$ for DEHA [27]. Regarding TBOEP, He *et al.* reported to have found this compound in 48% of samples (n=87) in a market study in Australia including cereals, fruits, vegetables, milk, meat, fish, seafood, eggs and beverages, with a concentration of 0.36 $ng.g^{-1}$ wet weight in the 95^{th} percentile [48]. Ding *et al.* performed a similar market study in China and found TBOEP in 100% of the samples (n=37), with a concentration between 0.08-1.2 $ng.g^{-1}$ fresh weight [49]. To the best of our knowledge TBOEP has never been reported in honey before.

An estimated maximum daily intake was calculated from the highest levels of DEHA and TBOEP in honey collected in the Canadian market to characterize the risks of adverse health effects for

a general adult consumer (**Table 5.4**). Using this extreme scenario, the daily intakes of DEHA and TBOEP corresponded to less than 1% of their respective Tolerable Daily Intake (TDI) or Reference Dose (RfD), indicating no health concern.

Table 5.4. Highest concentration (µg.kg^{-1}) and associated daily intake (µg.kg^{-1} bw.day^{-1}) of DEHA and TBOEP from honey compared their respective TDI or RfD values. A body weight of 70 kg was used for the calculation of the daily intake.

Compound	Sample	Concentration (µg.kg^{-1})	Calculated daily intake from honey (µg.kg^{-1} bw.day^{-1})	TDI or RfD (µg.kg^{-1} bw.day^{-1})
DEHA	H81	17.1	0.001	300 [50]
TBOEP	H73	164.7	0.006	1.5 [51]

5.4.4 Discrimination of honey samples through PCA based on the distribution of PRCs

Principal Component Analysis was applied to the 662 compounds tentatively identified as PRCs in the honey samples (**Figure 5.3**). This was done in order to obtain a first overview of the trends observed in the honey samples based on the presence of the tentatively identified PRCs. The 3 first principal components explained 16.43% of the variance of the data (PC1, PC2 and PC3 accounted for 7.42, 4.84 and 4.17% of the variance, respectively). The low percentage of the variance of the data explained by the first three principal components underlines the high variability of chemical composition among the honey samples in this study. Although most of the variability was not captured with these 3 principal components, there was a trend observed in the PCA as the honey samples seemed to be forming different groups. First, the type of jar (glass or plastic) was highlighted in the PCA but the material of the jar was not the major factor influencing the grouping of samples (**Figure 5.3-A** and **B**). The year of sampling was also highlighted, and samples showed some grouping based on this parameter (**Figure 5.3-C** and **D**), indicating that the sampling year or the storage time prior to analysis influenced the chemical signature of the honey samples.

In order to identify the compounds responsible for the clear variability observed in the data in PC1 and PC3 (**Figure 5.3-D**) the loading plot of PC1 and PC3 was examined (**Figure S5.3**). In this plot, among the compounds with an important loading in PC1, natural compounds such as coumarin and limonene were tentatively identified. These compounds are listed in the library Agilent Extractable & Leachable LC/QTOF PCDL because they are used in the plastic industry as additives or solvents [52, 53]. However, their presence in honey may not necessarily reflect their possible use in plastics. In fact, both coumarin and limonene occur naturally in honey from different floral origin, such as honey from mahaleb cherry tree, Polish sweet yellow clover honey and citrus honey [54-56]. In addition, limonene is found in essential oils used as biopesticides and for the treatment of American Foulbrood in honey bees, so its presence in honey could also be due to agricultural and beekeeping practices [57, 58]. It is important to note that natural compounds, such as limonene, may also degrade during the storage of the samples. A 17% degradation of limonene was measured, for example, in citrus-oil emulsions after only 15 days of storage at 25˚C [59]. Such a compound, occurring naturally in honey and having multiple possible anthropogenic origins, possibly degrading during storage, is likely responsible for the variability observed in the PCA plots (**Figure 5.3-C** and **D**) and segregation of samples based on the sampling year.

5.4.5 Comparison of the PRC profiles among the honey samples commercially sold in plastic or glass jars

One of the challenges of the non-targeted multivariate analysis is to apply the right data treatment to answer a specific question. A common situation when applying this approach to complex matrices, such as food, is to obtain a large list of compounds distributed among the samples with high variability [60, 61]. In this case, the key to obtaining meaningful results is to frame a concrete question and to then perform the necessary data treatment to answer it. **Table 5.5** summarizes the different approaches investigated in the present study to compare the PRC profiles among honey samples sold in plastic and in glass jars. As observed in **Table 5.5**, a slight

variation in the question may lead to somehow different results. This underlines the complexity of the non-targeted approach in the field of trace contaminants in food, and the importance of formulating the right question before starting to design and apply any data treatment. Individual results for each of the approaches are described in detail in the following sections.

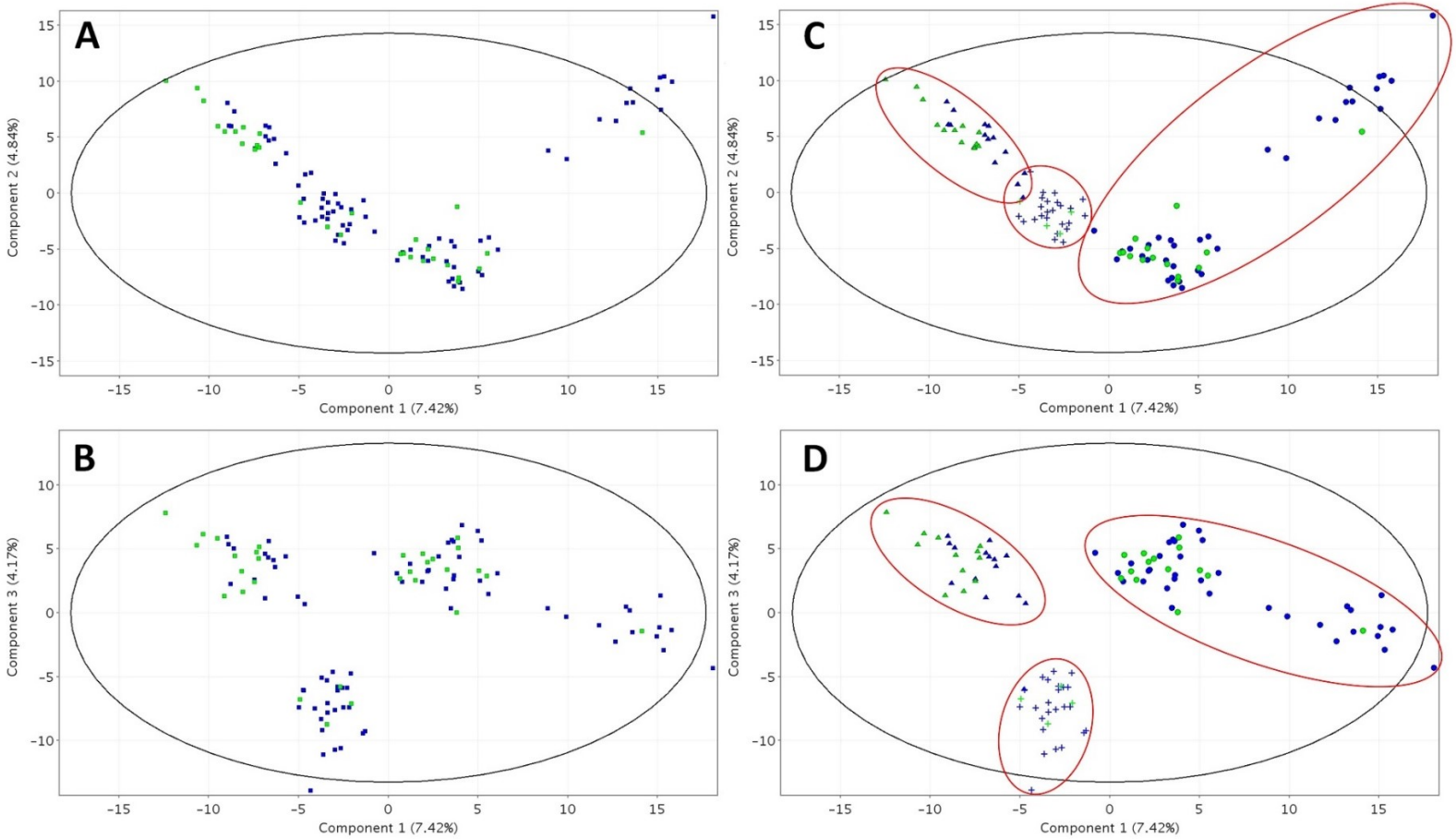

Figure 5.3. PCA plots of the PCRs in different types of honey samples: PCA with material of the jar (A: PC1 vs. PC2, B: PC1 vs. PC3), and PCA with material of the jar and sampling year (C: PC1 vs. PC2, D: PC1 vs. PC3). Legend for all: green = plastic, blue = glass. Legend for C and D: ▲ = 2016, **+** = 2017, ● = 2018.

Table 5.5. Comparison of different data treatment approaches for the comparison of PRC profiles among the honey samples commercially sold in plastic or glass jars.

Question	Data Treatment	Number of compounds	Section
Are there any compounds found in all samples sold in plastic jars and in none of the samples sold in glass jars? (or vice versa)	Unique entity analysis (100% detection vs. 0% detection)	0	5.4.5.4
Are there any compounds whose overall signal is significantly higher in all the honey samples sold in plastic or glass jars?	Volcano plot $p>0.05$	2 (P1 and G1)	5.4.5.2
Are there any compounds more frequently detected in honey samples sold in plastic or glass jars?	Frequency of detection	40 in plastic and 13 in glass	5.4.5.3
Are there any compounds found only in honey samples sold in plastic or glass jars? (Regardless of the frequency of detection in that type of jar)	Unique entity analysis (any % detection vs. 0% detection)	6 in plastic and 3 in glass	5.4.5.3

5.4.5.1 Unique Entity Analysis

First, a unique entity analysis was performed to investigate whether some compounds were exclusive to honey sold in one specific type of jar. This type of analysis usually searches for compounds that are present in 100% of the samples of one group and absent in 100% of the samples of the other group [62]. The results showed that none of the 662 PRCs was unique in one of the two groups (plastic and glass jars). Considering the diversity of jar materials and origin of the honey samples, it was somehow expected that the jars in this study would come from different producers using different ingredients in their formulations. This explains probably why there is no PRC common to all the honeys in plastic or glass jars.

5.4.5.2 Volcano plot of statistical significance versus fold change

The volcano plot is a widely used statistical tool in metabolomics to detect compounds whose intensities are significantly different among two groups of samples [63, 64]. In the present study, a volcano plot was produced for all the 662 compounds based on the fold change (glass vs. plastic) and *p*-value (**Figure 5.4**). Since no compound was found unique to one type of jar (section 5.4.5.1), the goal of this plot was to investigate whether some compounds were characteristic of one type of jar (glass or plastic) while not being exclusive to it. The results of the fold change analysis performed as part of the volcano plot analysis (**Figure 5.4**), showed that 285 out of the 662 detected PRCs were higher in honey samples sold in plastic jars (down-regulated, left hand-side of the volcano plot), and that 377 PRCs were higher in honey samples sold in glass jars (up-regulated, right hand-side of the volcano plot). However, when looking at the compounds with statistically significant differences, only one single compound was significantly higher ($p<0.05$) in honeys sold in plastic jars (P1, top left corner of the volcano plot), and another single compound was significantly higher ($p < 0.05$) in honeys sold in glass jars (G1, top right corner of the volcano plot). In other words, very few compounds differentiated the two groups according to the volcano plot (using $p < 0.05$). As with the unique entity analysis, this result can be explained from

the diversity of honey samples tested and the low frequency of contamination of honey (**Figure 5.2-B**).

Some attempts were made to further investigate the identity of compounds P1 and G1. A detailed explanation of the identification process is presented in the supplementary information. In summary, the identity of P1 and G1 remain unknown since no structure in any of the consulted libraries matched the recorded spectra for these compounds in honey. Further identification of these compounds could be attempted in future studies using other libraries or trying to correlate some of the observed peaks in the MS/MS spectra with common fragments and functional groups.

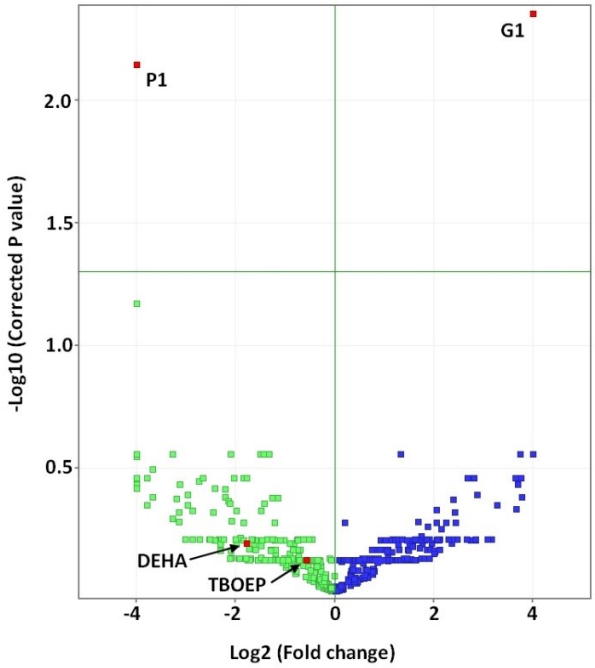

Figure 5.4. Volcano plot of the comparison between honey sold in glass and plastic jars. Compounds with a positive value in the X-axis (blue) have an average concentration across all glass samples which is higher than that of the plastic samples, and compounds with a negative value in the X-axis (green) have an average concentration across all plastic samples which is higher than that of the glass samples. Selected compounds (red) were those whose identity was further studied.

5.4.5.3 Frequency of detection in each group

Since the signals for 660 out of the 662 PRCs were not statistically different for honey samples sold in either plastic or glass jars, a different approach based on the frequency of detection was taken. A Pearson's Chi-squared test was performed for the 662 PRCs based on their rates of detection in the honey samples sold in either glass and plastic jars. In total, 40 PRCs were detected more frequently in honey samples from plastic jars and 13 PRCs were detected more frequently in honey samples from glass jars (p<0.05 in both cases). Therefore, more PRCs with statistically higher frequency of detection were found in honey samples in plastic jars than in glass jars. The tentative identification of these 53 compounds based only on their exact mass and MS spectra (level 4 of confidence) is presented in **Table S5.2** and **Table S5.3**. DEHA, one of the compounds identified in section 5.4.2, was statistically detected more frequently in honey samples in plastic than in glass jars (68% detection in glass jars and 87% in plastic jars, Pearson's Chi-squared test p=0.0478). At least 15 out of the 27 samples from plastic jars in which DEHA was detected were made of PET (**Table S5.1**). DEHA is commonly used as a plasticizer for PET, which could explain its high frequency of detection among the honey samples sold in plastic jars [44].

Although section 5.4.5.1. showed that there were no unique entities in either of the groups (i.e. entities found in 100% of the samples sold in plastic jars and none of the samples sold in glass jars or vice versa), the results of the Pearson's Chi-squared test (**Table S5.2** and **Table S5.3**) showed that some compounds were only found in samples of one type of jar. Indeed, 6 compounds were only found in samples from plastic jars and 3 compounds were found only in samples from glass jars. These compounds, however, had frequencies of detection in the range of 6 to 19%, which is the reason why the unique entity analysis (section 5.4.5.1) did not categorize them as being unique to one group. Nonetheless, the fact that these compounds were only detected in samples from glass or plastic jars could make them characteristic of that type of jar, and their presence in a honey sample could be used as an indicative marker of the material of the jar the sample was sold in. Oleamide, for example, was tentatively identified in 3 of the honey

samples from plastic jars and in none of the samples from glass jars. All 3 jars were made with LDPE, for which oleamide is used as an additive, so the detection of this compound could potentially be correlated with the material of the jar [65]. However, as mentioned before, there are other possible sources of contamination with PRCs.

5.4.5.4 Discussion on suspect screening of contaminants in food samples

Tools such as unique entities analysis or volcano plot are commonly applied to identify differential metabolites in controlled exposure experiments [66]. The comparison of food samples based on their contaminant load is however more complex, mostly because of the relatively low frequency of these residues in food. As observed in **Figure 5.3**, PCA using even a reduced list of features based on a library of compounds does not provide a straightforward comparison of samples, especially since some of the compounds driving the PCA may have multiple origins (e.g. both natural and anthropogenic). As illustrated in **Table 5.5**, questions about the contamination of honey samples with PRCs may be formulated in different ways, resulting in an overall different list of contaminants. Unique entity analysis with 100% detection and volcano plot with $p < 0.05$ found 0 and 2 compounds, respectively, while in the third approach based on the differential frequency of detection the number of relevant compounds increased to 13 in glass and 40 in plastic. Among the compounds found with this last approach, 6 were unique to honey samples sold in plastic jars and 3 were unique to honey samples sold in glass jars.

Unique entity analysis, despite being used in other studies to find compounds characteristic to one group of samples, relies on these compounds having a high rate of detection [62]. In a controlled exposure experiment this can easily be achieved, since all the samples from the exposed group will have received the same treatment. The distribution of contaminants among actual food products from the market is however quite complex. In this case, applying this type of analysis can lead to finding no unique entities, even when some compounds are detected only in one group. A unique entity analysis without any frequency restrictions, focusing only on the

absence of compounds in one group regardless of the frequency in the other group, is recommended in this situation.

Volcano plots with Student t-test are also widely used to compare two groups, e.g. in metabolomics. This type of analysis identifies significant differences among the average signal/concentration for each compound in each group. In the present study, however, the distribution of the majority of compounds among the samples in each group was not normal, but instead the few detected signals for each compound represented outliers within the group. Achieving statistical difference in this situation would be very difficult. For this reason, only one compound was detected as being significantly higher in each group of samples. Therefore, an approach considering the frequency of detection in each group instead of the concentration of contaminant would provide more information on the compounds being characteristic to one group of samples.

5.5 CONCLUSIONS

A non-targeted workflow was applied to the screening of PRCs in 104 honey samples from the Canadian market, and a total of 662 compounds were detected using a library of leachable and extractable compounds. The identity of two of these compounds, namely DEHA and TBOEP, was further confirmed with standards. Their concentrations were assessed in the honey samples, and the estimated daily intake of these compounds is expected to be far below the tolerable daily intake.

With regards to the comparison of the chemical burden in honey samples sold in either glass or plastic jars, various data treatment approaches were used which resulted in different lists of relevant contaminants. Because of the low frequency of detection of the 662 PRCs found in honey in the present study, unique entity analysis with 100% detection and volcano plot with $p < 0.05$ found none and 2 compounds, respectively. When applying the third approach based on the differential frequency of detection, however, the number of relevant compounds increased to

13 in glass and 40 in plastic. In conclusion, the relatively low frequency of contaminants in food needs to be accounted for when selecting the appropriate data treatment tool to compare groups of samples.

Among the compounds found with the last approach, 6 were unique to honey samples sold in plastic jars and 3 were unique to honey samples sold in glass jars. Controlled migration studies should be performed in order to determine to which extent the material of the jar plays a role in the presence of these compounds in honey.

5.6 ACKNOWLEDGMENTS

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5.8 SUPPLEMENTARY INFORMATION

Detailed identification of compounds

C1

C1 was initially identified with a confidence level of 4 as DEHA ($C_{22}H_{42}O_4$, *m/z* 370.3083, total score of 74.09%). MS/MS information for this compound was available in the Agilent Extractable & Leachable LC/QTOF PCDL, so a manual comparison between this and the spectra obtained from the honey samples using the All Ions MS/MS mode was possible. According to the library, the three main peaks in the MS/MS spectrum for positive ionization mode at a collision energy of 10 V were *m/z* 129.0546 (base peak), *m/z* 111.0441 (24% of the base peak) and *m/z* 147.0652 (23% of the base peak). All three peaks were observed in the honey samples, although due to the potential differences between the acquisition methods used in the present study and in the creation of the library the observed relative abundances of *m/z* 111.0441 and *m/z* 147.0652 were different than in the library. The chromatogram of a standard of DEHA was acquired in order to clarify this identification. After seeing that the difference in retention time between the standard and the honey samples was within ± 0.15 min, it was proceeded to identify DEHA following the IPs system established in the 2002/657/EC European Commission Decision [1]. The results of this identification are presented in **Table 5.3**. Using two ions, the parent ion (MS1) and one fragment (MS2), 4 IPs were achieved with an error in the measured MS2/MS1 ratio below the maximum tolerance limits (**Tablele 5.2**). Therefore, C1 was successfully identified as DEHA with a confidence level of 1, which is the highest level in the identification system proposed by Schymanski *et al.* [2].

C2

C2 was initially identified with a confidence level of 4 as TBOEP (C18H39O7P, *m/z* 398.2433, total score of 95.26%). MS/MS information for this compound was available in the Agilent Extractable

& Leachable LC/QTOF PCDL, so a manual comparison between this and the spectra obtained from the honey samples with All-Ions mode was possible. According to the library, the three main peaks in the MS/MS spectrum for positive ionization mode at a collision energy of 10 V were *m/z* 399.2506 (base peak), *m/z* 299.1618 (92.28% of the base peak) and *m/z* 199.0730 (56.75% of the base peak). All three peaks were observed in the honey samples, although the relative abundances of *m/z* 299.1618 and *m/z* 199.0730 were different than in the library (35.45% and 17.81%, respectively). The chromatogram of a standard of TBOEP was acquired in order to clarify this identification. After seeing that the difference in retention time between the standard and the honey samples was within ± 0.15 min, it was proceeded to identify TBOEP following the same IPs system as with DEHA. The results of this identification are presented in **Table 5.3**. Using two ions, the parent ion (MS1) and one fragment (MS2), 4 IPs were achieved with an error in the measured MS2/MS1 ratio below the maximum tolerance limits. Therefore, C2 was successfully identified as TBOEP with a confidence level of 1.

G1

The initial identification of G1 with the extractable and leachable library yielded three possible candidates with equal score: methyl-N-hydroxybenzimidate, 2-amino-5-methylbenzoic acid and acetaminophen (C8H9NO2, *m/z* 151.0633, total score of 99.16%). The compound database used for the identification of the PRCs had MS/MS information for only acetaminophen, so a manual comparison between the spectra in the library and those obtained with All-Ions mode from the honey samples was possible for this compound. According to the library, the main peaks in the MS/MS spectrum of acetaminophen using positive ionization mode at a collision energy of 20 V are *m/z* 110.0600 (base peak), *m/z* 93.0335 (30% of the base peak) and *m/z* 152.0706 (6% of the base peak). However, in the honey samples only the peak with *m/z* 152.0706 was observed. Missing the main peaks of the MS/MS for this compound would seem to indicate that the detected compound was not acetaminophen. To further clarify this identification, as well as to try to obtain more information on the identity of the other two candidates, the MS/MS spectra

of these three compounds was acquired using a targeted MS/MS method. The recorded MS/MS spectra of this compound in honey were processed with the software Molecular Structure Correlator to see whether the structure of any of these 3 candidates would correlate with the MS/MS data. The results showed that the only candidate whose structure would be possible with the MS/MS data from the honey samples was 2-amino-5-methylbenzoic acid. Therefore, the chromatogram of a standard of this compound was acquired in order to verify the identification. Unfortunately, the retention time difference between the standard and the compound found in honey was over 7 min, so the initial identification of G1 was also erroneous. Since the only structure proposed by the Molecular Structure Correlator was proved to be erroneous, the identity of G1 remains unknown.

P1

Compound P1 was initially identified with a confidence level of 4 as ethinylcyclohexanol ($C_8H_{12}O$, *m/z* 124.0888, total score of 76.73%). Since the library used for the identification had no MS/MS information for this compound, the recorded MS/MS spectra were processed with the Molecular Structure Correlator software to see if any of the proposed structures would match that of ethinylcyclohexanol. Unfortunately, no structures were found in any of the searched libraries that would match the MS/MS spectra recorded for this compound. The misidentification of this compound was finally confirmed by comparison with an ethinylcyclohexanol standard, which presented a difference in retention time of over 1.5 min. The identity of P1 remains unknown since no structures in any of the consulted libraries matched the recorded spectra for this compound in honey.

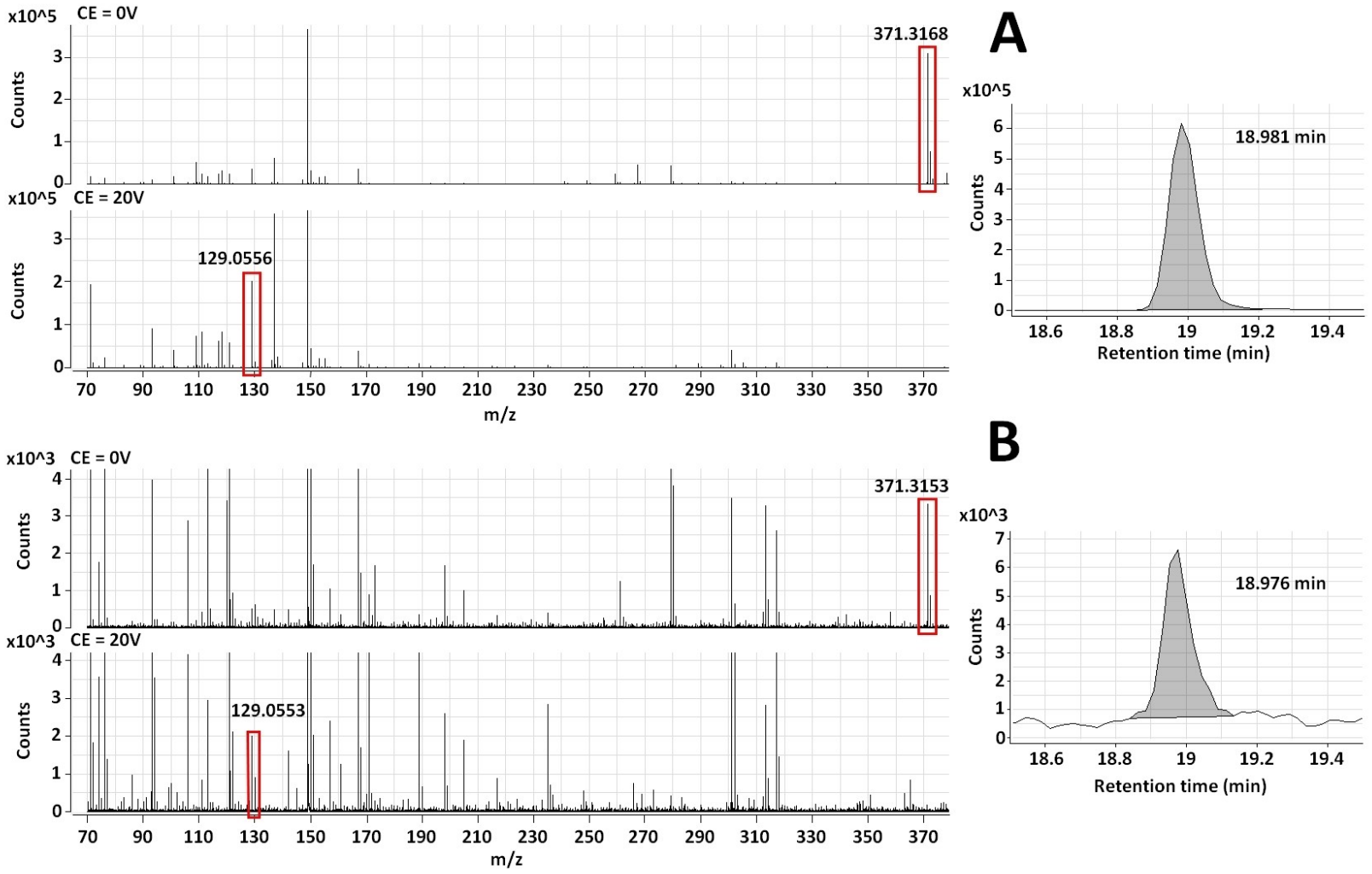


Figure S5.1. Chromatographic peak and MS/MS spectra of DEHA in standard solution (A) and in one of the honey samples in which it was detected (B)

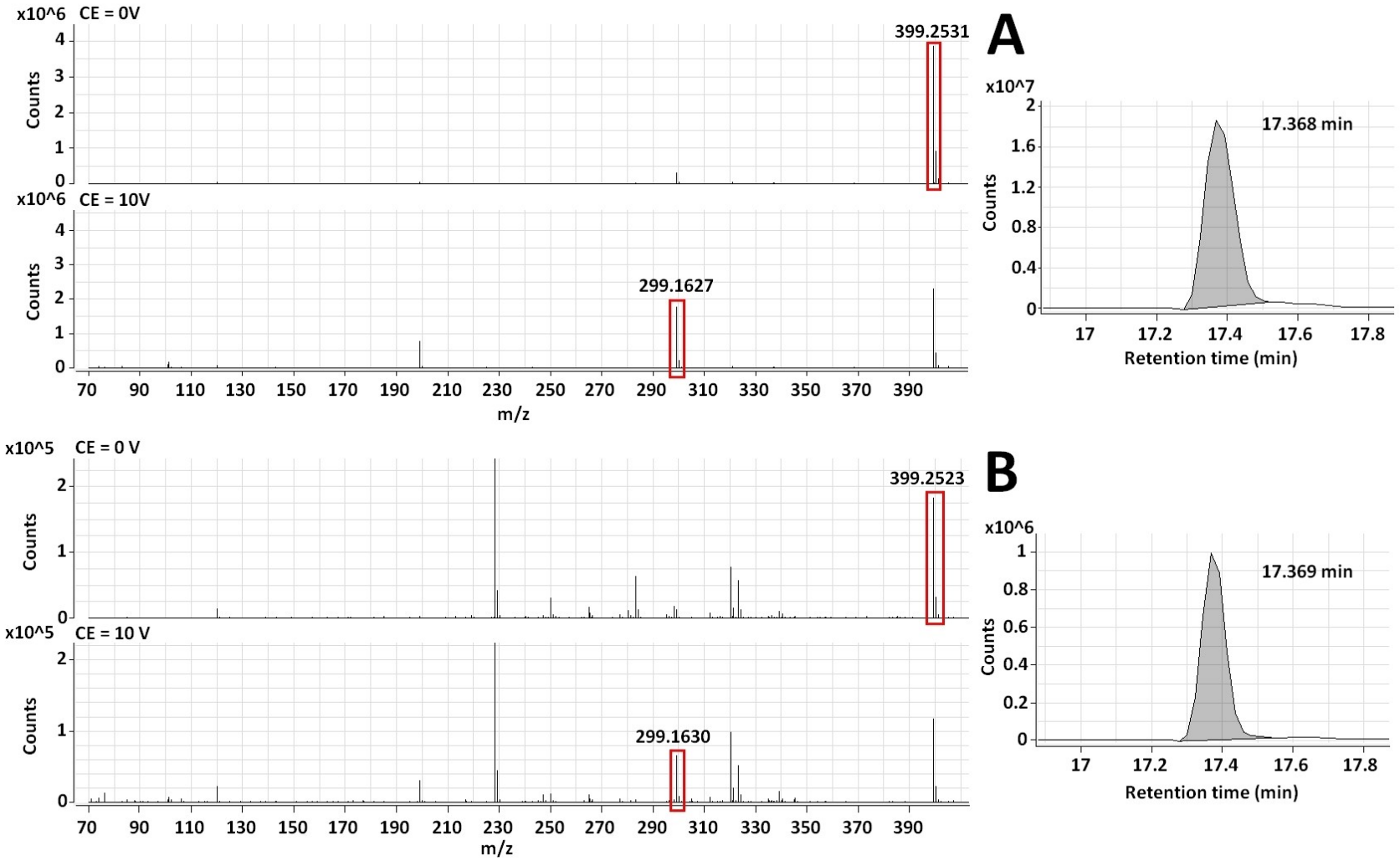


Figure S5.2. Chromatographic peak and MS/MS spectra of TBOEP in standard solution (A) and in one of the honey samples in which it was detected (B)

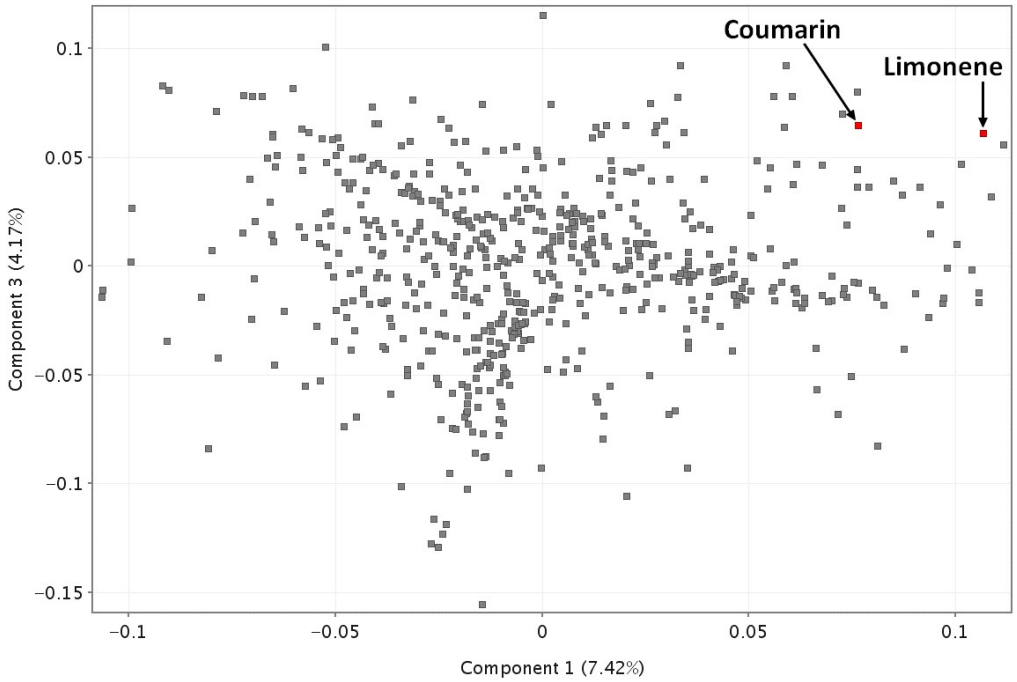


Figure S5.3. Loading plot of PC1 and PC3 of the PCA presented in **Figure 5.3**.

Table S5.1. List of honey samples and detailed information as obtained from their labels.

Sample	Type of container	Sampling year	Colour[a]	Type of farming	Botanical origin	Geographical origin
H1	Glass	2016	Amber	Non-organic	Wildflowers	USA
H2	Glass	2016	White	Non-organic	Clover	Canada
H3	Glass	2016	White	Non-organic	Acacia	France
H4	Glass	2016	White	Non-organic	Clover	Canada
H5	Glass	2016	Golden	Non-organic	Meadow and wildflowers (vetch, sweet clover, clover, milkweed)	Canada
H6	Glass	2016	Dark	Non-organic	Unknown	Canada
H7	Glass	2016	Golden	Organic	Unknown	Canada
H8	Glass	2016	Golden	Non-organic	Clover	Canada
H9	Glass	2016	Golden	Non-organic	Forest honey	Canada
H10	Glass	2016	Golden	Organic	Summer flowers (white clover, melilot, raspberry bush, alfalfa, linden, Wildflowers)	Canada
H11	Glass	2016	White	Non-organic	Unknown	Canada
H12	Glass	2016	White	Non-organic	Wildflowers	Canada
H13	Glass	2016	White	Non-organic	Clover	Canada
H14	Glass	2016	White	Organic	Wildflowers	Canada
H15	Glass	2017	Golden	Non-organic	Wildflowers	Bulgaria, Guatemala, Mexico, Spain,

						Thailand and Ukraine
H16	Glass	2017	Golden	Non-organic	Orange blossom	Spain
H17	Glass	2017	Golden	Non-organic	Unknown	Canada
H18	Glass	2017	Dark	Non-organic	Buckwheat	Canada
H19	Glass	2017	White	Non-organic	Meadow flowers (spring flowers)	Canada
H20	Glass	2017	White	Non-organic	Unknown	Canada
H21	Glass	2017	Amber	Non-organic	Wildflowers (fall flowers)	Canada
H22	Glass	2017	Amber	Non-organic	Wildflowers	Canada
H23	Glass	2017	White	Non-organic	Clover	Canada
H24	Glass	2017	White	Non-organic	Clover	Canada
H25	Glass	2017	White	Non-organic	Clover, alfalfa, and Wildflowers (summer flowers)	Canada
H26	Glass	2017	White	Non-organic	Clover	Canada
H27	Glass	2017	Golden	Non-organic	Goldenrod	Canada
H28	Glass	2017	Golden	Organic	Acacia	Brazil
H29	Glass	2017	Golden	Non-organic	Unknown	Canada
H30	Glass	2017	Golden	Non-organic	Wildflowers	Canada
H31	Glass	2017	White	Non-organic	Blueberry	Canada
H32	Glass	2017	White	Non-organic	Wildflowers	Canada
H33	Glass	2017	Golden	Non-organic	Lavender	Portugal
H34	Glass	2017	Amber	Non-organic	Unknown	India
H35	Glass	2017	White	Non-organic	Acacia	Poland
H36	Glass	2017	Golden	Non-organic	Clover	Canada
H37	Glass	2017	Amber	Non-organic	Fall flowers	Canada

H38	Glass	2017	White	Non-organic	Unknown	Canada
H39	Glass	2018	Amber	Non-organic	Wildflowers	Canada
H40	Glass	2018	Amber	Non-organic	Unknown	Greece
H41	Glass	2018	Golden	Non-organic	Wildflowers	Canada
H42	Glass	2018	Dark	Non-organic	Buckwheat	Canada
H43	Glass	2018	White	Non-organic	Buckwheat	Canada
H44	Glass	2018	Golden	Non-organic	Wildflowers	Canada
H45	Glass	2018	Dark	Non-organic	Buckwheat	Canada
H46	Glass	2018	Golden	Non-organic	Apple blossom	Canada
H47	Glass	2018	White	Non-organic	Clover	Canada
H48	Glass	2018	White	Non-organic	Clover	Canada
H49	Glass	2018	Golden	Non-organic	Sunflower	Canada
H50	Glass	2018	Golden	Non-organic	Clover	Canada
H51	Glass	2018	Golden	Non-organic	Goldenrod honey	Canada
H52	Glass	2018	White	Non-organic	Clover	Canada
H53	Glass	2018	Amber	Non-organic	Blueberry	Canada
H54	Glass	2018	Amber	Non-organic	Wildflowers	Canada
H55	Glass	2018	Golden	Non-organic	Multiflora	Canada
H56	Glass	2018	Golden	Non-organic	Multiflora	Canada
H57	Glass	2018	Amber	Non-organic	Eucalyptus	Spain
H58	Glass	2018	Amber	Non-organic	Eucalyptus	Italy
H59	Glass	2018	White	Organic	Acacia	Italy
H60	Glass	2018	Golden	Non-organic	Summer flowers	Canada
H61	Glass	2018	Dark	Organic	Buckwheat, goldenrod, aster, milkweed	Canada
H62	Glass	2018	White	Non-organic	Raw summer honey	Canada
H63	Glass	2018	Golden	Non-organic	Wildflowers	Canada

H64	Glass	2018	Dark	Non-organic	Buckwheat	Canada
H65	Glass	2018	Amber	Non-organic	Spring flowers	Canada
H66	Glass	2018	Golden	Non-organic	Camelina	Canada
H67	Glass	2018	Golden	Non-organic	Tree of heaven	Italy
H68	Glass	2018	Golden	Non-organic	Coriander	Italy
H69	Glass	2018	Golden	Non-organic	Tree of heaven	Italy
H70	Glass	2018	Golden	Non-organic	Apple	Italy
H71	Glass	2018	Amber	Non-organic	Forest honey	Italy
H72	Glass	2018	White	Non-organic	Unknown	Canada
H73	Glass	2018	White	Non-organic	Unknown	Canada
H74	Plastic (PET[b])	2016	Golden	Non-organic	Clover	Canada
H75	Plastic (PET)	2016	Amber	Non-organic	Unknown	Australia
H76	Plastic (Unknown)	2016	Golden	Organic	Unknown	Brazil
H77	Plastic (Unknown)	2016	Amber	Organic	Wildflowers	Brazil
H78	Plastic (Unknown)	2016	White	Non-organic	Acacia	Hungary
H79	Plastic (PET)	2016	Amber	Organic	Unknown	Australia and Brazil
H80	Plastic (PET)	2016	Golden	Non-organic	Unknown	Canada
H81	Plastic (Unknown)	2016	Dark	Organic	Beechwood	New Zealand
H82	Plastic (Unknown)	2016	White	Non-organic	Clover	Canada

H83	Plastic (PET)	2016	Amber	Organic	Unknown	Brazil
H84	Plastic (LDPE[c])	2016	White	Non-organic	Clover	Canada
H85	Plastic (PET)	2016	Dark	Non-organic	Manuka	Australia and New Zealand
H86	Plastic (PET)	2017	Amber	Organic	Unknown	Brazil
H87	Plastic (PET)	2017	White	Non-organic	Unknown	Canada
H88	Plastic (PET)	2017	Amber	Non-organic	Eucalyptus	Brazil
H89	Plastic (Unknown)	2017	Amber	Non-organic	Unknown	India
H90	Plastic (PET)	2017	Golden	Non-organic	Unknown	Hungary
H91	Plastic (Unknown)	2018	Golden	Non-organic	Unknown	Canada
H92	Plastic (LDPE)	2018	Dark	Non-organic	Buckwheat	Canada
H93	Plastic (LDPE)	2018	Golden	Non-organic	Wildflowers	Canada
H94	Plastic (LDPE)	2018	Golden	Non-organic	Clover	Canada
H95	Plastic (LDPE)	2018	Golden	Non-organic	Blueberry	Canada
H96	Plastic (PET)	2018	Amber	Non-organic	Unknown	Canada

H97	Plastic (PET)	2018	Amber	Non-organic	Blueberry	Canada
H98	Plastic (PET)	2018	Golden	Non-organic	Clover	Canada
H99	Plastic (PET)	2018	Golden	Non-organic	Unknown	Canada
H100	Plastic (PET)	2018	Golden	Non-organic	Clover	Canada
H101	Plastic (PET)	2018	Golden	Non-organic	Blueberry	Canada
H102	Plastic (PET)	2018	Golden	Non-organic	Orange	Australia and Brazil
H103	Plastic (PET)	2018	Amber	Organic	Unknown	Brazil, Canada and Mexico
H104	Plastic (PET)	2018	Dark	Non-organic	Buckwheat	Canada

[a] In the cases where the colour of the honey was not specified in the label, this was determined by visual comparison with other samples of similar colour whose colour was specified by the manufacturer in the label.

[b] PET = Polyethylene terephthalate

[c] LDPE = Low-density polyethylene

Table S5.2. List of 40 features with higher frequency in honey samples sold in plastic jars along with their proposed formula, compound name and total score after screening with the Agilent Extractable & Leachable LC/QTOF PCDL database.

Exact mass (Da)	Retention time (min)	Proposed formula	Proposed compound name	Score (%)	% Detection in plastic	% Detection in glass	p-value
85.0889	4.344	C5H11N	Piperidine	85.97	6	0	0.0286
115.0634	4.253	C5H9NO2	N-Methylolmethacrylamide	85.77	13	1	0.0119
122.0366	5.438	C7H6O2	Benzoic acid	76.99	23	7	0.0216
122.0371	9.061	C7H6O2	Benzoic acid	81.48	23	7	0.0216
122.0727	11.36	C8H10O	2,4-Xylenol	85.51	61	29	0.0018
122.0732	13.289	C8H10O	2-Ethylphenol	76.02	13	3	0.0420
124.0877	12.453	C8H12O	Ethinylcyclohexanol	76.73	39	7	< 0.0001
126.0321	6.044	C6H6O3	Pyrogallol	84.86	42	21	0.0245
132.0938	16.927	C10H12	Dicyclopentadiene	82.55	10	0	0.0070
136.0888	14.952	C9H12O	α-α-Dimethylbenzenemethanol	98.94	42	18	0.0093
138.1045	15.233	C9H14O	3,5,5-Trimethylcyclohexenone	93.83	39	18	0.0225
139.0997	4.051	C8H13NO	Vinylcaprolactam	86.74	32	7	0.0007
146.0366	8.761	C9H6O2	Coumarin	79.48	16	1	0.0031
148.0531	7.222	C9H8O2	Cinnamic acid	84.11	94	68	0.0062
150.1039	12.713	C4H14O	4-tert-Butylphenol (PTBP)	85.26	55	25	0.0029

150.1044	13.347	C4H14O	4-tert-Butylphenol (PTBP)	75.17	29	10	0.0119
151.0627	5.022	C8H9NO2	Acetaminophen	97.97	42	22	0.0373
162.1029	11.374	C11H14O	4’-Isopropylacetophenone	71.50	39	16	0.0137
162.1267	12.682	C8H18O3	Butyl carbitol	80.21	55	29	0.0116
162.1401	13.765	C12H18	1,3-Diisopropylbenzene	80.95	10	1	0.0439
164.0828	14.982	C10H12O2	2-tert-Butyl-1,4-benzoquinone	70.47	39	16	0.0137
166.0990	12.306	C10H14O2	tert-Butylhydroquinone (TBHQ)	77.50	16	3	0.0127
168.0421	5.369	C8H8O4	Dehydroacetic acid	86.05	23	8	0.0428
176.1191	10.986	N.I. [a]	N.I.	N.I.	13	1	0.0119
180.0797	13.059	C10H12O3	Isopropyl 4-hydroxybenzoate	81.59	10	1	0.0439
180.1149	11.260	C11H16O2	3-tert-Butyl-4-hydroxyanisole (BHA)	84.78	45	16	0.0020
182.0729	13.916	C13H10O	Benzophenone	75.24	10	0	0.0070
208.0165	5.928	C6H12N2S3	Tetramethylthiuram monosulfide	93.38	19	5	0.0281
208.0168	6.086	C6H12N2S3	Tetramethylthiuram monosulfide	78.21	32	12	0.0161
208.1462	14.310	C13H20O2	Isobornyl acrylate	92.95	10	0	0.0070
218.1157	6.904	C10H18O5	2-Hydroxysebacic acid	78.5	10	0	0.0070
230.1521	16.290	C12H22O4	Diisopropyl adipate	98.12	39	19	0.0354
235.1552	9.515	N.I.	N.I.	N.I.	52	27	0.0176

236.1029	14.972	C13H16O4	Monopentyl phthalate	82.26	52	29	0.0260
281.2712	7.699	C18H35NO	Oleamide	81.97	10	0	0.0070
299.9496	4.812	C4HF9O3S	Perfluorobutanesulfonic acid (PFBS)	80.03	26	10	0.0313
370.3086	18.966	C22H42O4	DEHA [b]	74.09	87	68	0.0478
390.2784	18.969	C24H38O4	Di(2-ethylhexyl) terephthalate (DHT)	94.86	13	3	0.0420
414.2071	17.252	N.I.	N.I.	N.I.	13	3	0.042
446.3401	20.374	N.I.	N.I.	N.I.	29	11	0.0226

[a] N.I. = Not Identified (no formula proposed by the software with a score above 70%).

[b] The identity of this compound was confirmed with a standard with a confidence level of 1.

Table S5.3. List of 13 features with a higher frequency in honey samples sold in glass jars along with their proposed formula, compound name and total score after screening with the Agilent Extractable & Leachable LC/QTOF PCDL database.

Exact mass (Da)	Retention time (min)	Proposed formula	Proposed compound name	Score (%)	% Detection in plastic	% Detection in glass	p-value
108.0223	3.679	C6H4O2	Benzoquinone	77.64	3	18	0.0463
120.0933	11.895	C9H12	Cumene	77.96	6	25	0.0312
137.0846	3.414	C8H11NO	p-Phenetidine	86.05	6	32	0.0062
151.0634	4.939	C8H9NO2	Acetaminophen	99.16	3	49	< 0.0001
162.1053	12.556	C11H14O	4’-Isopropylacetophenone	76.02	61	81	0.0354
164.0835	9.295	C10H12O2	2-tert-Butyl-1,4-benzoquinone	86.93	29	55	0.0161
166.0265	15.368	C8H6O4	1,3-Benzenedicarboxylic acid	96.18	29	56	0.0113
166.0631	7.017	C9H10O3	Ethylparaben	78.10	0	12	0.0408
186.1162	9.973	C12H14N2	N-1-Naphthylehtylenediamine (NEDA)	98.45	0	15	0.0223
208.1483	8.774	C13H20O2	Isobornyl acrylate	71.81	0	19	0.0088
228.0790	17.006	C14H12O3	Oxybenzone	95.36	10	29	0.0346
256.0733	16.279	C15H12O4	Monobenzyl phthalate	99.30	74	93	0.0075
530.4704	21.764	C35H62O3	Irganox 1076	84.12	10	29	0.0346

5.9 SUPPLEMENTARY INFORMATION REFERENCES

[1] Commission Regulation of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC), Official Journal of the European Union, (2002) 8-36.

[2] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P. Singer, J. Hollender, Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence, Environ. Sci. Technol., 48 (2014) 2097-2098.

CONNECTING PARAGRAPH

The main steps of the non-targeted workflow for the analysis of trace organic contaminants in honey were studied in detail and optimized in Chapters 3 to 5. Having completed this, Chapter 6 uses the method developed in the previous chapters for the study of the thermal degradation of tylosin A, a veterinary drug, in honey, as well as the determination of its potentially unknown degradation products. Additionally, the possibility of a semi-quantification of tylosin B using its parent compound, tylosin A, is assessed, which closes the non-targeted workflow development. Chapter 6 will be submitted for publication in Food Research International: A. von Eyken, S. Bayen; *Non-targeted study of the thermal degradation of tylosin in honey and water.*

CHAPTER 6: NON-TARGETED STUDY OF THE THERMAL DEGRADATION OF TYLOSIN IN HONEY AND WATER

6.1 ABSTRACT

Tylosin A, a macrolide antibiotic used in beekeeping, is known to undergo thermal degradation in aqueous solution and in honey. Its degradation kinetics in these matrices after different treatments have been described before, leading to the formation of tylosin B as the only known degradation product in honey. However, the degradation studies in honey were performed following a targeted analysis approach (i.e. monitoring only the evolution of tylosin A and tylosin B concentrations using standards), which did not allow the identification of any unknown degradation products. The aim of the study was to characterize the behaviour of tylosin A in honey after heating and during storage, and to identify its degradation products using a non-targeted approach. In addition, the possibility of a semi-quantification of tylosin B using tylosin A was assessed as a case study for the semi-quantification of degradation products using the parent compounds. The results showed significant degradation of tylosin A in aqueous solution (~96%) as well as in spiked and incurred honey (~50% and ~29%, respectively) after heating at 100˚C during 90 min. However, at a lower heating temperature of 70˚C, degradation was only observed in water (~31%). When stored at room temperature (27˚C) for one year, tylosin A degraded significantly (~47%) in an incurred honey sample. Tylosin B, the only reported degradation product of tylosin A in honey so far, increased significantly in aqueous solution under all treatments, but it only increased in spiked honey after heating at 100˚C. Two new degradation products, namely OMT and lactenocin, were tentatively identified in water and spiked honey after heating at 100˚C. The results of the present study reinforce the conclusion that relying only on the water model or spiked food matrix is not sufficient to understand the thermal degradation of antibiotics in food matrices. Finally, a semi-quantification of tylosin B with a relative error of 20% in an incurred honey sample was possible using the response factor of tylosin A, its parent compound. The results of this study prove that a semi-quantification using the parent compound to quantify its degradation compound can provide satisfactory results, but this will be analyte-dependent.

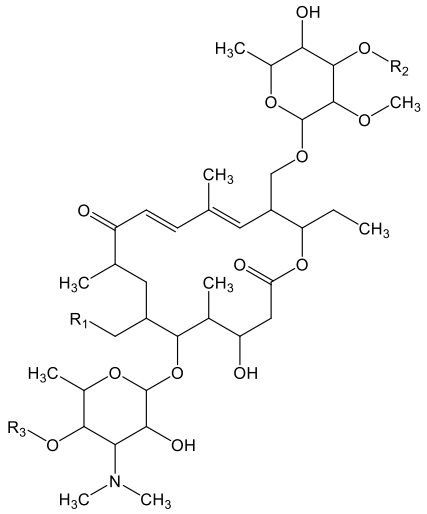
6.2 INTRODUCTION

Tylosin is a macrolide antibiotic produced by fermentation from a strain of the soil microorganism *Streptomyces fradiae* [1]. As shown in **Figure 6.1**, tylosin consists of one major component, tylosin A, and three minor components: desmycosin (tylosin B), macrocin (tylosin C) and relomycin (tylosin D). While most of the microbiological activity resides with tylosin A, tylosin B, C and D have approximately 83%, 75% and 35% of the activity of tylosin A, respectively [2]. In honey bees, tylosin is used for the control of American foulbrood, a highly contagious disease caused by the sporeforming bacterium *Paenibacillus larvae*, which is the most virulent brood disease known in bees [3]. Tylosin A is the major component in the tylosin commercial products applied to the honeybees [4].

Regarding the potential toxicity to humans, tylosin has been found to be relatively non-toxic in mammal models, leading to an acceptable daily intake of 0-30 $\mu g.kg^{-1}$ bw [5]. However, the World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance classified tylosin as a critically important antibiotic for human medicine, as it is the veterinary analog to some human medicines that are part of a limited available therapy to treat several diseases, and also because some of the pathogens that are treated with tylosin may be transmitted to humans from non-human sources [6]. This underlines the need to fully characterize the occurrence and the stability of tylosin A all along the food supply chain.

Residues of tylosin A and B are commonly reported in honey samples. The Canadian Food Inspection Agency (CFIA), in their 2013-2014 National Chemical Residue Monitoring Program report, detected tylosin A in up to 10.05% of the tested domestic honeys and 6.10% of honeys imported to Canada [7]. In the same report, tylosin B was detected in 19.74% of domestic honeys and was the most prevalent residue in imported honeys (14.71% detection). It should be however highlighted that the concentration of tylosin reported by the CFIA rarely exceeded the maximum

residue limit (MRL) for these substances in honey, which is 0.2 $mg.kg^{-1}$ for the sum of tylosin A and B [8].



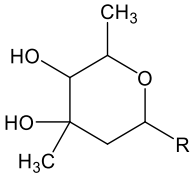
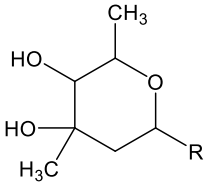
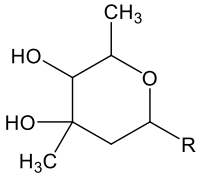
	Tylosin A	Tylosin B	Tylosin C	Tylosin D
R_1	−CHO	−CHO	−CHO	$-CH_2OH$
R_2	$-CH_3$	$-CH_3$	−H	$-CH_3$
R_3		−H		

Figure 6.1. Chemical structures of tylosin components.

Some information on the physicochemical and biological degradation of tylosin A has been reported in the literature. Mitchell *et al.* studied the degradation of tylosin A in aqueous solutions and reported half-lives for the hydrolysis of tylosin A at 60˚C of 1.1 days at pH 4, and 3.5 days at pH 9 [9]. Based on tandem mass spectrometry (MS/MS) data, the presumed degradation pathways of tylosin A under these conditions were proposed, leading to 3 degradation products other than tylosin B (see **Table S6.1**) [9]. In animal models, ingested tylosin is metabolized primarily in the liver into four major metabolites and several minor metabolites, the major metabolic pathway being the reduction of tylosin A to tylosin D [10]. In addition, tylosin A has

been found to be the only macrolide antibiotic able to absorb sunlight and undergo photochemical degradation, resulting in the formation of the photoisomer γ/δ-*cis*-tylosin [11]. **Table S6.1** summarizes the molecular formula, the exact mass and the structure of tylosin A and all its related compounds described in the literature to date, including the degradation products and biosynthesis precursors.

There are also some reports on the degradation of tylosin A in honey. These studies were performed following a targeted analysis approach, i.e. they monitor only the evolution of tylosin A and tylosin B concentrations using analytical standards. For example, Kochansky *et al.* studied the stability of tylosin A and B in honey during storage in the dark at 34˚C, approximating hive conditions, and found that tylosin A had a half-life time of 130 days under these conditions [12]. The concentration of tylosin B increased over the same period of time, indicating that tylosin B is stable in honey for up to 9 months. In a similar study conducted at higher temperatures, tylosin A in honey was found to degrade into tylosin B following a first-order kinetic model, with half-lives of 9 days at 50˚C, 9 h at 80˚C, and 48 min at 110˚C [13]. In honey stored at 80˚C and 110˚C, tylosin B was found to decay into unknown products [13]. To-date, no comprehensive non-targeted study of the behaviour of tylosin A in honey has been reported.

When performing degradation studies with a non-targeted approach, the two main steps of the data treatment that need to be optimized are the identification and quantification of new substances. Although progresses have been made on the identification of non-targeted substances in food matrices, to date providing quantitative information in the absence of a pure analytical standard remains a challenge as the response factor (RF) is unknown [14, 15]. Three approaches have been proposed to address this issue and provide a semi-quantification of the concentration of the analytes: *in silico* ionization prediction [16, 17], semi-quantification based on chemical similarity [18-20] and semi-quantification based on similar chromatographic behaviour [21].

In the case of degradation products of known contaminants, some authors suggested a semi-quantification of the degradation products using the response factor of the parent compound. This method has been applied to estimate the levels of the degradation products of chlortetracycline and demeclocycline in agricultural soils and drainage waters [22]. Although this approach seems to lead to some reasonable estimates of the concentrations, to date no validation of this approach has been reported.

The aim of the study was to characterize the behaviour of tylosin A in honey after heating and during storage, and to identify its degradation products using a non-targeted approach. In addition, the semi-quantification of tylosin B using tylosin A was assessed as a case study for the semi-quantification of degradation products using the parent compounds in non-targeted analysis.

6.3 MATERIALS AND METHODS

6.3.1 Chemicals and reagents

An analytical standard of tylosin A was purchased from Sigma-Aldrich (St Louis, MO, USA). Tylosin B was purchased from Toku-E (Bellingham, WA, USA). HPLC grade solvents (water, methanol and acetonitrile), as well as LC/MS grade formic acid were all purchased at Fisher Scientific (Pittsburgh, PA, USA). Stock standards of tylosin A and B were prepared in methanol. LC/MS grade acetic acid and sodium hydroxide (99.99% purity) were purchased from Sigma-Aldrich (St Louis, MO, USA).

6.3.2 Honey Samples

Four honey samples (H1-H4) were obtained from different stores in Canada in May 2016. All 4 samples were preliminary tested for tylosin A and B, and the results confirmed their absence in samples H1, H2 and H3, while sample H4 had detectable amounts of both tylosin A and B [23].

For this reason, samples H1-H3 were selected as matrix blanks for the validation of the semi-quantification approach, and sample H4 was used for the degradation studies. In addition, H1 was also used as a matrix blank for the degradation studies. The pH of samples H1 and H4 was measured following the method described by the International Honey Commission [24], which gave a value of 4.05 and 4.36 for samples H1 and H4, respectively. All samples were transferred from their original container to 40 mL amber glass vials and kept at -18^{o}C until analysis.

6.3.3 Sample Preparation

6.3.3.1 Thermal degradation in water (70 and 100˚C)

A summary of all the degradation experiments realized in the present study in aqueous solution and honey samples is presented in **Figure 6.2**. Separate working solutions of tylosin A (25.2 nmol.L^{-1}) and tylosin B (25.9 nmol.L^{-1}), which would correspond to the concentration after extraction of a honey sample 10 times above the MRL [8], were made up from the stock standard using HPLC grade water. pH was adjusted to 3.60 with acetic acid and sodium hydroxide to mimic the usually acidic pH of honey [25]. Aliquots (1 mL, n=5) were transferred into 2 mL amber glass vials and were heated in a water bath. Two temperatures (70 and 100°C) were selected as they simulate the use of honey as a sweetener in hot beverages and in baked goods. The selected heating time for this and the other heating experiments in the present study was 90 min, since according to the literature at least some degradation should be observed for both temperatures in both water and honey after this time [9, 13, 26]. Some of the reported studies on the degradation of tylosin extended the heating time to up to several hours [13]. However, when thinking of the degradation of tylosin in food matrices during heating in a real-life scenario, such long cooking times would rarely happen. For this reason, 90 min were selected as a compromise between achieving some degradation and being somehow representative of a real cooking situation.

After 90 minutes, samples were cooled down rapidly in cold water, and were kept at -18°C prior to LC-MS analysis. Five replicates of the same solutions were prepared as above but were not heated. Ten procedural blanks (5 heated and 5 non-heated) were prepared as above without any tylosin. Additionally, 5 Quality Control (QC) samples were prepared by mixing equal volumes of all the extracted samples. Such QC samples are used in non-targeted analysis as indicators of the quality of the analysis and to detect any possible instrumental artifacts such as any drifts in mass accuracy or retention time (RT). Post analysis, the results of the QC samples can be examined visually, generally through an unsupervised method such as Principal Component Analysis (PCA), to give an indication of the reproducibility of the analysis [27].

6.3.3.2 Thermal degradation in honey (70 and 100˚C)

For this experiment, 0.2 g of honey sample (H1 and H4) were dissolved in 3.6 mL of water to obtain a 5.6% (w/v) solution of honey. This ratio corresponds approximately to the concentration of honey when used as sweetener in hot beverages (about 2 teaspoons per cup). Another solution was prepared as above using honey sample H1 spiked with a mixture of tylosin A and B standards (442.6 and 526.6 $nmol.kg^{-1}$ in honey, respectively). Aliquots (1 mL, n=5) of each of these solutions ("H1", "spiked H1" and "H4") were transferred into 2 mL amber glass vials and were heated in a water bath (70 and 100°C). After 90 min, samples were cooled down rapidly in cold water. The sample preparation from here was adapted from a previous study [23]. Briefly, 1 mL of acetonitrile was added to obtain a water/acetonitrile (1:1 v/v) solution. Prior to injection into the HPLC system, extracts were further diluted with water to a final concentration corresponding to 1% of honey (w/v). Five replicates of each honey sample (H1, spiked H1 and H4) were prepared following the same procedure but without heating, along with 5 heated and 5 non-heated procedural blanks. Sample H1 was used as a matrix blank for both spiked H1 and H4 because it was not possible to acquire sample H4 non-incurred (which would constitute a true matrix blank). Five QCs were also prepared as explained in section 6.3.3.1.

6.3.3.3 Degradation in honey during storage

For the storage experiment, 1 mL of honey samples H1 and H4 were transferred into 2 mL amber glass vials (n=5 for each honey) and stored in an oven maintained at 27 ± 1°C for one year. After this time, approximately 0.2 g of honey was weighed in a glass conic tube and 2 mL of a mixture of acetonitrile and water 1:1 was added. Samples were vortexed for about 2 min, or until the honey was completely dissolved, and then filtered through a 0.22 µm PTFE filter from Chrom4 (Thüringen, Germany). Prior to the injection into the HPLC system, the extract was further diluted with water to a final concentration corresponding to 1% of honey (w/v). Sample H1 was used as a matrix blank for sample H4. Five QCs were also prepared as explained in section 6.3.3.1.

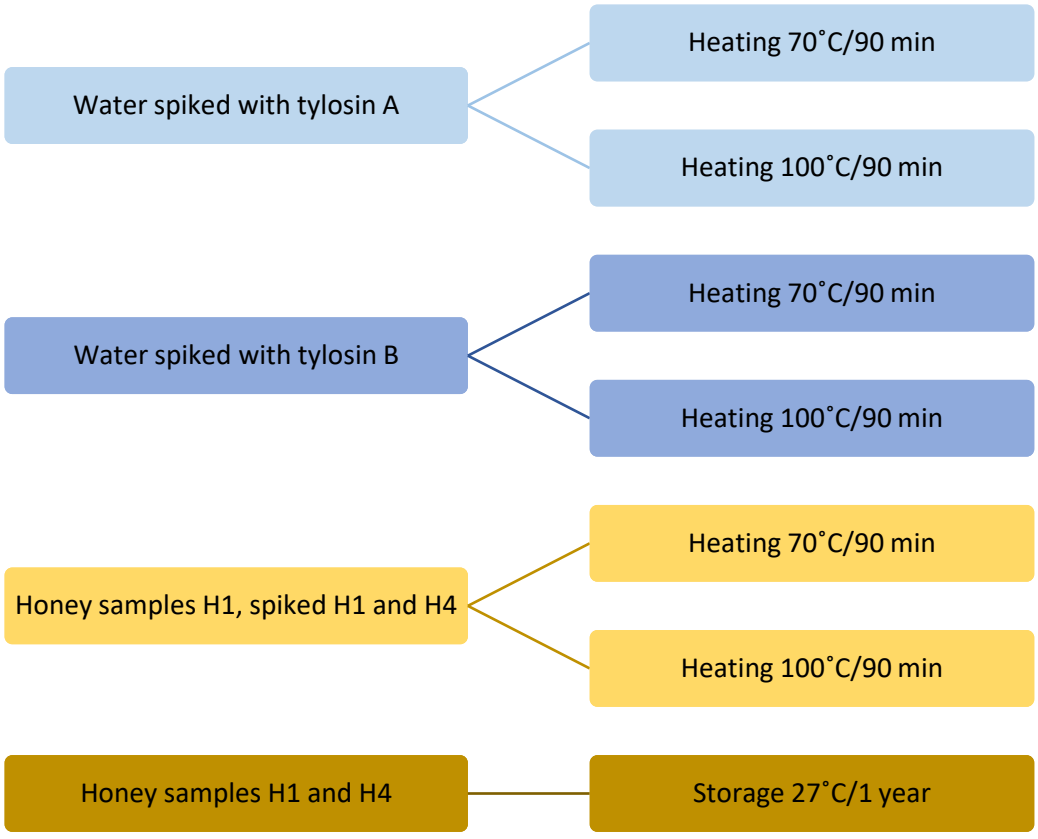


Figure 6.2. Summary of the degradation experiments conducted in water and in honey.

6.3.3.4 Semi-quantification of tylosin B

Honey samples H1-H3 spiked with tylosin A and B were used as a case study for the semi-quantification approach. Approximately 0.2 g of honey was weighed in a glass conic tube and 2 mL of a mixture of acetonitrile and water 1:1 was added. Samples were vortexed for about 2 min, or until the honey was completely dissolved, and then filtered through a 0.22 µm PTFE filter from Chrom4 (Thüringen, Germany). Prior to the injection into the HPLC system, the extract was further diluted with water to a final concentration corresponding to 1% of honey (w/v). Aliquots (1 mL) of each of these diluted extracts were spiked with 50 µL of a mixture of tylosin A and B at 7 different levels corresponding to 0.007-3.4 $\mu g.g^{-1}$ honey, in order to evaluate the semi-quantification approach at different concentrations.

6.3.4 Instrument analysis

Samples were analyzed following a previously developed method [23]. A 1290 series LC system from Agilent Technologies (Santa Clara, CA, USA) equipped with an InfinityLab Poroshell 120 Phenyl Hexyl (3.0 x 100 mm, 2.7 µm) column fitted with an InfinityLab Poroshell 120 Ec-C18 (3.0 x 5 mm, 2.7 µm) guard column, both from Agilent Technologies. The mobile phase consisted in water (solvent A) and methanol (solvent B), both with 0.1% formic acid, at a flow rate of 0.2 $mL.min^{-1}$. The mobile phase gradient profile was as follows: 1 min 5% B, from 1 to 15 min gradient to 100% B, from 15 to 20 min 100 % B, from 20 to 20.10 min gradient to 5% B and from 20.10 to 25 min 5% B. The injection volume was 20 µL and the column temperature was set to 20°C. The LC system was coupled to a 6545 series Q-TOF from Agilent Technologies (Santa Clara, CA, USA) equipped with a Dual AJS ESI ion source operating in positive ionization mode. Drying gas temperature was 325°C with a flow of 5 $L.min^{-1}$, sheath gas temperature was 275°C with a flow of 12 $L.min^{-1}$, the pressure on the nebulizer was 20 psi, the capillary voltage was 4000 V, the fragmentor voltage was 175 V, the skimmer voltage was 65 V and the nozzle voltage was 2000 V. All Ions MS/MS data was collected as MS scans between *m/z* 100 and 1700 at a scan rate of 3 $spectra.s^{-1}$ for four different collision energies (0, 10, 20 and 40 V). As validated in a previous

study, the first 3 min of elution were diverted to waste to prevent the entry of sugars and matrix-related polar compounds into the detector [23]. Samples were kept at 4°C in the multisampler compartment.

6.3.5 Data treatment

6.3.5.1 Targeted quantification of tylosin A and B

Antibiotic concentrations were calculated using Mass Hunter Workstation Software – Quantitative Analysis B.07.01 (Agilent Technologies). In the degradation studies, tylosin A and B were quantified based on external calibration (n=7, ranging 0.08 to 44 nmol.L^{-1}) following a method developed in a previous study [23]. Peaks with a signal-to-noise ratio below 10 were considered below the limit of quantification. An extraction window of ± 10 ppm was used, and matrix effect of each sample was taken into account when calculating antibiotic concentrations based on a previous study [23]. The statistical significance of the changes of concentration for both tylosin A and tylosin B were determined in each experiment through performing a Student t-test with $p < 0.05$.

6.3.5.2 Non-targeted identification of degradation products of tylosin A

LC-MS data were processed with the *Molecular Feature Extraction* algorithm of MassHunter Profinder B.08.00 (Agilent Technologies) to perform the peak alignment in the chromatograms and the extraction of molecular features. The software parameters were selected as follows based on an earlier optimization study [15]: a peak filter of 200 counts, a RT tolerance of 0.00% + 0.30 min, a mass tolerance of 10 ppm + 2.00 mDa, and a post-processing filter of 1000 counts. Adducts with H^+, Na^+ and K^+ were considered. Molecular feature extraction was limited to compounds present in at least 80% of the samples in at least one group of samples. This threshold corresponded to the presence of up to one outlier in each group.

After peak alignment, the comparison of the compounds among the various samples was performed using the software Mass Profiler Professional B.14.8 (Agilent Technologies). In this software, all data was normalized with 75.0 percentile shift. First, a PCA of all samples was performed to investigate the grouping of the QC samples. Their grouping in a PCA plot reflects the quality of the LC-MS analysis [27]. Then, volcano plots were created to compare the various groups of samples in each experiment, and to identify molecular features related to the degradation of tylosin A. The statistical analysis of the volcano plot consisted in a moderated Student t-test, followed by a Benjamini-Hochberg test (*p*-value threshold set at 0.05). For each experiment, the treated (i.e. heating or storage) spiked or incurred samples were compared with the same samples before treatment as well as with the treated blanks. Once relevant features had been identified with the algorithms *Find by Molecular Feature,* molecular formulas were assigned to each compound using the *Generate Formulas* algorithm (Agilent Mass Hunter Workstation Software – Qualitative Analysis B.07.00). Only formulas with a total score above 70% were considered, which is what is commonly done for this type of non-targeted analysis [15, 28]. Total scores reflect the probability that a feature has been correctly identified, a score of 100% being a perfect fit [29]. Finally, Agilent MassHunter Molecular Structure Correlator B.07.00 was used to create a list of potential candidate structures for each of the compounds for which a formula with a total score above 70% had been assigned. In order to do so, since this software only recognizes MS/MS spectra obtained through targeted or auto MS/MS (but not All-Ions MS/MS data), targeted MS/MS spectra were acquired for each of the compounds. The search for structures with similar MS/MS spectra was done using the ChemSpider library [30]. The criteria used to choose the degradation product candidates among the proposed structures was similarity with tylosin A structure, considering the central tylactone as the minimum requirement for similarity.

6.3.5.3 Semi-quantification of tylosin B

Tylosin B was first quantified in three selected honey samples using external calibration based on the response of its $[M+H]^+$ ion fragment *m/z* 772.4483 [31] and the use of pure analytical standard (absolute quantification). The semi-quantification of tylosin B was then performed in the same samples using the response factor of tylosin A measured from 6 matrix matched calibration standards. In this case, the signal obtained for various characteristic ion fragments of tylosin A and B were used for this purpose. Since data were acquired in the All Ions MS/MS mode for four different collision energies, many combinations of major ion fragments/collision energies can be potentially used for semi-quantification. In each test the collision energy at which both tylosin A and B fragments were recorded was the same. For each of these combinations, a relative error on the quantification was calculated as shown in **Equation 1,** where C_{SQ} is the concentration obtained through semi-quantification and C_{AQ} is the concentration obtained through absolute quantification:

$$Relative\ error\ (\%) = \left(\frac{C_{SQ}}{C_{AQ}} - 1\right) \times 100 \qquad \textbf{Equation 1}$$

To further assess the suitability of the approach, the semi-quantification method with the lowest relative error was then applied to sample H4. This final semi-quantification approach was also applied to estimate the concentration of the potential degradation products identified during the study of the thermal degradation of tylosin in honey.

6.4 RESULTS AND DISCUSSION

6.4.1 QA/QC

First, the linearity of the calibration curves used for the quantification of tylosin A and B during the different experiments was calculated as the relative standard deviation (RSD) of the RFs. In all the experiments, the response of the instrument was linear, with RSD below 10%.

Next, the performances of the method were assessed in terms of RT, reproducibility and mass accuracy. RT reproducibility was calculated for tylosin A (RT: 14.41 min) and B (RT: 13.69 min) as the RSD of the RT across all the samples in each experiment in which they were detected. RTs for these two compounds were very reproducible, with RSD values systematically below 0.2% in each experiment. Then, the mass accuracy was evaluated throughout the different experimental runs by calculating the mass error of the exact mass measured for tylosin A and B across all the samples in each experiment in which they were detected. The mass accuracy obtained was acceptable, with mass errors in the different experiments between 1.5 ± 0.3 and 4.4 ± 0.9 ppm for tylosin B (*m/z* 772.4483) and between 1.7 ± 0.1 and 4.1 ± 1.6 ppm for tylosin A (*m/z* 916.5270).

QC samples were also run in each batch to assess the overall variability of the LC-MS analysis. PCA plots were produced for each experiment in which a statistically significant degradation of tylosin A had been observed. As shown in **Figure S6.1**, QC samples were grouped together in all the PCA plots, indicating that the LC-MS based analysis was reproducible [27].

6.4.2 Thermal degradation of tylosin A in water

The concentration of tylosin A in water decreased significantly from its initial value (25.2 nmol.L^{-1}) after 90 min of heating (**Table 6.1**), reaching concentrations of 17.4 ± 2.3 and 1.1 ± 0.1 nmol.L^{-1} at 70 and 100˚C, respectively (**Figure 6.3-A**). This represented a mean degradation percentage of ~31 and ~96% at 70 and 100˚C, respectively. These results are in line with other hydrolysis

studies of tylosin at high temperatures [9, 26]. The concentration of tylosin B increased significantly from its initial value as an impurity of the tylosin A standard (0.4 nmol.L^{-1}) after heating the aqueous solutions containing tylosin A (**Table 6.1**). The concentrations of tylosin B after heating were of 5.8 ± 0.6 and 15.2 ± 1.4 nmol.L^{-1} at 70 and 100˚C, respectively (**Figure 6.3-B**). Tylosin B has been reported in the literature as the main (known) degradation product of tylosin A in water [26]. The mass balance reported by Paesen *et al.* was close to 100% when the percentages of decrease of tylosin A and increase of tylosin B at pH 4.0 and 70˚C were combined [26]. However, in the present study, done at a slightly more acidic pH of 3.60, tylosin A showed a decrease of 7.8 ± 2.3 and 24.1 ± 0.1 nmol.L^{-1} at 70 and 100˚C, respectively, while the increase of tylosin B was lower (5.4 ± 0.6 and 14.8 ± 1.4 nmol.L^{-1} at 70 and 100˚C, respectively), indicating the potential formation of other degradation products.

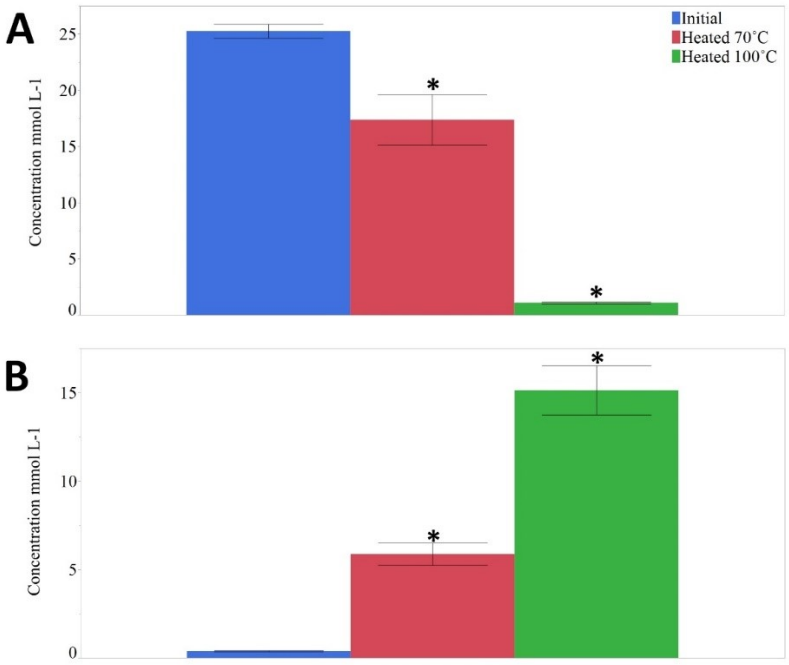


Figure 6.3. A: Concentration of tylosin A in aqueous solutions (pH 3.60, heating time of 90 min). B: Concentration of tylosin B in aqueous solutions spiked with tylosin A. Significant ($p < 0.05$) changes are indicated with *.

Volcano plots were created to compare the different pairs of groups of samples and their outcomes are summarized in **Table 6.2**. An example of the volcano plot corresponding to the comparison of aqueous solution of tylosin A unheated vs. heated at 100˚C is presented in **Figure S6.2**. Twelve molecular features increased significantly (p<0.05) and with S/N>3 in heated spiked aqueous solutions. The *Find by Molecular Feature* and *Generate Formulas* algorithms were used to assign molecular formulas to these potential degradation products. Tylosin B was confirmed as one of the molecular features increasing after heating and, among the 11 other compounds, 6 were assigned a molecular formula with a total score above 70% using (**Table 6.3)**. Co-eluting compounds, a limitation of this clean up-free method which could impact mass accuracy and formula assignment, could be a cause for the lack of assigned formula in the remaining 5 compounds. Targeted MS/MS spectra were acquired for the 6 compounds with molecular formula, and Agilent MassHunter Molecular Structure Correlator was used to propose possible structures. Two compounds, CPD19 and CPD28, were assigned a putative compound whose structure is related to tylosin A, and were tentatively identified as 5-O-mycaminosyltylonolide (OMT) and lactenocin respectively (**Figure 6.4**).

Table 6.1. Summary of the Student t-test results for tylosin A and B in all the experiments.

Experiment	Sample	Temperature	Tylosin A	Tylosin B
Heating	Water with tylosin A	70˚C	DEGRADATION ($p = 6.47 \times 10^{-4}$)	INCREASE ($p = 4.13 \times 10^{-5}$)
		100˚C	DEGRADATION ($p = 1.11 \times 10^{-7}$)	INCREASE ($p = 1.90 \times 10^{-5}$)
	Water with tylosin B	70˚C	n.a.[a]	NO CHANGE ($p = 4.90 \times 10^{-1}$)
		100˚C	n.a.	NO CHANGE ($p = 2.45 \times 10^{-1}$)
	Sample H4	70˚C	NO CHANGE ($p = 9.65 \times 10^{-1}$)	NO CHANGE ($p = 3.13 \times 10^{-1}$)
		100˚C	DEGRADATION ($p = 3.22 \times 10^{-2}$)	NO CHANGE ($p = 4.62 \times 10^{-1}$)
	Sample H1 spiked	70˚C	NO CHANGE ($p = 1.70 \times 10^{-1}$)	NO CHANGE ($p = 1.05 \times 10^{-1}$)
		100˚C	DEGRADATION ($p = 5.65 \times 10^{-6}$)	INCREASE ($p = 3.39 \times 10^{-4}$)
Storage	Sample H4	27˚C	DEGRADATION ($p = 6.78 \times 10^{-6}$)	NO CHANGE ($p = 9.93 \times 10^{-1}$)

[a] n.a. = Not applicable

Table 6.2. Summary of compounds whose level is increasing significantly for each of the thermal degradation experiments

Experiment	**Number of significantly increasing compounds with S/N > 3**
Water spiked with tylosin A heated at 100˚C	12
Spiked H1 heated at 100˚C	10
H4 heated at 100˚C	9
H4 stored at 27˚C	12

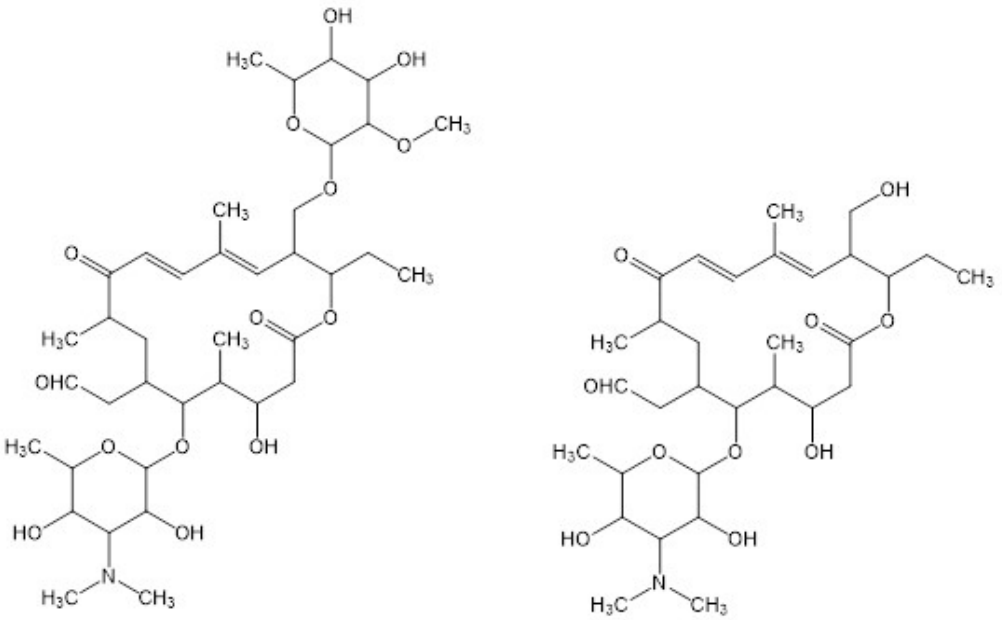


Figure 6.4. Structures of the two tentatively identified compounds related to tylosin A.

Table 6.3. Neutral mass, RT and suggested formula for tylosin A and its potential degradation products observed in the four degradation experiments. Legend for the symbols: ×: not detected, ↑: increasing significantly ($p < 0.05$) after treatment, = : detected but not changing significantly ($p < 0.05$) after treatment, ↓: decreased significantly ($p < 0.05$) in treated samples.

Name	Neutral mass (Da)	RT (min)	Suggested formula [a]	Heating water with tylosin A 100˚C	Heating Spiked H1 100˚C	Heating H4 100˚C	Storage H4 27˚C
Tylosin A	915.5192	14.41	$C_{46}H_{77}NO_{17}$	↓	↓	↓	↓
Tylosin B	771.4402	13.69	$C_{39}H_{65}NO_{14}$	↑	↑	=	=
CPD1	215.9952	4.67	$C_{13}N_2O_2$ (71%)	×	×	↑	×
CPD2	262.0003	4.67	$C_{14}H_2N_2O_4$ (77%)	×	×	↑	×
CPD3	212.0665	6.64	$C_5H_{12}N_2O_7$ (77%)	×	↑	×	×
CPD4	191.0951	8.01	$C_{11}H_{13}NO_2$ (98%)	×	=	↑	=
CPD5	188.0467	8.29	$C_{11}H_8O_3$ (96%)	×	×	×	↑
CPD6	504.8450	8.59	No formula found	↑	×	×	×
CPD7	440.1363	8.82	$C_{24}H_{26}NO_3S_2$ (71%)	×	↑	×	×
CPD8	112.0517	9.08	$C_4H_6N_3O$ (73%)	×	×	=	↑

CPD9	230.0690	9.14	$C_{12}H_{10}N_2O_3$ (82%)	✗	✗	↑	✗
CPD10	283.0833	9.34	$C_{14}H_{11}N_4O_3$ (81%)	✗	=	↑	↑
CPD11	292.0552	9.44	$C_9H_{12}N_2O_9$ (87%)	✗	=	↑	↑
CPD12	124.0879	9.58	$C_6H_{10}N_3$ (84%)	✗	✗	✗	↑
CPD13	394.8610	9.88	$C_{19}H_{20}Cl_2N_2O_3$ (86%)	✗	=	=	↑
CPD14	394.8610	10.13	$C_{19}H_{20}Cl_2N_2O_3$ (87%)	✗	=	=	↑
CPD15	168.0692	10.76	$C_{11}H_8N_2$ (96%)	✗	↑	↑	↑
CPD16	214.0749	10.89	$C_{12}H_{10}N_2O_2$ (85%)	✗	=	↑	↑
CPD17	472.1266	11.79	$C_{23}H_{22}NO_{10}$ (85%)	✗	↑	✗	✗
CPD18	629.3754	12.02	$C_{32}H_{55}NO_{11}$ (95%)	↑	↑	=	=
CPD19	597.3501	12.03	$C_{31}H_{51}NO_{10}$ (81%)	↑	↑	=	=
CPD20	224.1399	12.47	$C_{13}H_{20}O_3$ (87%)	✗	↑	✗	✗
CPD21	789.4476	13.04	No formula found	↑	✗	✗	✗
CPD22	771.4447	13.43	No formula found	↑	✗	✗	✗

CPD23	774.4569	13.65	$C_{35}H_{70}N_2O_{14}S$ (79%)	↑	↑	=	↑
CPD24	556.1003	13.74	No formula found	✗	✗	=	↑
CPD25	771.4388	13.96	$C_{39}H_{65}NO_{14}$ (96%)	↑	↑	↑	=
CPD26	817.4756	14.10	No formula found	↑	✗	✗	✗
CPD27	805.4792	14.21	$C_{40}H_{71}NO_{15}$ (71%)	↑	✗	✗	✗
CPD28	757.4586	14.46	$C_{38}H_{63}NO_{14}$ (85%)	↑	✗	✗	✗
CPD29	771.4388	15.48	No formula found	↑	✗	✗	✗
CPD30	302.2058	18.26	No formula found	✗	=	=	↑

[a] Total score of the suggested formula calculated by the software Mass Profiler Professional B.14.8 is presented between parenthesis

OMT and lactenocin are two intermediates in the biosynthesis of tylosin A that have been found in the fermentation media during tylosin A production [26, 32]. Since tylosin B, the main known degradation product of tylosin A, is also an intermediate in the biosynthesis of tylosin A, the present results suggest that tylosin A could degrade into OMT and lactenocin [32]. To the best of our knowledge, these two compounds have not been reported as degradation products of tylosin A in the literature.

Tylosin A is known to contain other tylosin species, such as tylosin C, in its commercial formulations [2, 4]. In the present study, a peak with the same exact mass of tylosin C was observed in the unheated standard solutions (901.5035 Da, RT: 14.32 min). This peak decreased significantly ($p < 0.05$) after heating at 100˚C. Based on its structure, lactenocin (**Figure 6.4**) could come from the degradation of tylosin A but also from traces of tylosin C present in the aqueous solution. This hypothesis, however, could not be confirmed in the present study due to the absence of standards. The identity of OMT and lactenocin, and the exact degradation pathway taking place in each case should be confirmed in further studies based on the availability of analytical standards.

The two main concerns of the presence of unknown or unexpected antibiotics in food are their potential toxic effects on the consumer and their residual antimicrobial activity, which could contribute to the development of antimicrobial resistance [33, 34]. The only data on the possible antimicrobial activity of OMT and lactenocin was reported in 1995 by Zuzulova *et al.*, who studied the antimicrobial activity of tylosin A and five other 16-membered macrolides against *Ureaplasma urealyticum*, and found that both OMT and lactenocin showed antimicrobial activity [35]. According to this study, the aldehyde group in these compounds would be the main responsible for their antimicrobial activity [35]. Were the identity of the two compounds found in the present study to be confirmed with their standards, the results from Zuzulova *et al.* would suggest that some antimicrobial activity could be retained after the degradation of tylosin A. To the best of our knowledge, no data was found regarding the toxicity of OMT and lactenocin.

6.4.3 Thermal degradation of tylosin B in water

The possible thermal degradation of tylosin B was also studied (experimental conditions as in section 6.4.2) to fully interpret the thermal degradation results of tylosin A in water. No significant degradation of tylosin B was observed after heating a diluted solution of the compound at 70 and 100˚C for 90 min (**Table 6.1**). This result was expected, since tylosin B appears to be a stable degradation product of tylosin A and its degradation has only been observed at longer heating times or higher temperatures (i.e. above 10 h in honey at 80˚C, above 1.5 h in honey at 110˚C) [13, 26].

6.4.4 Degradation of tylosin A in honey at high temperatures

Based on the literature, tylosin A degrades in honey both at high temperatures (50, 80 and 110˚C), and at temperatures resembling hive conditions (34˚C) [12, 13]. In the present study, the thermal degradation of tylosin A in honey was studied in two different situations common to food products: heating and storage.

The degradation of tylosin A in honey after heating was studied using one incurred sample (H4) and one matrix blank (H1) spiked with a mixture of tylosin A and tylosin B. Tylosin A concentrations did not change when heated at 70°C but decreased significantly in the all treatments at 100˚C (**Table 6.1**). Indeed, in spiked H1 samples (n=5) treated at 100˚C for 90 min., the mean concentration of tylosin A decreased from 442.6 to 223.0 ± 28.4 nmol.kg^{-1} (~50% decrease) (**Figure 6.5-A**), while the mean concentration of tylosin B increased significantly ($p < 0.05$) from 526.6 to 782.3 ± 72.2 nmol.kg^{-1} (**Figure 6.5-B**). In other words, the amount of tylosin A degraded (219.6 ± 28.4 nmol.kg^{-1}) was proportionally equal to the amount of tylosin B produced (255.7 ± 72.2 nmol.kg^{-1}) in spiked honey. Under the same conditions, the mean concentration of tylosin A in incurred H4 samples decreased from 24.6 ± 1.6 to 17.4 ± 5.2 nmol.kg^{-1} (~29% decrease), while the concentration of tylosin B did not significantly change (**Table 6.1**). This

observation suggests that degradation products other than tylosin B were formed in incurred honey heated at 100˚C.

Following a data treatment workflow similar to the one reported in section 3.2, volcano plots were created to identify the potential degradation products of tylosin A (**Figures S6.3 and S6.4**). Ten compounds were found to significantly increase ($p < 0.05$) with a S/N > 3 in spiked sample H1 heated at 100˚C (**Table 6.2**). In the case of the incurred honey sample (H4), 9 compounds were found to increase significantly in sample H4 heated at 100˚C (**Table 6.2**). The identity of both groups of compounds was further studied using the *Find by Molecular Feature* and *Generate Formulas* algorithms, as in section 3.2. **Table 6.3** summarizes the information obtained for the 10 compounds found in spiked H1 and the 9 compounds found in H4. Targeted MS/MS spectra were acquired for those compounds that were assigned a formula with a total score above 70% and Agilent MassHunter Molecular Structure Correlator was used to propose possible structures. However, none of the suggested chemical structures could be directly related to tylosin A, except for OMT (CPD19).

Although the same heating conditions were applied to all the samples, different suites of degradation products of tylosin A were obtained in water, spiked honey and incurred honey. These findings are in line with the study by Tian *et al.*, which identified different suites of degradation products of chloramphenicol in model solutions, spiked mussel tissues and incurred mussel tissues [36]. Similarly, Franje *et al.* studied the thermal degradation of three amphenicols in different matrices (chicken muscle, soybean sauce and water) and that the degradation kinetics and degradation products were different for all these matrices [37]. The present results reinforce the conclusion that relying only on the water model or spiked food matrix is not sufficient to understand the fate, and notably the thermal degradation of antibiotics in food.

6.4.5 Degradation of tylosin A in honey during storage

A storage experiment was performed for an incurred sample (H4) using sample H1 as a matrix blank. Tylosin A concentration was found to decrease significantly ($p < 0.05$) from 24.6 ± 1.6 to 13.0 ± 2.2 nmol.kg^{-1} (i.e. ~47% degradation) after one year of storage at room temperature (**Table 6.1**, **Figure 6.5-A**). Tylosin B levels did not change significantly ($p > 0.05$) (**Table 6.1, Figure 6.5-B**), suggesting a degradation of tylosin A into other compounds. Alternatively, this result could be the simultaneous degradation of tylosin A into tylosin B and of the tylosin B already present at the beginning of the storage experiment into other products. In the present study tylosin B did not degrade significantly in water (Section 6.4.3). However, Kochansky *et al.* reported the simultaneous degradation of tylosin A and B in honey stored at 34˚C after 250 days [13], which supports the idea of a hypothetical degradation of tylosin B in honey during storage at 27˚C in the present study. Further studies with honey samples containing only tylosin B are needed in order to verify this hypothesis.

The comparison of initial and stored samples, following the same data treatment workflow as used in sections 6.4.2 and 6.4.4 (**Figure S6.5**), led to 12 compounds increasing significantly in sample H4 stored for one year (**Table 6.2**). No new structures potentially related to tylosin A were suggested for any of these compounds.

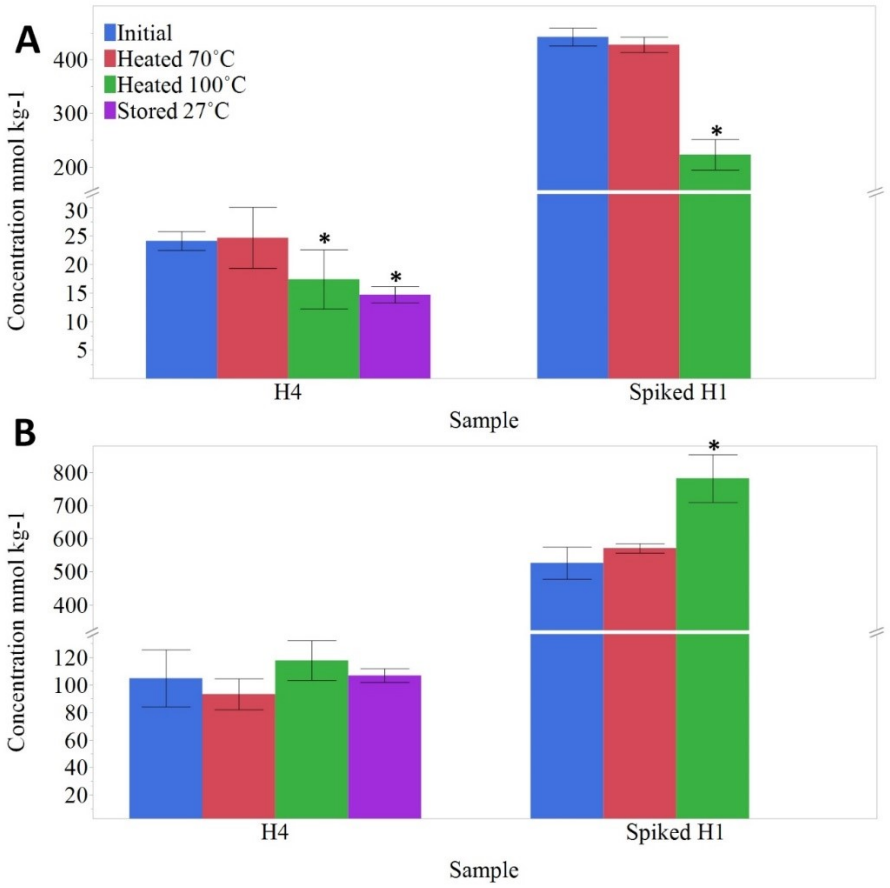


Figure 6.5. A: Concentrations of tylosin A in H4 and spiked H1 after heating (90 min) and storage (1 year). B: Concentrations of tylosin B in H4 and spiked H1 after heating and storage. Statistical significance ($p < 0.05$) is indicated with *.

6.4.6 Semi-quantification of tylosin B and other degradation products

The feasibility of the semi-quantification of tylosin B using its parent compound, tylosin A, was assessed using three honey samples spiked with both compounds. The results (**Table 6.4**) show that in many cases the concentration obtained with semi-quantification (C_{SQ}) was very different from the one obtained using an absolute quantification (C_{AQ}). Indeed, relative errors above 100% were obtained in many cases. However, in the tests where both ions were the parent ions (test 1) at low collision energy, or when they both were the smallest and most stable fragment (test

6) at high collision energy, the relative errors decreased dramatically, reaching levels of accuracy comparable to those of conventional targeted quantification [23]. Despite being similar molecules, the fragmentation patterns of tylosin A and B and the relative stabilities of their fragments could be different. This would explain, for example, why using the same ion *m/z* 772.4483 for both tylosin A and B (test 3) gave such high errors at all the tested collision energies.

The results of the semi-quantification using both parent ions (**Table 6.4**, test 1) at 0 V were equivalent to those at 10 V, and also to those obtained with the smallest fragments (**Table 6.4**, test 6) at 40 V. However, it was decided to select the ions from the first test at 0 V for the semi-quantification of the incurred honey samples because, being the only successful case that does not need MS/MS, it would have higher applicability in further studies.

Table 6.4. Mean absolute values of relative error (%) for the semi-quantification of tylosin B for each test at different collision energies.

Test #	**Ion of tylosin B used to semi-quantify tylosin B**	**Ion of tylosin A used to calculate the RF for the semi-quantification**	**Relative error (%)**			
			0 V	**10 V**	**20 V**	**40 V**
1	*m/z* 772.4483	*m/z* 916.5270	18 ± 4	11 ± 3	45 ± 9	89 ± 2
2	*m/z* 174.1130	*m/z* 916.5270	98 ± 1	81 ± 27	15 ± 2	> 100
3	*m/z* 772.4483	*m/z* 772.4483	> 100	> 100	> 100	> 100
4	*m/z* 174.1130	*m/z* 772.4483	> 100	> 100	> 100	> 100
5	*m/z* 772.4483	*m/z* 174.1130	> 100	> 100	> 100	99 ± 0
6	*m/z* 174.1130	*m/z* 174.1130	> 100	88 ± 40	> 100	17 ± 2

The concentration of tylosin B in the incurred sample H4 was calculated using this semi-quantification approach in order to see its results in a real case scenario. The estimated concentration of tylosin B in this sample, obtained with the semi-quantification method, was 96.9 $ng.g^{-1}$. The actual concentration derived from the RF of tylosin B standard was 80.9 $ng.g^{-1}$, which

lead to a relative error of 20% for the semi-quantification in this sample. The total sum of tylosin A and B in this sample obtained through semi-quantification of tylosin B was 119.5 $ng.g^{-1}$, while the actual concentration calculated using both standards was 103.4 $ng.g^{-1}$. If this semi-quantification approach were to be applied for regulatory screening purposes, this sample would have been correctly classified as compliant because its estimated concentration calculated through semi-quantification would still be below the MRL of 200 $ng.g^{-1}$ of total tylosin (A+B) [8]. This sample, however, presented levels of tylosin A and B that, being representative of what is commonly found in market studies [7], were much lower than the regulatory limit. Further studies need to be conducted to assess the impact that using a semi-quantification approach for tylosin B would have in the classification of samples with concentrations around the regulatory limit. The range of concentrations in which this semi-quantification would have an acceptable uncertainty would need to be defined in order to consider the application of semi-quantification for regulatory purposes.

Finally, this semi-quantification approach was used to estimate the concentration of the two compounds suspected to be degradation products of tylosin A (OMT and lactenocin). The concentration of OMT in aqueous solutions heated at 100˚C was estimated to be 1.3 ± 0.1 $nmol.L^{-1}$, while in spiked H1 heated at 100˚C it was estimated to be 24.6 ± 2.7 $nmol.kg^{-1}$. Lactenocin was only detected in the water experiment, with an estimated concentration of 0.12 ± 0.01 $nmol.L^{-1}$ in spiked water heated at 100˚C. **Figure 6.6** shows the peaks of OMT and lactenocin in the four different degradation experiments performed in the present study.

The contribution of OMT and lactenocin to the total mass balance of tylosin A and its degradation products is presented in **Table 6.5**. In the water experiment, the total increase of tylosin B, OMT and lactenocin after heating at 100˚C was still lower than the decrease of tylosin A. Two main conclusions could be derived from this result: (i) either tylosin A degraded into more unknown products that were not quantified in the present study or (ii) the error induced by the semi-quantification of these products was greater than the error of the semi-quantification of tylosin

B (20%), and their actual concentration is higher. On the other hand, in the case of spiked honey H1 heated at 100˚C the addition of OMT could complete the mass balance of tylosin A, potentially resulting in its only degradation product besides tylosin B. The calculation of these mass balances is, however, only an estimation. Further studies with OMT and lactenocin standards are needed in order to obtain their absolute quantification and thus evaluate their semi-quantification and contribution to mass balance in the present study.

Table 6.5. Mass balances of tylosin A, tylosin B, OMT and lactenocin for the experiments where OMT and/or lactenocin increased significantly ($p < 0.05$).

Compound	**Heated water with tylosin A 100˚C (nmol.L^{-1})**	**Heated Spiked H1 100˚C (nmol.kg^{-1})**
Tylosin A	-24.1 ± 0.1	-219.6 ± 28.4
Tylosin B	14.8 ± 1.4	255.7 ± 72.2
OMT	1.3 ± 0.1	24.6 ± 2.7
Lactenocin	0.12 ± 0.01	Not detected

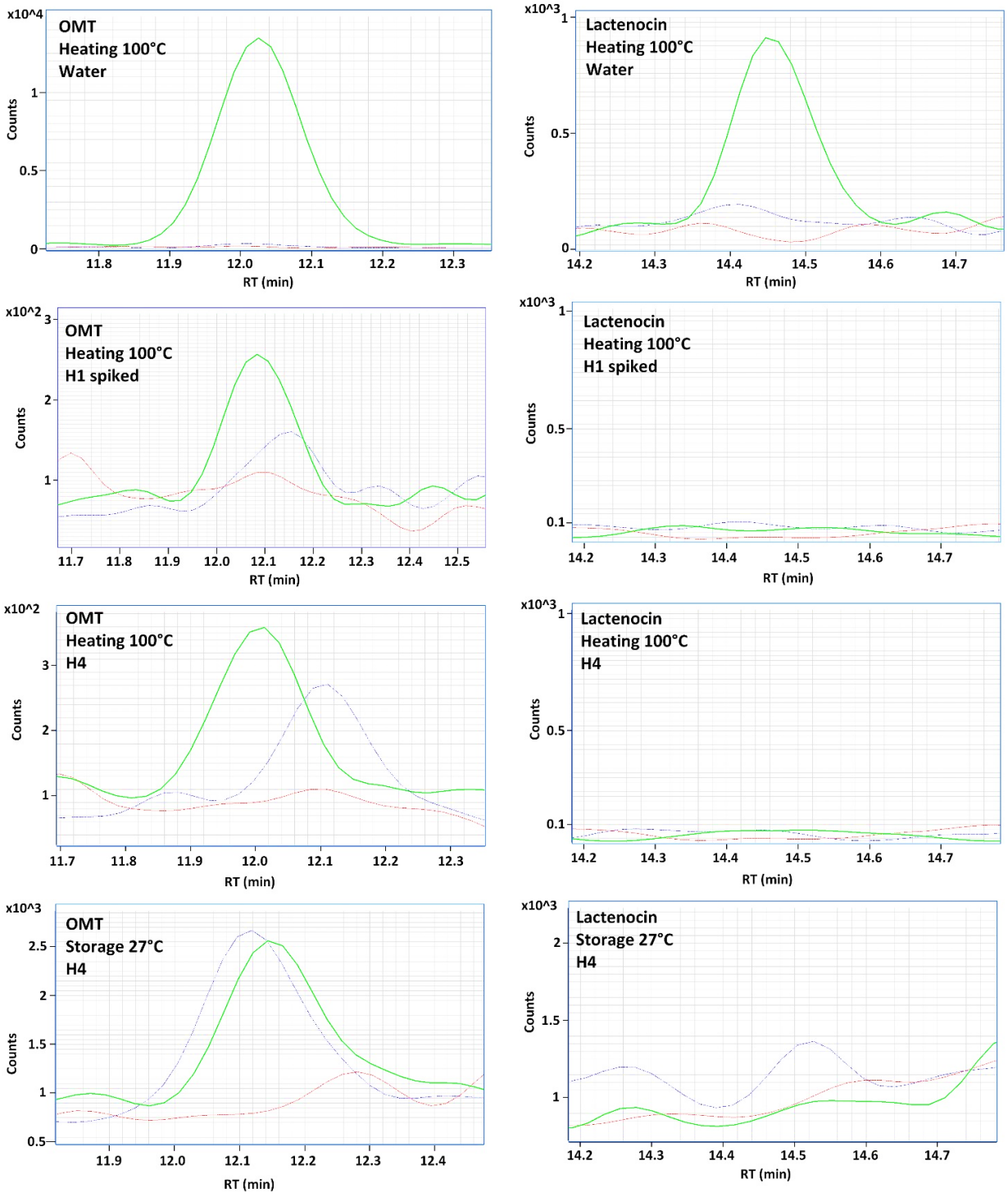


Figure 6.6. Peaks of OMT and lactenocin in each degradation experiment. Legend: green = spiked/incurred treated sample, blue = spiked/incurred non-treated sample, red = treated blank.

6.5 CONCLUSIONS

The degradation of the veterinary drug tylosin A in water and honey after different thermal treatments was studied. Tylosin A degraded significantly in aqueous solution as well as in spiked and incurred honey after heating at 100˚C during 90 min. However, at a lower heating temperature of 70˚C, degradation was only observed in water. When stored at room temperature (27˚C) for one year, tylosin A degraded significantly in an incurred honey sample. Tylosin B, the only reported degradation product of tylosin A so far, increased significantly in aqueous solution under all treatments, but it only increased in spiked honey after heating at 100˚C. Overall, these results confirm what had been previously reported by other authors.

The novelty of the present study is the characterization of the behaviour of tylosin A using a non-targeted approach, which led to the identification of two tentative new degradation products, namely OMT and lactenocin. Both compounds increased significantly in aqueous solution after heating at 100˚C, and OMT also increased significantly in spiked honey heated at 100˚C. The rates of degradation of tylosin A as well as the potential degradation products detected in each of the studied matrices (i.e. water, spiked honey and incurred honey) under the same heating conditions were different. This supports the conclusion that relying only on the water model or spiked food matrix is not sufficient to understand the thermal degradation of antibiotics in food matrices. However, only one incurred honey sample was available at the time of this study, so further experiments including more incurred samples are needed in order to confirm the differences between using a model based on spiked honey or a real incurred sample.

To the best of our knowledge, lactenocin and OMT have not been reported as degradation products of tylosin A. The identity of these compounds and the exact degradation pathway taking place in each case should be confirmed in future studies based on the availability of analytical standards. In addition, while the potential antimicrobial activity of OMT and lactenocin has been reported, no information regarding their toxicity is available. Therefore, further studies are

required in order to characterize the hazards and assess the potential risks associated with the presence of these compounds in food.

Regarding the semi-quantification approach, the results of this study prove that a semi-quantification using the parent compound to quantify its degradation compound can have satisfactory results, and thus it could be a good strategy for the semi-quantification of degradation compounds in non-targeted analysis. However, the applicability of this semi-quantification approach for regulatory purposes still needs to be explored, as further studies are needed to define the range of concentration with acceptable uncertainty.

6.6 ACKNOWLEDGMENTS

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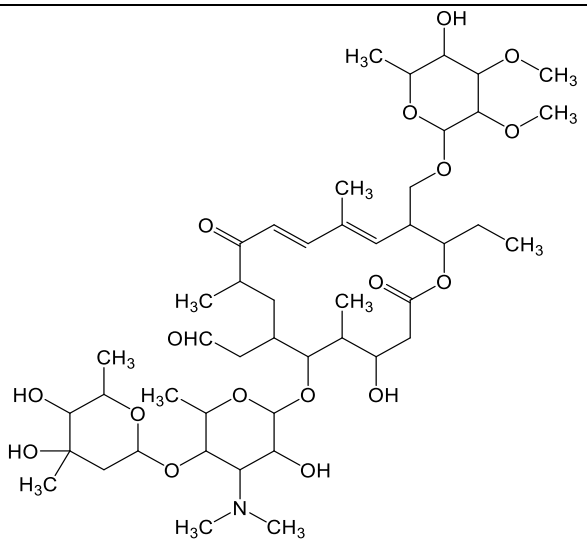
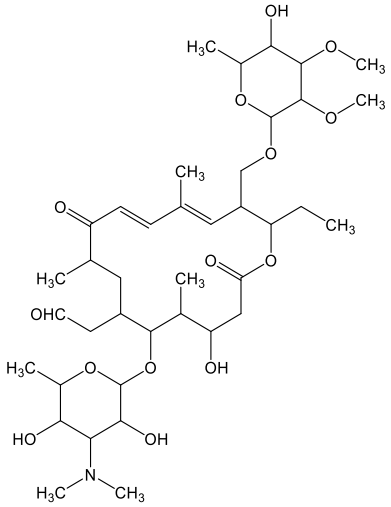
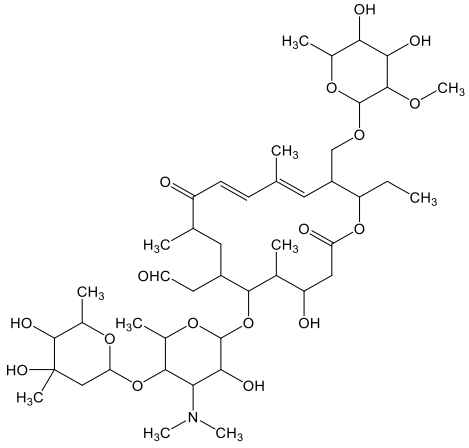
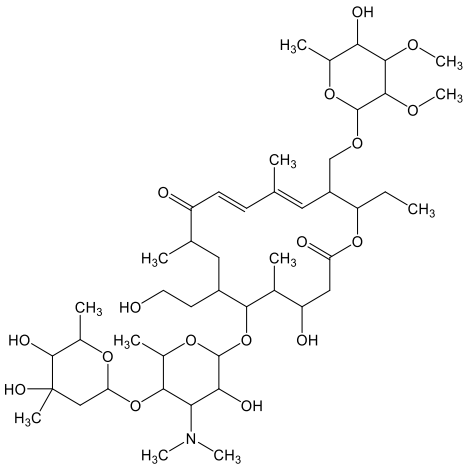
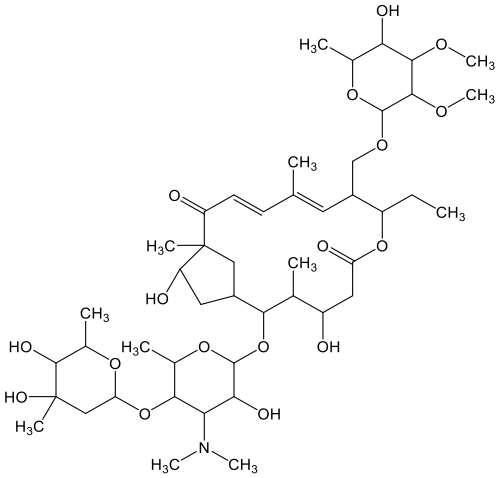
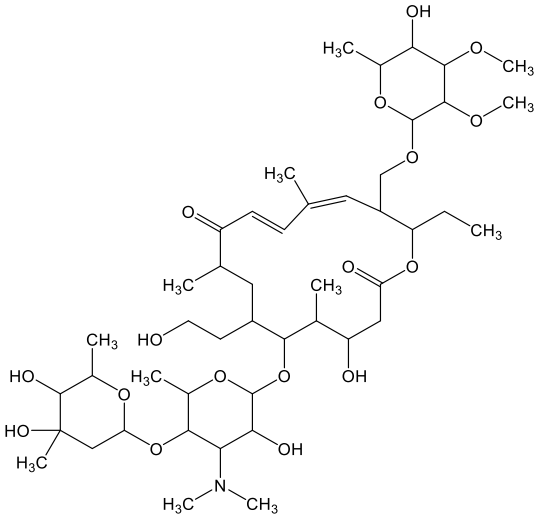
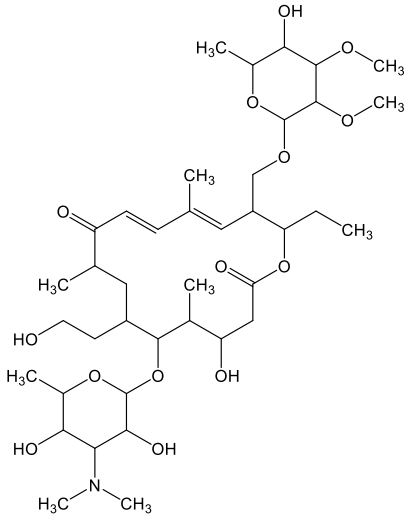
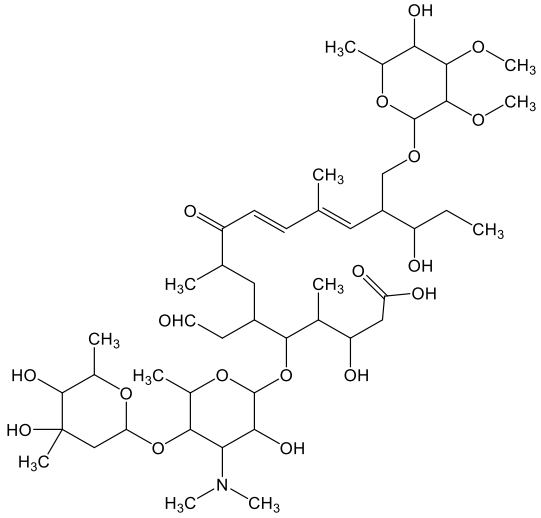
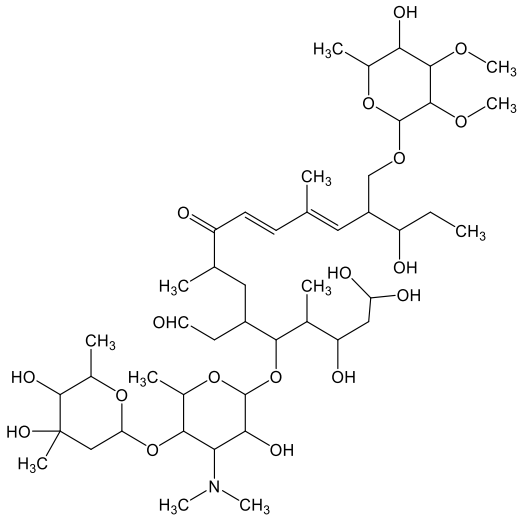
6.8 SUPPLEMENTARY INFORMATION

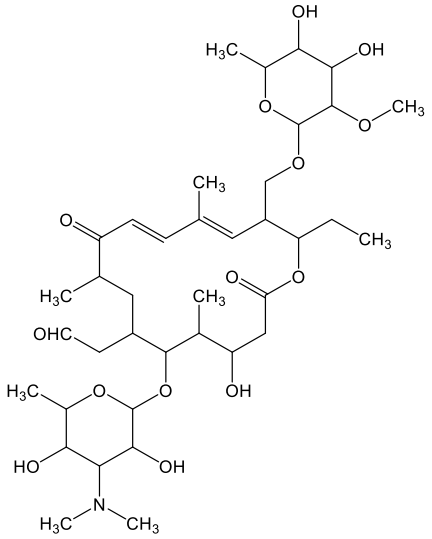
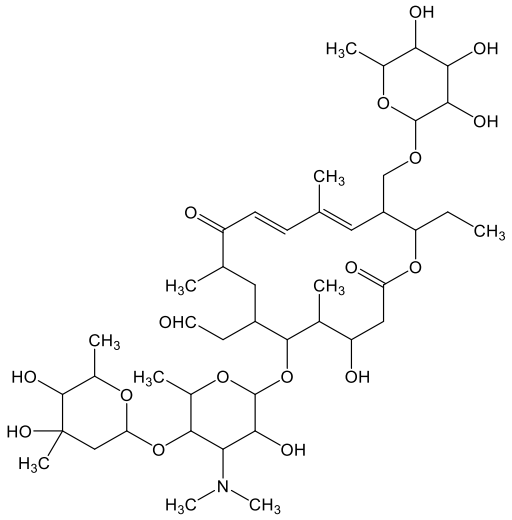
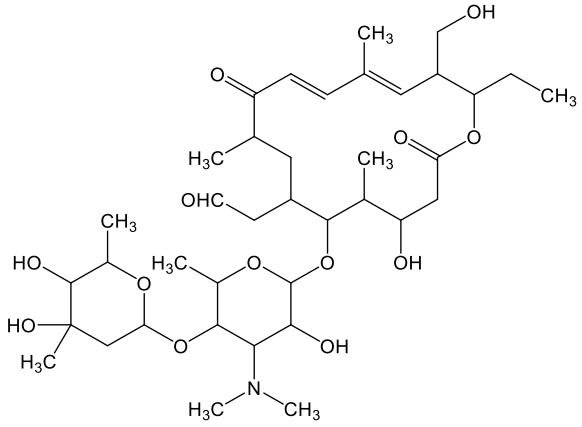
Table S6.1. Name, molecular formula, exact mass and structure of tylosin A and all its related products (degradation products and/or biosynthesis precursors) described to-date.

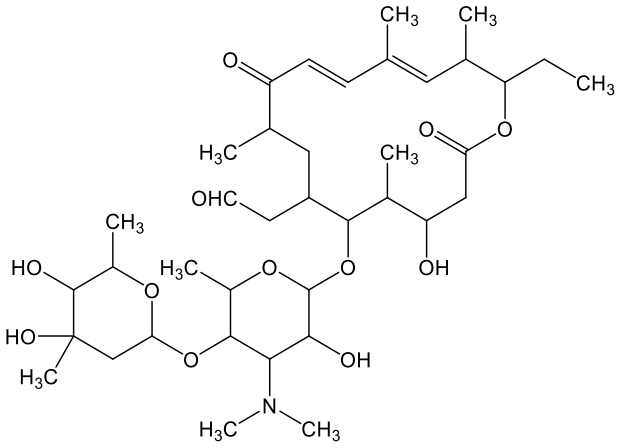
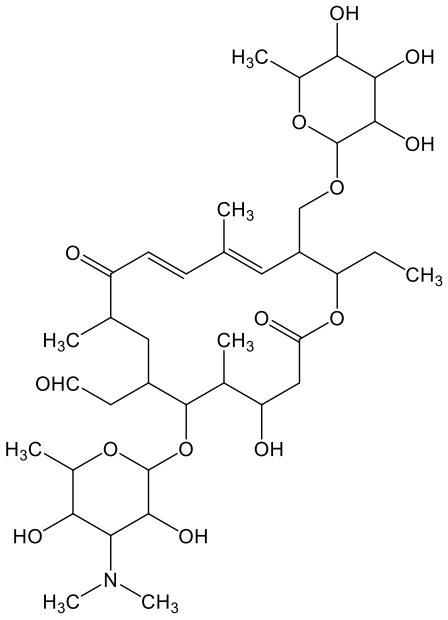
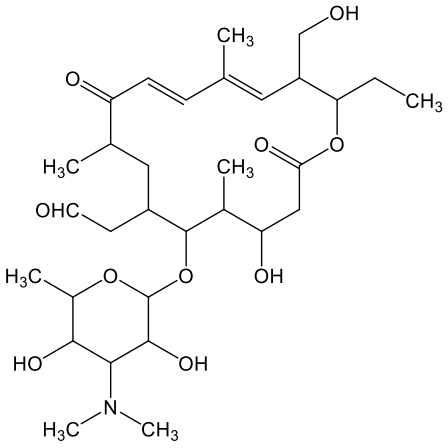
Name	**Formula**	**Exact mass (Da)**	**Structure**	**Ref.**
Tylosin A	$C_{46}H_{77}NO_{17}$	915.5192		[1]

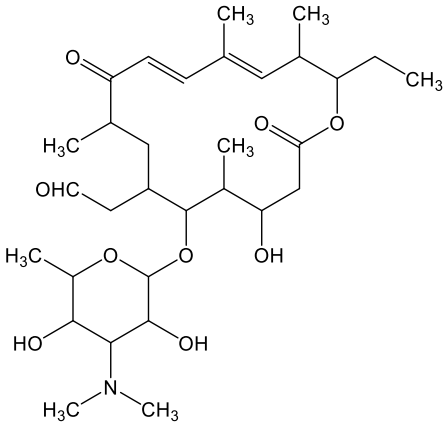
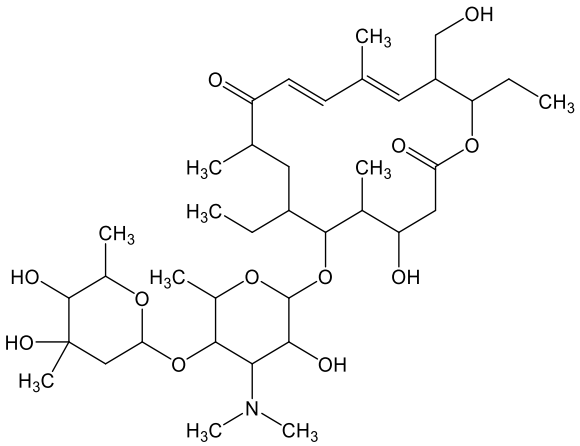
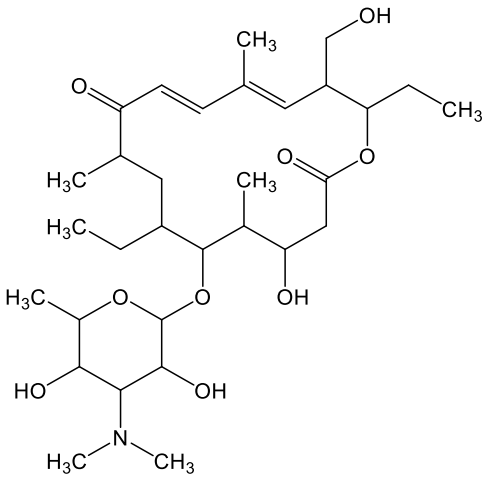
Tylosin B (desmycocin)	$C_{39}H_{65}NO_{14}$	771.4405		[1]
Tylosin C (macrocin)	$C_{45}H_{75}NO_{17}$	901.5035		[1]

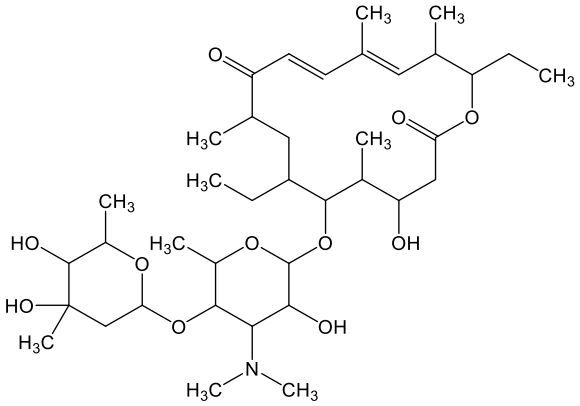
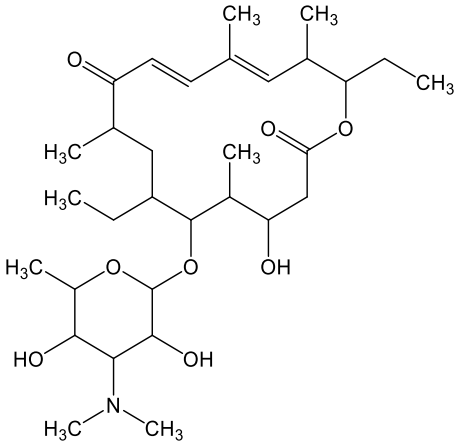
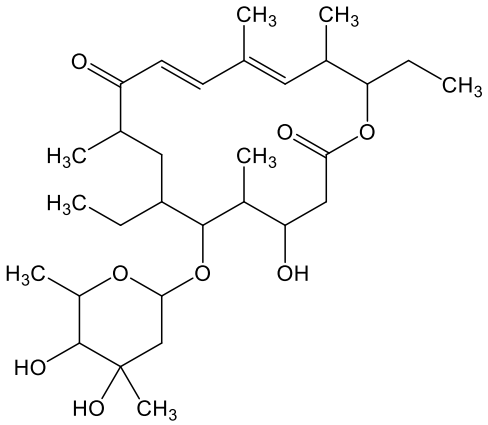
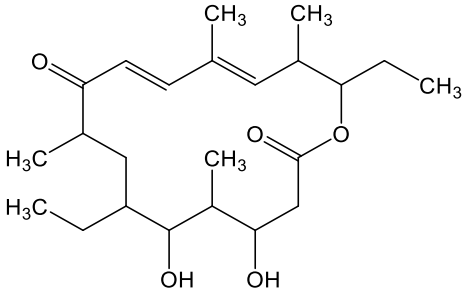
Tylosin D (relomycin)	$C_{46}H_{79}NO_{17}$	917.5348		[1]
Tylosin A aldol	$C_{46}H_{77}NO_{17}$	915.5192		[1]
Isotylosin A alcohol	$C_{46}H_{79}NO_{17}$	917.5348		[2]

Dihydrodesmycosin	$C_{39}H_{67}NO_{14}$	773.4562		[3]
Unknown degradation product 1	$C_{46}H_{79}NO_{18}$	933.5297		[4]
Unknown degradation product 1	$C_{46}H_{81}NO_{18}$	935.5454		[4]

Lactenocin	$C_{38}H_{63}NO_{14}$	757.4249		[1]
O-Demethylmacrocin (DOMM)	$C_{44}H_{73}NO_{17}$	887.4879		[5]
Demcycinosyltylosin (DMT)	$C_{38}H_{63}NO_{13}$	741.4299		[5]

23-Deoxydemycinosyltylosin (DMOT)	$C_{38}H_{63}NO_{12}$	725.4350		[5]
O-Demethyllactenocin (DOML)	$C_{37}H_{61}NO_{14}$	743.4092		[5]
5-O-Mycaminosyltylonolide (OMT)	$C_{31}H_{51}NO_{10}$	597.3513		[5]

23-Deoxy-5-o-mycaminosyltylonolide (DOMT)	$C_{31}H_{51}NO_9$	581.3564		[5]
20-Deoxy-20-dihydrodemycinosyltylosin (DODMT)	$C_{38}H_{65}NO_{12}$	727.4507		[5]
20-Deoxy-20-dihydro-5-O-mycaminosyltylonolide (DOOMT)	$C_{31}H_{53}NO_9$	583.3720		[5]

20,23-Dideoxy-20-dihydrodemycinosyltylosin (DODMOT)	$C_{38}H_{65}NO_{11}$	711.4558		[5]
5-O-Mycaminosyltylactone (DODOMT)	$C_{31}H_{53}NO_8$	567.3771		[5]
5-O-Mycarosyltylactone	$C_{30}H_{50}O_8$	538.3506		[5]
Tylactone	$C_{23}H_{38}O_5$	394.2719		[5]

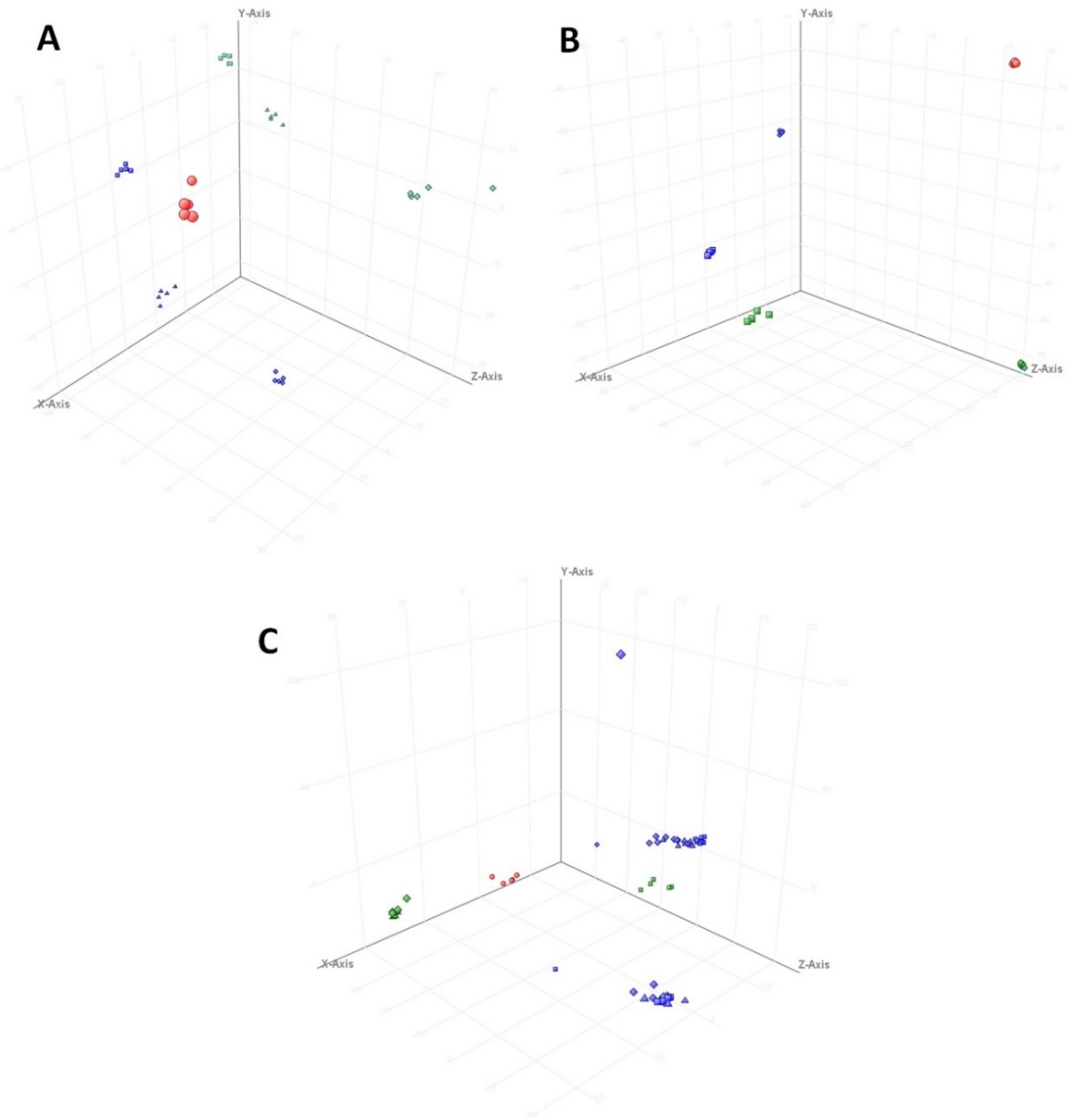


Figure S6.1. PCA plots of all samples in each experiment, including the water or honey samples containing tylosin, the procedural or matrix blanks and the QC samples. A: Degradation of tylosin A in water (legend: green = procedural blanks, blue = spiked water, red = QCs). B: Degradation of tylosin A in honey during storage (legend: green = sample H1, blue = sample H4, red = QCs). C: Degradation of tylosin A in honey after heating (legend: green = sample H1, blue = sample H4 and H1 spiked, red = QCs).

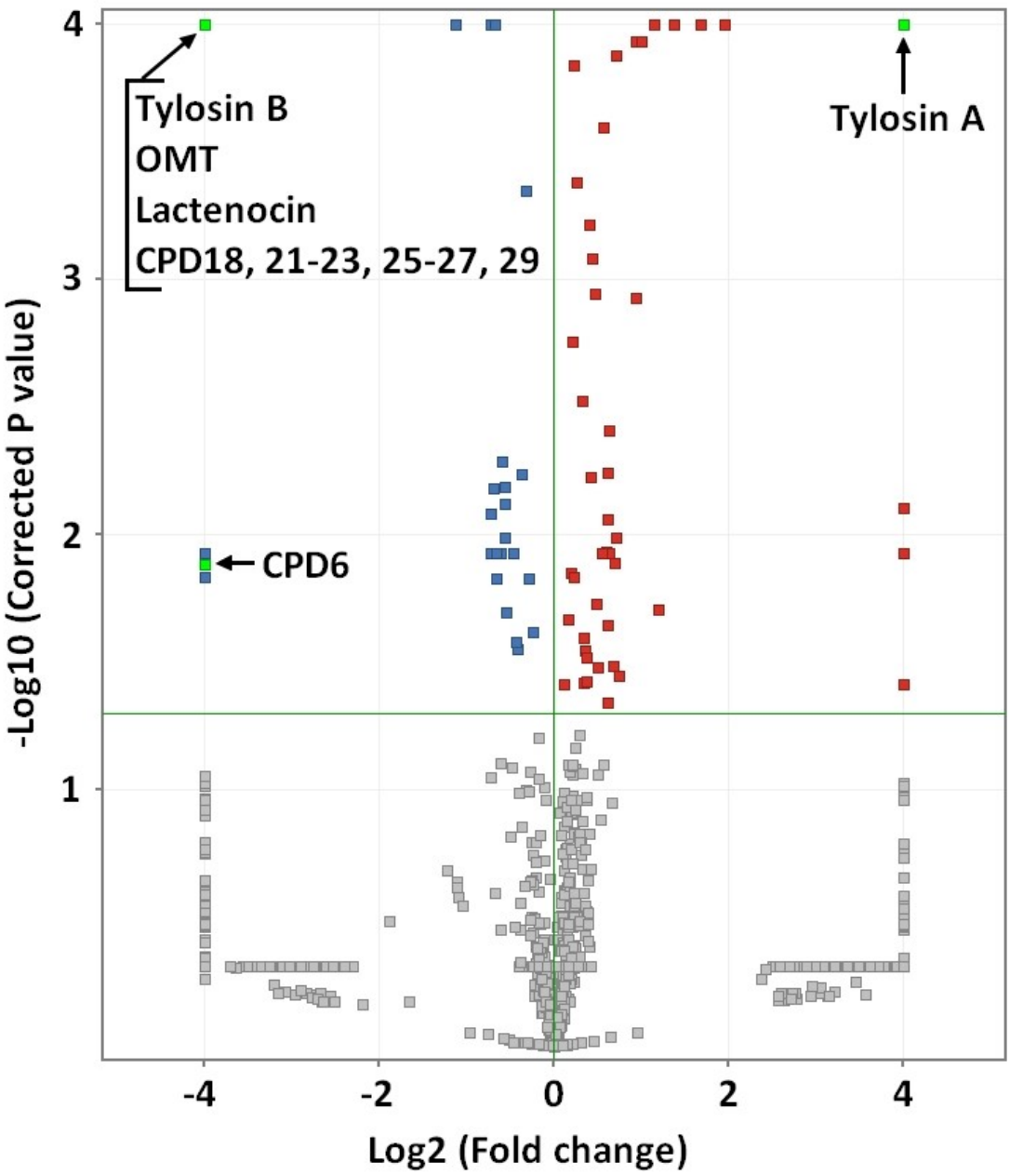


Figure S6.2. Volcano plot of the comparison between water spiked with tylosin A unheated and heated at 100˚C. Legend: red = compounds significantly decreasing after heating ($p<0.005$), blue = compounds significantly increasing after heating ($p<0.05$), grey = compounds without a significant change after heating ($p>0.05$), green = compounds changing significantly ($p<0.05$) and identified in **Table 6.3**.

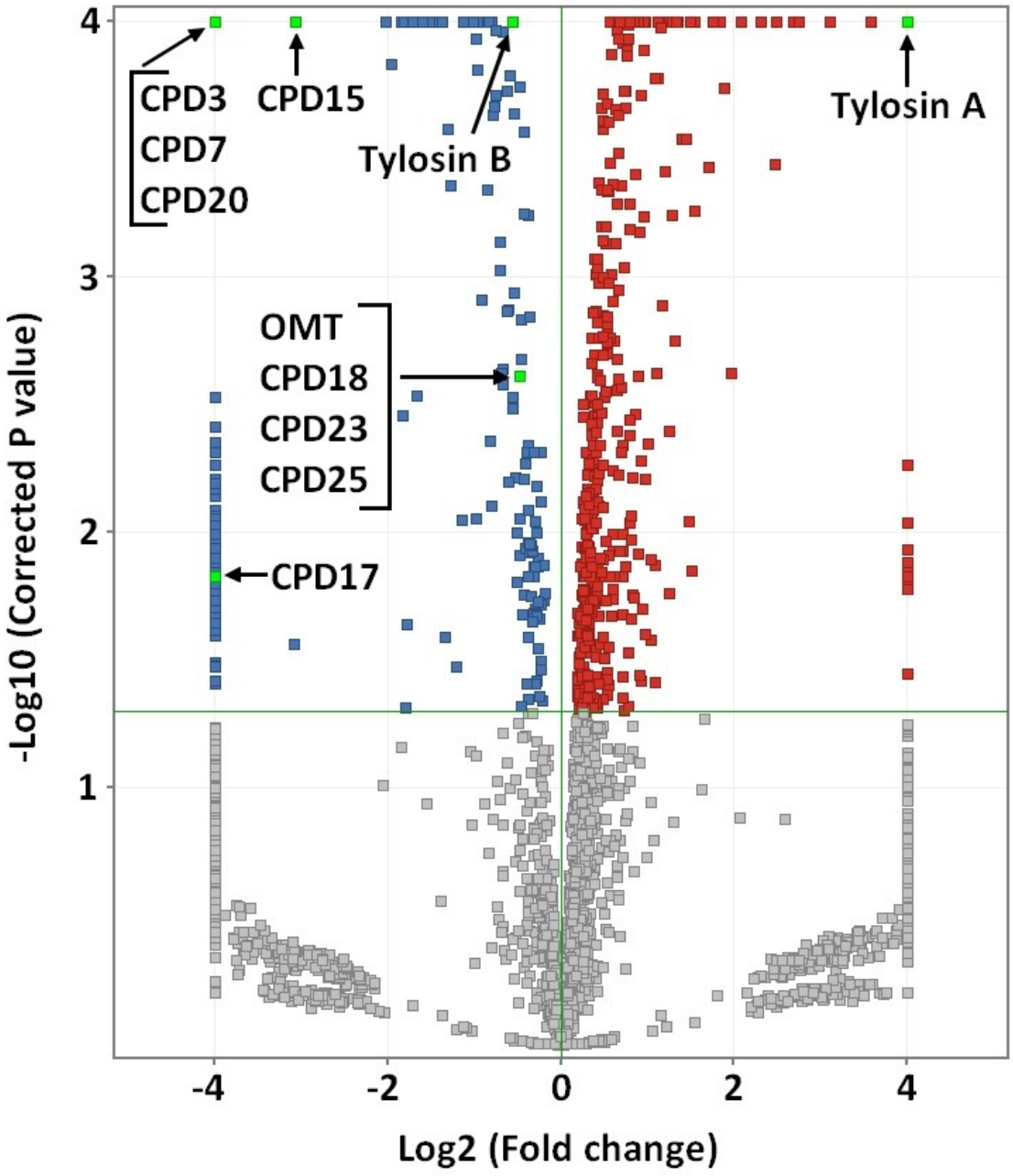


Figure S6.3. Volcano plot of the comparison between spiked H1 unheated and heated at 100˚C. Legend: red = compounds significantly decreasing after heating ($p<0.005$), blue = compounds significantly increasing after heating ($p<0.05$), grey = compounds without a significant change after heating ($p>0.05$), green = compounds changing significantly ($p<0.05$) and identified in **Table 6.3**.

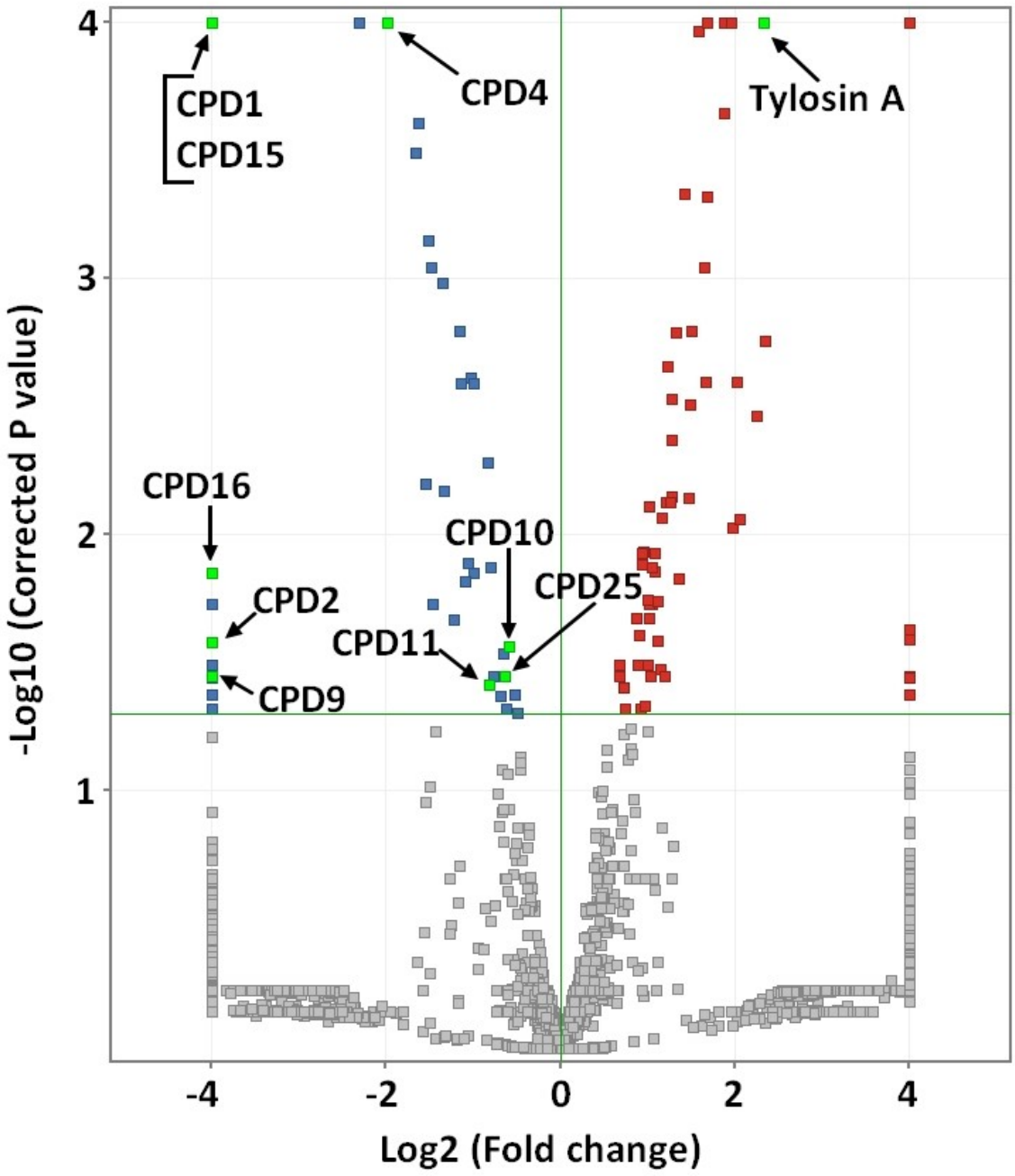


Figure S6.4. Volcano plot of the comparison between H4 unheated and heated at 100˚C. Legend: red = compounds significantly decreasing after heating ($p<0.005$), blue = compounds significantly increasing after heating ($p<0.05$), grey = compounds without a significant change after heating ($p>0.05$), green = compounds changing significantly ($p<0.05$) and identified in **Table 6.3**.

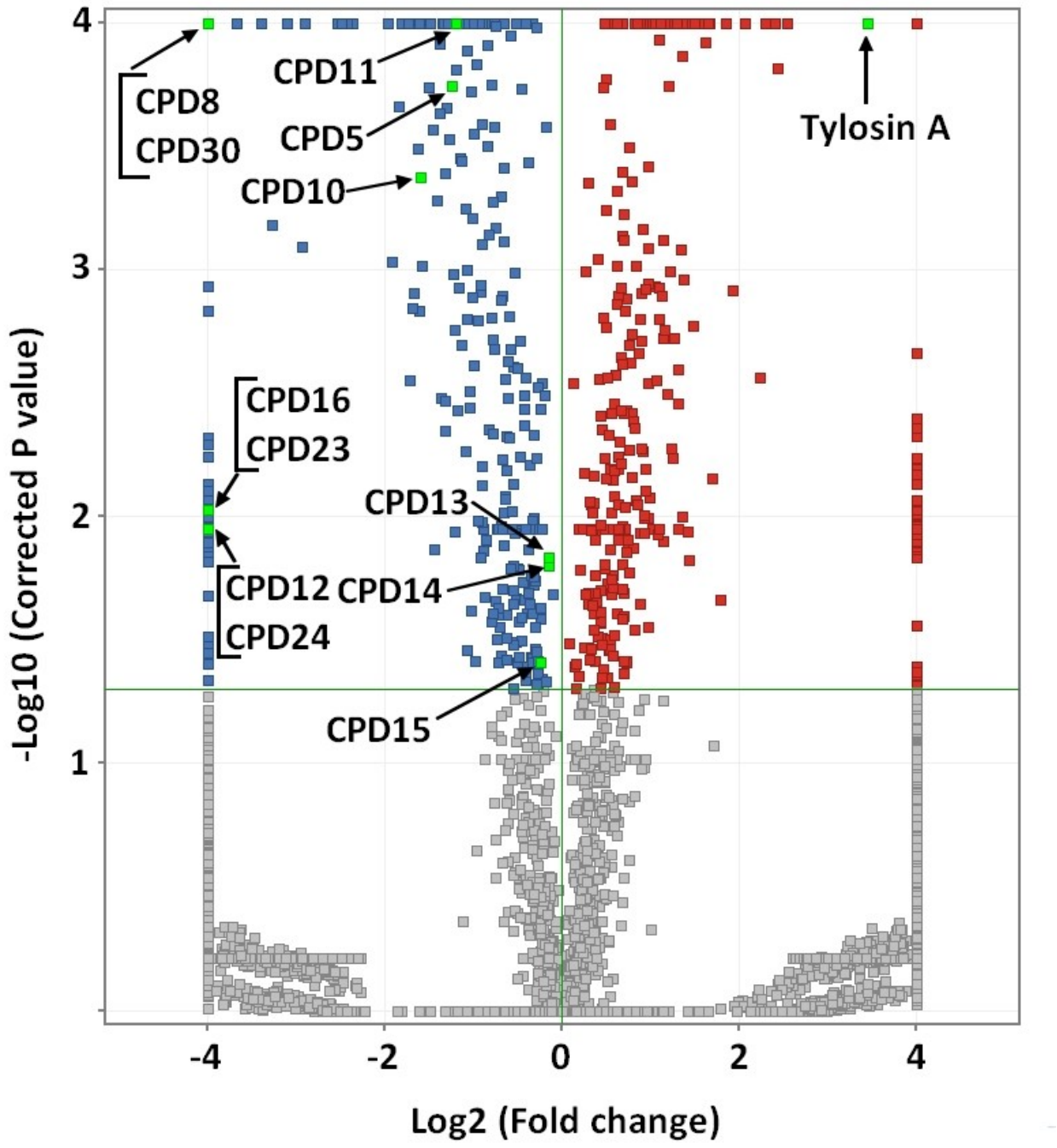


Figure S6.5. Volcano plot of the comparison between H4 initially and stored at 27˚C. Legend: red = compounds significantly decreasing after storage ($p<0.005$), blue = compounds significantly increasing after storage ($p<0.05$), grey = compounds without a significant change after storage ($p>0.05$), green = compounds changing significantly ($p<0.05$) and identified in **Table 6.3.**

6.9 SUPPLEMENTARY INFORMATION REFERENCES

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CHAPTER 7: GENERAL CONCLUSIONS

7.1 CONCLUSIONS

In this work, a non-targeted method was developed for the analysis of multiple trace organic contaminants in honey. Each step of the non-targeted workflow was studied and optimized to allow for the screening of different contaminant families in this complex matrix.

First, a fast screening and quantification method was successfully developed and validated for the targeted analysis of 7 veterinary drug residues in honey, using direct injection HPLC-QTOF-MS. Even though the sample preparation consisted of a dilute-and-shoot approach with no clean-up, the selected veterinary drug residues were detected at levels approximately 20 to 100 times lower than the actual regulatory limits, with acceptable recoveries, linearity and repeatability. Furthermore, thanks to the use of direct injection, the total analysis time was only 45 min per sample, including sample preparation and instrumental runtime. A data-independent All-Ions MS/MS mode was used to continuously record MS and MS/MS data at four different collision energies, allowing for the confirmation of the identity of the target analytes. This first study demonstrated the non-targeted potential of this method.

Next, the data pre-treatment steps for the non-targeted identification of trace organic contaminants in honey were studied using the same 7 veterinary drugs as case study. The impact of the match mass tolerance, the mass extraction window, the isotope abundance score, the peak filter absolute height, the average of spectra included, the exclusion of TOF spectra and the post-processing peak filters on the correct identification of the target compounds was assessed. The expansion window for chromatogram extraction and the average scans included in the spectra influenced the identification results significantly. The optimized identification workflow was used to screen 55 honey samples from the Canadian market using a library of 43 honey-related compounds, including veterinary drugs, pesticides and other contaminants, which led to the detection of tylosin A and HMF among these samples. These results showed that data pre-treatment parameters can affect the identification of trace contaminants in food. In other words,

an assessment of data pre-treatment parameters should be systematically conducted before applying non-targeted workflows to the identification of trace contaminants in food matrices.

Then, the optimized non-targeted workflow was applied to the screening of plastic-related compounds in 104 honey samples from the Canadian market. A total of 662 compounds were tentatively detected using a library of leachable and extractable compounds. The identity of two of these compounds, namely DEHA and TBOEP, was further confirmed with pure analytical standards, and their estimated daily intake was found to be far below the tolerable daily intake. The chemical burden in honey samples sold in either glass or plastic jars was compared using 3 data treatment approaches, each of which resulted in a different list of relevant contaminants. Among the compounds discovered based on the differential frequency of detection, 6 were unique to honey samples sold in plastic jars and 3 were unique to honey samples sold in glass jars. These results showed that some of the most commonly used data treatments in metabolomics need to be carefully selected when it comes to identifying trace contaminants in food. In particular, the relatively low frequency of contaminants in food needs to be accounted for when selecting the appropriate data treatment tool.

Finally, the degradation of the veterinary drug tylosin A in water, spiked honey and incurred honey after different thermal treatments was studied using the optimized non-targeted method. The results, in terms of rates of degradation of tylosin A and increase of tylosin B (the only degradation product reported so far) were in agreement with the literature. However, the non-targeted approach used for this study led to the tentative identification of two new degradation products, namely OMT and lactenocin. Although the thermal treatment applied was the same, the degradation products identified in water, spiked honey and incurred honey appeared to be different. These results further support that relying only on the water model or spiked food matrix is not sufficient to understand the thermal degradation of antibiotics in food matrices. The possibility of a semi-quantification of tylosin B using tylosin A, its parent compound, was also assessed. The present results proved that a semi-quantification using the parent compound to

quantify its degradation compound can be a suitable strategy for the semi-quantification of degradation compounds obtained through non-targeted analysis.

7.2 CONTRIBUTION TO KNOWLEDGE

The work presented in this thesis reported for the first time:

1. The applicability of direct injection combined with HRMS in the All Ions MS/MS mode for the combined targeted and non-targeted screening of food contaminants in honey. Notably, this new method made possible the detection of glucose-sulfonamide conjugates, which had not been previously reported in honey. These results open the door to the analysis of sulfonamides in food without the need of an extra acid hydrolysis step.

2. The systematic assessment of the influence of 7 data pre-treatment parameters (i.e. the match mass tolerance, the mass extraction window, the isotope abundance score, the peak filter absolute height, the average of spectra included, the exclusion of TOF spectra and the post-processing peak filters) on the non-targeted identification of model compounds (veterinary drugs) in honey.

3. A novel non-targeted analysis method capable of detecting plastic-related compounds in honey. This method allowed for the detection of the flame retardant TBOEP, which had not been previously reported in honey.

4. The non-targeted characterization of the thermal degradation of tylosin A in honey, which led to the identification of two new degradation compounds: OMT and lactenocin.

5. A case study supporting the applicability of the semi-quantification of degradation products based on the response factor of the parent compound (with LC-MS).

7.3 FUTURE RESEARCH RECOMMENDATIONS

After the completion of this thesis, some recommendations for future research have been identified. These include:

1. Complementing the approach with negative ionization to allow for the analysis of additional trace organic contaminants that ionize better in negative mode in electrosprays (e.g. chloramphenicol).

2. The quantitative analysis of glucose-sulfonamide conjugates in food using direct injection LC-MS.

3. The assessment of the influence of data pre-treatment parameters on the non-targeted identification of other families of compounds and other matrices.

4. Developing migration studies to determine to which extent the material of the jars plays a role in the presence of plastic-related compounds in honey.

5. The confirmation of the identity of OMT and lactenocin as the two detected new degradation products of tylosin A using pure analytical standards, and the determination of the degradation pathway that could produce them. The characterization of the hazards and the assessment of the potential risks associated with the presence of these compounds in food.

6. The determination of the range of concentrations in which the semi-quantification of tylosin B based on the response factor of tylosin A provides an acceptable uncertainty, to assess the applicability of this approach for regulatory purposes.

GENERAL REFERENCE LIST

NOTE: In accordance with the Guidelines for Thesis Preparation, each of the manuscript chapters (i.e. Chapters 3-6) contain their own reference list. Hence, the following reference list corresponds to the references included in the remaining chapters of the thesis (i.e. Chapters 1 and 2).

[1] M. Cesen, D. Lambropoulou, M. Laimou-Geraniou, T. Kosjek, U. Blaznik, D. Heath, E. Heath, Determination of bisphenols and related compounds in honey and their migration from selected food contact materials, J. Agric. Food Chem., 64 (2016) 8866-8875.

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