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# Uptake and metabolization of antibiotics in crops

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# Uptake and metabolization of antibiotics in crops

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*Dedicated to all my Masters*

*Naham karta*

"Do not ignore the laws of nature.
Never disregard nature or nature will take its retribution."

*Bhagwan Sri Deep Narayan Mahaprabhuji*

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## Abstract

The discovery of antibiotics (ABs) and their mass production brought a revolution in medicine worldwide during the middle of the XX century. Infectious diseases with high morbidity and mortality suddenly become treatable. In the years that followed ABs were widely (mis) used in human and veterinary applications, consequently ABs become the victims of their own success. AB bacterial resistance, although an ancient phenomenon, become one of the biggest threats to global health and food safety. AB pollution has been reported worldwide and in different environmental compartments. The presence of ABs has been reported in wastewaters (WW), surface waters, groundwater, manure, agricultural soils, animal food (meat and dairy products) and more recently in vegetables. In recent years several studies conducted in greenhouse conditions showed that ABs can be taken up and translocated in crops.

The base of this Thesis is analytical methodology development and its application for the determination of several classes of incurred antibiotics in crops. Namely, analysis of ABs is a challenging task due to the complexity of vegetable matrices that entangle their quantification, especially if they are present at a relatively low ng $g^{-1}$ level. Thus Chapter II presents an analytical method for the target analysis of 10 ABs and 6 of their metabolites. The developed method relies on liquid chromatography coupled to mass spectrometry in combination with liquid extraction and cleanup workflows. Strikingly, in 73% of all samples analysed, the concentration of a transformation product (TP) was higher than the concentration of its parent compound.

Despite the numerous studies reporting the uptake and translocation of ABs by vegetables in controlled conditions or simulated real field scale conditions, there is a lack of data regarding the occurrence of ABs and its transformation products in vegetables under real field-scale conditions to assess the potential risk associated with their consumption. Thus, holistic sampling campaigns were performed (Chapter III) where four different vegetable types were included. Moreover, different agricultural practices were involved and all collected samples represent real, commercially available vegetables intended for human consumption. Farming plots where manure was applied as fertilizer and water from Llobregat River was used for irrigation showed the highest ABs loads. Crop type was another important factor that determined the level of ABs occurrence. In real agricultural environments variations of many different factors are creating complex and specific interactions, thus have to be observed as such.

Another unknown and poorly studied area that draws attention is related to ABs biotransformation pathways in pore water-plant systems. An innovative workflow for non-target screening allowed us annotation and identification of 11 metabolites of ofloxacin (OFL), of which five metabolites were reported for the first time in plants, suggesting the need to be included in surveys if they possess antimicrobial activity and/or toxicity (Chapter IV). Major transformation pathways were tentatively proposed revealing the major OFL metabolization pathways in lettuce.

Finally, in Chapters V and VI, general discussion of all results and conclusions is given summing up the main ideas and hypothesis validation.

# Resumen

El descubrimiento de antibióticos (AB) y su producción masiva trajo una revolución en la medicina mundial a mediados del siglo XX. En ese momento, las enfermedades infecciosas con alta morbilidad y mortalidad fueron tratables. A partir de entonces, se ab-usó de los AB tanto en aplicaciones medicinales como veterinarias y, en consecuencia, se convirtieron en víctimas de su propio éxito. La resistencia bacteriana a los AB, aunque es un fenómeno antiguo, se ha convertido en una de las mayores amenazas para la salud a escala global, así como en la seguridad alimentaria. La contaminación ambiental por AB es ubícua y en diferentes compartimentos. Así pues, se ha detectado la presencia de AB en aguas residuales, aguas superficiales, aguas subterráneas, estiércol, suelos agrícolas, alimentos de origen animal (carne y productos lácteos) y vegetales. En los últimos años, varios estudios realizados en invernaderos mostraron que los AB pueden ser absorbidos y translocados en cultivos.

En esta Tesis se aborda el desarrollo y aplicación de una metodología analítica para la determinación de ABs y sus productos de transformación en vegetales. Resulta conocido que el análisis de ABs es una tarea muy compleja debido a la composición de las matrices vegetales implicadas es su cuantificación, especialmente si los ABs están presentes en un nivel relativamente bajo, inferior de ng $g^{-1}$. Por lo tanto, el Capítulo II presenta un método analítico para la determinación de 10 ABs diana y 6 de sus metabolitos. El método se basa en la cromatografía líquida acoplada a la espectrometría de masas en combinación con un flujo de trabajo que implica la extracción y purificación del extracto obtenido. Sorprendentemente, en el 73% de las muestras analizadas, la concentración de un producto de biotransformación fue mayor que la de su compuesto original.

A pesar de que hay numerosos estudios que confirman la absorción y la translocación de AB en verduras bajo condiciones controladas o condiciones de campo simuladas, faltan datos sobre la presencia de AB en condiciones de producción a escala real para evaluar el riesgo a la salud humana que su consumo presenta. Por lo tanto, se realizó una campaña de muestreo integral (Capítulo III) donde se incluyeron cuatro tipos diferentes de vegetales. Además, se utilizaron diferentes prácticas agrícolas y todas las muestras recolectadas representan verduras disponibles comercialmente y destinadas al consumo humano. Las mayores cargas de AB se detectaron en las parcelas agrícolas donde se aplicó estiércol como fertilizante y se utilizó el agua del río Llobregat para riego. El tipo de cultivo fue otro factor importante que determinó el nivel de AB. En entornos agrícolas reales, las variaciones de

muchos factores diferentes crean interacciones complejas y específicas, por lo que deben observarse como tales.

Otra área desconocida y poco estudiada está relacionada con las vías de biotransformación de AB en el sistema agua intersticial-planta. El flujo de trabajo innovador presentado para el análisis no dirigido, nos permitió la anotación e identificación de 11 metabolitos de ofloxacina (Capítulo IV), de los cuales cinco metabolitos fueron reportados por primera vez en plantas, que deberían incorporarse en la vigilancia si se demuestra su toxicidad y/o actividad antimicrobiana. Las principales vías de transformación revelan cómo la ofloxacina, así como otros productos químicos relacionados se metabolizan en la lechuga.

Finalmente, en los capítulos V y VI, se presenta una discusión general de todos los resultados y conclusiones resumiendo las ideas y la confirmación de las hipótesis principales.

# Resum

El descobriment d'antibiòtics (AB) i la seva producció a gran escala va donar lloc a una revolució de la medicina a mitjans del segle XX a escala mundial. Tot seguit, les malalties infecciones amb alta morbiditat i mortalitat es van tornar tractables. A partir de llavors, els AB es van fer servir excessivament (malament) i, conseqüentment, es van tornar en víctimes del seu propi èxit. La resistència bacteriana als AB, tot i que és un fenomen antic, s'ha convertit en una de les majors amenaces per a la salut a escala mundial i la seguretat alimentària. La contaminació per AB s'ha registrat en tot el món i en diversos entorns. També s'ha detectat la presencia d'AB en aigües residuals, aigües superficials, aigües subterrànies, fems, sòls agrícoles, aliments d'origen animal (carn i productes làctics) i verdures. Els últims anys, diversos estudis realitzats en hivernacles han demostrat que els AB es poden absorbir i translocar a cultius.

En aquesta Tesi s'ha desenvolupat i aplicat una metodologia analítica per la detecció d'ABs i els seus productes de transformació en cultius vegetals. Se sap que l'anàlisi d'AB és una tasca complexa degut a la composició de les matrius vegetals implicades en la seva quantificació, especialment si els AB són presents a un nivells de concentració relativament baixos, inferior a ng $g^{-1}$. Per tant, el Capítol II presenta un mètode analític per la determinació de 10 ABs diana i 6 dels seus metabòlits. El mètode es basa en la cromatografia líquida acoblada a l'espectrometria de masses en combinació amb fluxes de treball que incorporen l'aïllament i purificació dels extractes. Sorprenentment, en el 73% de les mostres analitzades, la concentració d'un producte de biotransformació va ser superior que la del compost original.

Tot i que hi ha nombrosos estudis que confirmen l'absorció i la translocació d'AB en vegetals en condicions controlades o condicions de camp simulades, falten dades sobre la seva presència en vegetals en condicions de camp a escala real. Per tant, es va realitzar una campanya de mostreig integral (Capítol III) on es van incloure quatre tipus diversos de verdures. A més, es van considerar diverses practiques agrícoles i totes les mostres recol·lectades representen verdures reals disponibles comercialment i destinades al consum humà. Les majors càrregues d'AB es van quantificar en les parcel·les agrícoles on es van aplicar fems com a fertilitzant i es va fer servir aigua del riu Llobregat per regar. El tipus de cultiu va ser un altre factor important que va determinar el nivell d'AB. En entorns agrícoles reals, les variacions de diversos factors creen interaccions complexes i especifiques, que s'han d'observar com a tals.

Una àrea poc coneguda que necessita dedicar-li atenció està relacionada amb les vies de biotransformació d'AB en el sistema aigua intersticial-planta. El flux de treball innovador presentat per a l'anàlisi no dirigida ens va permetre l'anotació i identificació d'11 metabòlits de l'ofloxacina (Capítol IV), dels quals cinc metabòlits es van informar per primera vegada en plantes, que s'haurien d'incorporar en la vigilància si es demostra la seva toxicitat i/o activitat antimicrobiana. Les vies principals de transformació proposades revelen com l'ofloxacina i d'altres productes químics relacionats estructuralment, es metabolitzen en l'enciam.

Finalment, als Capítols V i VI, es presenta una discussió general de tots els resultats i conclusions resumint les idees i hipòtesis principals.

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## Key to Abbreviations

| | |
|---|---|
| **AB(s)** | Antibiotic(s) |
| **ADI(s)** | Acceptable Daily Intake(s) |
| **APB** | Aqueous Phosphate Buffer |
| **ARB** | Antibiotic Resistant Bacteria |
| **ARG(s)** | Antibiotic-Resistant Gene(s) |
| **ASE** | Accelerated Solvent Extraction |
| **BW** | Body Weight |
| **CCA** | Canonical-Correspondence Analysis |
| **CID** | Collision-Induced Dissociation |
| **CLI** | Clindamycin |
| **DDD** | Defined Daily Doses |
| **DI** | Daily Intake |
| **dw** | Dry Weight |
| **EDI** | Estimated Daily Intake |
| **ENR** | Enrofloxacin |
| **ESI(+)** | Electrospray Ionization (in positive mode) |
| **FISh** | Fragment Ion Search |
| **FOD** | Frequency Of Detection |
| **fw** | Fresh Weight |
| **HCA** | Hierarchical Agglomerative Clustering Analysis |
| **HCD** | Higher-Energy Collisional Dissociation |
| **HI** | Hazard Index |
| **HLB** | Hydrophilic-Lipophilic Balance |
| **HQ** | Hazard Quotient |
| **HRMS** | High Resolution Mass Spectrometry |
| **IDL(s)** | Instrumental Detection Limit(s) |
| **IQL(s)** | Instrumental Quantification Limit(s) |
| **LC-MS/MS** | Liquid Chromatography Tandem Mass Spectrometry |
| **MDL(s)** | Method Detection Limit(s) |

| | |
|---|---|
| **MIC** | Minimum Inhibitory Concentration |
| **MQL(s)** | Method Quantification Limit(s) |
| **MRM** | Multiple Reaction Monitoring |
| **MSC** | Minimal Selective Concentration |
| **MW** | Molecular Weight |
| **OFL** | Ofloxacin |
| **PC(s)** | Principal Component(s) |
| **PCA** | Principal Component Analysis |
| **RDB** | Ring Double Bond |
| **RSD** | Relative Standard Deviation |
| **RT** | Retention Time |
| **RWW** | Reclaim Wastewater |
| **SDG(s)** | Sustainable Development Goal(s) |
| **SDI** | Sulfadiazine |
| **SDM** | Sulfadimethoxine |
| **SMT** | Sulfamethizole |
| **SMX** | Sulfamethoxazole |
| **SMZ** | Sulfamethazine |
| **SPE** | Solid-Phase Extraction |
| **STZ** | Sulfathiazole |
| **TMP** | Trimethoprim |
| **TP(s)** | Transformation Product(s) |
| **UAE** | Ultrasound-Assisted Solvent Extraction |
| **WHO** | World Health Organization |
| **WW** | Wastewater |
| **WWTPs** | Wastewater Treatment Plants |

## Motivation

The occurrence and fate of ABs in vegetables and their pathways of entering into the food web were of particular interest to me. Due to the population growth and limited natural resources (clean irrigation water, nutrients), the application of certain agricultural practices becomes necessary. For instance, irrigation with reclaimed WW is a commonly accepted practice in arid and semiarid regions, which is one of the inputs of ABs in agricultural ecosystems. Another important input pathway of ABs is the application of manure as fertilizer. In addition, the circular economy is getting more and more implemented, thus we have to monitor and develop strategies in order to mitigate AB pollution in our environment and food products. Thus, it is of paramount importance to find a way to use reclaimed WW in a safe manner, alongside manure application as soil amendment in agriculture. There should be a comprise neither in food production nor in mitigating AB resistance spreading. The outcome of this research contributes to Sustainable Development Goals (SDGs) (Goals 2, 3 & 6). Accordingly, my main motivation arose from the need to address these SDGs by solving a piece of the problem that will reduce blurriness and shed light on the occurrence and fate of ABs in vegetables. Addressing these challenges will provide knowledge for the future research related to holistic approaches in the environmental and human health risk assessment and contribute to the sustainability, food safety and mitigate post-AB era.

## Thesis outline

The first task was to develop a robust analytical method for the monitoring of ABs residues and their metabolites in crops. The presence of ABs and their metabolites was confirmed in the edible parts of vegetable collected from different farms. Only a limited number of metabolites, namely the ones included in the target list, can be determined by applying target screening approach. Thus, a bench-scale experiment was performed in order to investigate and identify other possible metabolites, consequently ABs biodegradation pathways.

**Chapter I** constitutes an introduction to Thesis subject, and provides an state-of-the-art overview of the ABs history. Topics discussed are their occurrence and pathways of entering in the agricultural environments, hence food; factors and mechanisms affecting their uptake; current approaches used in health risk assessment and analytical methodologies applied in this Thesis.

**Chapter II** develops an analytical method for determination of 10 ABs and 6 of their metabolites at low ng $g^{-1}$ level in four different vegetable matrices (i.e., lettuce leaves, tomato fruits, cauliflower inflorescences, and broad bean seeds). The developed method is based on ultrasound extraction and solid-phase extraction clean-up, followed by liquid chromatography mass spectrometry in tandem (LC-MS/MS).

This Chapter is based on the article: Đorđe Tadić, Víctor Matamoros, Josep Maria Bayona, (2019). Simultaneous determination of multiclass antibiotics and their metabolites in four types of field-grown vegetables. Analytical and Bioanalytical Chemistry. 411, 5209–5222.

**Chapter III** presents a monitoring study of the occurrence of ABs in vegetables grown under real field-scale conditions. The achieved results provide a realistic insight since edible parts of different vegetables intended for human consumption were analyzed. In addition, effects of different agricultural practices on the ABs occurrence were studied.

This Chapter is based on the manuscript under review: Đorđe Tadić, Maria José Bleda Hernandez, Francisco Cerqueira, Víctor Matamoros, Benjamin Piña, Josep Maria Bayona. Occurrence and human health risk assessment of antibiotics and their metabolites in vegetables grown in field scale agricultural systems.

**Chapter IV** outlines the metabolization pathways of OFL in lettuce. High resolution mass spectrometry and novel computing tools were applied in metabolite annotation and elucidation. This Chapter aimed to provide an analytical methodology that will help in the identification of plant metabolites of other ABs, especially from, but not limited to, fluoroquinolone group.

This Chapter is based on the article: Đorđe Tadić, Michal Gramblicka, Robert Mistrik, Cintia Flores, Benjamin Piña, Josep Maria Bayona. (2020). Elucidating biotransformation pathways of ofloxacin in lettuce (*Lactuca sativa* L). Environmental Pollution. 260, 114002.

**Chapter V** and **Chapter VI,** provide general discussion and conclusions.

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# Chapter I: Introduction

## 1.1. Discovery, use, overuse and misuse of antibiotics

In 1929, Alexander Fleming published his work on penicillin's discovery reporting that mold extracts were able to kill a number of gram positive microorganisms. Penicillin had demonstrated its amazing curative properties in the clinical stage and later it was produced and distributed on a large scale. Encouraged by the commercial success of ABs, pharmaceutical companies were eager to discover their own. Thus a highly productive period was from the late 1940s until the 1970s during which many of the major AB classes were discovered [1]. ABs have been extensively used in human and veterinary medicine, as well as in aquaculture, for the purpose of preventing (prophylaxis) or treating microbial infections [2]. Consumption of ABs in animal feedstock is increasing worldwide approaching, or already surpassing, the volume consumed by humans [3]. Between 2000 and 2015, AB consumption, expressed as defined daily doses (DDD), increased 65% (21.1–34.8 billion DDDs), and the AB consumption rate increased 39% (11.3–15.7 DDDs per 1,000 inhabitants per day). Projections of global AB consumption in 2030, assuming no policy changes, were up to 200% higher than the 42 billion DDDs estimated in 2015. AB consumption is a primary driver of AB resistance, thus a growing global problem [4]. Antimicrobial resistance, the ability of microbes to evolve and withstand the effects of ABs, is recognized as one of the greatest threats to human health worldwide [5]. To address this threat, the World Health Organization (WHO) developed the Global Action Plan with the main goal of ensuring treatment and prevention of infectious diseases with quality-assured, safe and effective medicines [6]. Later on, the Tripartite (WHO, Food and Agriculture Organization of the United Nations, and World Organization for Animal Health) proposed a modular approach for developing the framework in order to directly address selected objectives of the Global Action Plan [7]. Moreover, technical experts from Canada, the European Union (EU), Norway, and the United States created the Transatlantic Taskforce on Antimicrobial Resistance to address the urgent threat of AB resistance. In a nutshell, ABs become the victims of their own success.

## 1.2. Classes of antibiotics. Applications

An AB class is defined by a characteristic core moiety, or pharmacophore, that is responsible for the observed antibacterial activity [1] and are constituted by a diverse group of chemicals [2]. Classification of ABs and their main applications is presented in the Table 1.1. Contribution of therapeutic use of ABs for human medication actually appears to be insignificant when compared to other type of use. According to United States Food and Drug Administration reports, only about 20% of the antimicrobials sold in the USA are used by humans while the rest 80% are used in animals, leading to their widespread occurrence. For instance, a whole water body may get contaminated with the ABs as a consequence of their therapeutic use in fish farms. Similar to animals, plants can also suffer from infections, which can be treated with ABs [8].

Table 1.1. AB classification and their applications [9]

| Class | Examples | Applications |
|---|---|---|
| Aminoglycosides | Neomycin, streptomycin, tobramycin, paramomycin | For infections from aerobic bacteria and Gram-negative bacteria |
| Glycopeptides | Teicoplanin, vancomycin | For infections from Gram-positive bacteria |
| Macrolides | Azithromycin, clarithromycin, erythromycin, telithromycin, spectinimycin, roxithromycin, tylosin | For streptococcal infections, respiratory infections, feed additive for animals |
| β-lactams Penicillins | Amoxicillin, ampicillin, carbenicillin, cloxacillin, penicillin, meticillin, ertapenem, | For wide range of infections, Streptococcal infections |
| β-lactams Carbapenems | Ertapenem, meropenem, doripenem | For infections from both Gram-positive and Gram-negative bacteria |
| β-lactams Cephalosporins | Cefalotin, cefamandole, cefoxitin, cefeprime, cefalexin, cefprozil, cefuroxime, bacitracin, | For infections from Gram-negative bacteria |
| Polypeptides | Bacitracin, colistin, polymyxin | For eye, ear and bladder infections |

(continuation) Table 1.1

| Class | Examples | Applications |
|---|---|---|
| Fluoroquinolones | Ciprofloxacin, enrofloxacin, ofloxacin, levofloxacin | For urinary tract infections, skin infections, respiratory infections |
| Sulfonamides + trimethoprim | Sulfamethizole, sulfanilamide, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfadiazine, sulfathiazole, sulfamethoxine | For urinary tract infections |
| Tetracyclines | Tetracyclines, chlortetracyclines, oxytetracyclines | For infections of respiratory tract, urinary tract |
| Phenicol | Chloramphenicol | Veterinary use |
| Lincosamides | Lincomycin | Veterinary use |

Physicochemical properties of ABs differ from physicochemical properties of pharmaceuticals from other therapeutic areas. For instance, they have always been considered as an exception to “Lipinski’s rules”, which represent a rule of thumb in correlation of the physicochemical properties of drugs with the predicted pharmacokinetics. More specifically, two major differences between antibacterial and other drugs are molecular weight (MW) and lipophilicity [10]. Moreover, ABs comprise structurally diverse groups. For instance, it is consisting synthetic drugs with relatively low MW such as the sulfa drugs, and fluoroquinolones as well as ABs derived from natural products with significant structural complexity such as macrolides and cephalosporins [1].

Table 1.2. Average physicochemical parameters for antibacterial classes [10]

| Class | MW (g $mol^{-1}$) | cLogP | PSA ($Å^2$) | H-donor | H-acceptor |
|---|---|---|---|---|---|
| Glycopeptides | 1740 | 1.3 | 586 | 22.8 | 37 |
| Macrolides | 790 | 3.5 | 189 | 3.6 | 15 |
| Penicillins | 413 | 1.4 | 149 | 2.8 | 8 |
| (Carba) Penems | 397 | -3.0 | 159 | 4.5 | 9 |
| Sulfa drugs | 273 | 0.6 | 112 | 3.1 | 6 |
| Fluoroquinolone | 371 | 1.3 | 82 | 2.1 | 6 |
| Tetracyclines | 481 | -0.7 | 184 | 7.1 | 10 |
| Aminoglycosides | 526 | -2.9 | 279 | 14.8 | 15 |

cLogP - calculated logarithm of the partition coefficient between n-octanol and water; PSA - polar surface area; H-hydrogen

The MW of the ABs range typically from 250 to 1700 Da and most of them are polar compounds. Accordingly, their log $K_{OW}$ values range from -7.5 (aminoglycosides) to 4.0 (macrolides). Most antimicrobials have acidic and/or basic functionalities; their ionization rate depends on acidic dissociation constants (i.e. $pK_a$ values) and is controlled by the solution's pH. These different chemical species (cationic, neutral, anionic) often own vastly different properties [11]. Differences in the physicochemical properties are numerically represented by listing average values for each of the major compound classes in Tables 1.2 and 1.3 [10,11].

Table 1.3. $pK_a$, log $K_{OW}$, $K_d$, and $K_{OC}$ values of various ABs [11,12]

| Class/ compound | $pK_a$ | log $K_{OW}$ | $K_d$ (L $kg^{-1}$) | $K_{OC}$ (L $kg^{-1}$) |
|---|---|---|---|---|
| **Aminoglycosides** | | | | |
| Streptomycin | $pK_{a1}$=11.2 | -7.53 | 8–290 | 580–11,000 |
| | $pK_{a2}$=13.1 | | | |
| | $pK_{a3}$=13.4 | | | |
| Gentamicin | $pK_{a1}$=12.5 | -1.88 | | |
| | $pK_{a2}$=10.2 | | | |
| Amikacin | $pK_{a1}$=8.1 | -7.4 | | |
| **β-lactams** | | | | |
| Amoxicillin | $pK_{a1}$=2.67 | 0.87 | | 865 |
| | $pK_{a2}$=7.11 | | | |
| | $pK_{a3}$=9.55 | | | |
| Penicillin G | $pK_{a1}$=2.74 | 1.83 | | 3 |
| Oxacillin | $pK_{a1}$=2.72 | 2.38 | | |
| Cefuroxime | $pK_{a1}$=2.5 | -0.16 | | 12–15 |
| **Macrolides** | | | | |
| Azithromycin | $pK_{a1}$=8.74 | 4.02 | 2 | 59,900 |
| Clarithromycin | $pK_{a1}$=8.99 | 3.16 | 262–400 | 150 |
| Tylosin | $pK_{a1}$=7.73 | 3.87 | 5–172,480 | 110–95,532 |
| Erythromycin | $pK_{a1}$=8.88 | 3.06 | 130 | 10 |
| **Glycopeptides** | | | | |
| Vancomycin | $pK_{a1}$=2.6 | | 0.3–0.7 | |
| | $pK_{a2}$=7.2 | | | |
| | $pK_{a3}$=8.6 | | | |
| | $pK_{a4}$=9.6 | | | |
| | $pK_{a5}$=10.5 | | | |
| | $pK_{a6}$=11.7 | | | |

(continuation) Table 1.3

| Class/ compound | $pK_a$ | log $K_{OW}$ | $K_d$ (L $kg^{-1}$) | $K_{OC}$ (L $kg^{-1}$) |
|---|---|---|---|---|
| **Fluoroquinolones** | | | | |
| Ciprofloxacin | $pK_{a1}$=6.09 | 0.28 | 427–4,844 | 1,127–61,000 |
| | $pK_{a2}$=8.74 | | | |
| Levofloxacin | $pK_{a1}$=5.59 | -0.39 | | |
| | $pK_{a2}$=9.94 | | | |
| Ofloxacin | $pK_{a1}$=5.97 | -0.39 | 1,471–4,325 | 44,140 |
| | $pK_{a2}$=9.28 | | | |
| Norfloxacin | $pK_{a1}$=6.34 | 0.46 | 591–5,791 | 310 |
| | $pK_{a2}$=8.75 | | | |
| **Sulfonamides and trimethoprim** | | | | |
| Sulfamethoxazole | $pK_{a1}$=1.6 | 0.89 | 0.6–4.9 | 1.8–31 |
| | $pK_{a2}$=5.7 | | | |
| Sulfamethazine | $pK_{a1}$=2.65 | 0.19 | 0.23–206 | 60–208 |
| | $pK_{a2}$=7.65 | | | |
| Sulfapyridine | pKa=8.43 | 0.35 | 1.60–7.40 | 80–308 |
| Trimethoprim | $pK_{a1}$=7.12 | 0.91 | 7.4 | 4,600 |
| **Tetracyclines** | | | | |
| Doxycycline | $pK_{a1}$=3.09 | -0.02 | | |
| | $pK_{a2}$=7.44 | | | |
| | $pK_{a2}$ =9.27 | | | |
| Oxytetracycline | $pK_{a1}$=3.27 | -0.90 | 417–1,026 | 2,872–93,317 |
| | $pK_{a2}$=7.32 | | | |
| | $pK_{a3}$=9.11 | | | |
| Tetracycline | $pK_{a1}$=3.30 | -1.30 | 417–1,026 | 400–93,320 |
| | $pK_{a2}$=7.68 | | | |
| | $pK_{a3}$=9.69 | | | |
| **Lincosamides** | | | | |
| Clindamycin | $pK_{a1}$=7.8 | 2.16 | | 70 |
| Lincomycin | $pK_{a1}$=7.6 | 0.2 | | 59 |

## 1.3. Occurrence in the environment and agricultural practices as inputs of antibiotics

ABs can take several routes into the environment (Figure 1.1). Consumed ABs are continuously discharged into the natural ecosystems via excretion after a short time of residence in the human and animal organisms [13].

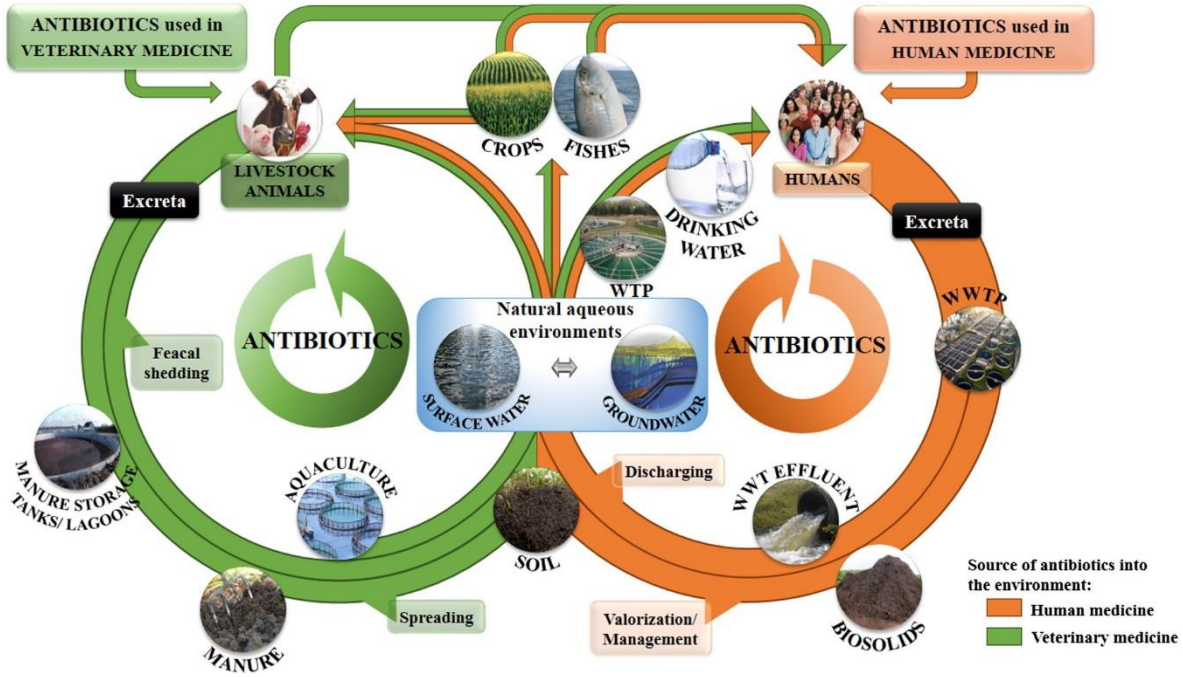



Figure 1.1. Input pathways of ABs in the environment [13]

ABs are frequently detected in reclaimed wastewater (RWW), biosolids and manure, consequently their applications are labeled as the most important inputs of ABs in agricultural environments [14,15]. AB occurrence in food chain, e.g., in meat, eggs, milk, vegetables, and grains, has been confirmed as well [16]. Following the new policies of circular economy and the new arising needs, RWW and manure usage are increasingly included in the agricultural practices.

Amending soil with livestock manure provides essential nutrients in agriculture. However, the major contribution of veterinary ABs is manure application in agricultural practices [13]. As mentioned, in many jurisdictions worldwide, except in the EU, livestock are fed with veterinary ABs at sub-therapeutic levels in order to promote growth [17]. Consequently, llong-term and frequent applications of animal manure in vegetable farms leads to AB pollution of groundwater, surface water (runoff), agricultural soils and vegetables [18]. As reported by Pan and Chu [19], ABs can be detected in μg $g^{-1}$ level in manure and biosolids (Figure 1.2). The

categories of the manure include livestock manure, thermophilically digested and composted manure, and organic fertilizers, which were produced through microbial fermentation using manure and farm byproducts. The biosolids presented in Fig. 1.2 included samples collected from wastewater treatment plants (WWTPs), which meet the criteria for land application, and dewatered anaerobically digested biosolids. The oxytetracycline was reported as the most abundant in unprocessed manure (184 μg $g^{-1}$), as well as ciprofloxacin, with 3.26 μg $g^{-1}$ in anaerobically digested biosolids.

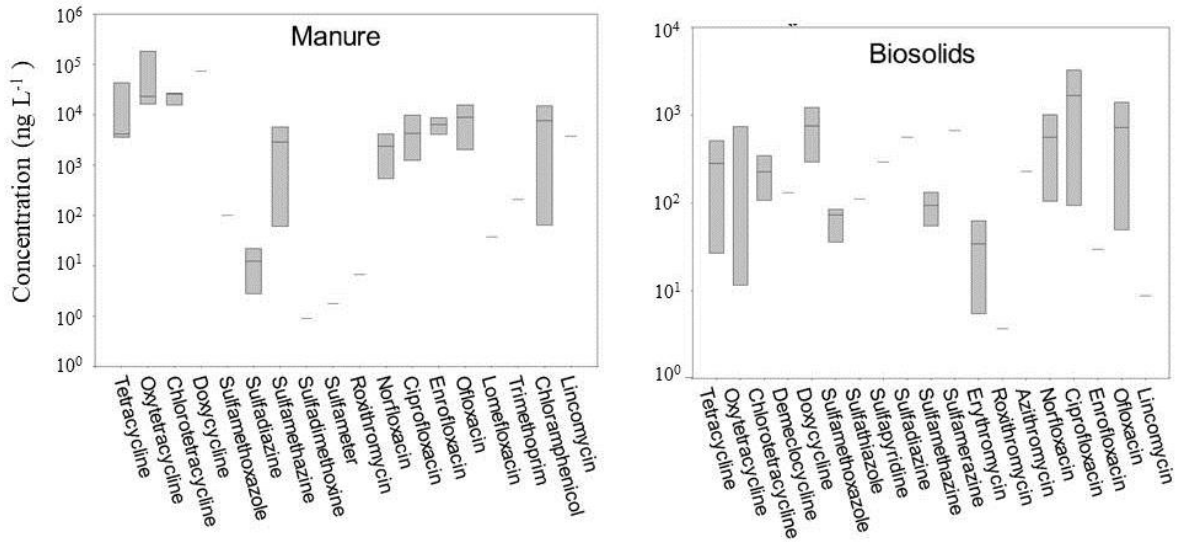



Figure 1.2. Concentrations of ABs in manure and biosolids [19]

In the future, higher food demand is expected due to the population increase, with a direct effect on agricultural water usage which can have serious consequences on the environment [20,21]. Moreover, many areas of the world are affected by water scarcity by increasing the drought periods. Some of the reasons for water scarcity are the misuse of water resources, the lack of infrastructures to water supply and climate change [21]. Since the imbalance between water demand and water availability has reached critical levels, the reuse of RWW is a widely accepted concept. The presence of ABs used in human medicine in sewage is mainly attributed to the discharge of RWW from WWTPs, since conventional WW treatment processes do not effectively remove these compounds [13]. Additionally, in environmental terms, in most arid and semiarid areas the main amount of water flowing in a river could be RWW, especially in the lower basins [22]. For instance, a significant volume of the water withdrawn from the Llobregat River (NE Spain) has already been treated by WWTPs (more than 60 WWTP discharges). The treated effluents are returned to the river itself, defining a major urban loop in the fate of the water as it travels along the basin [23]. Mean and maximum concentrations of ABs in surface waters and treated effluents in Asia and Europe

are presented in Figure 1.3. There is a huge variability in the concentrations found at different regions. For example, in the Patancheru industrial area near Hyderabad, India, enormously high levels of ciprofloxacin were detected in WWTP effluents (up to 14,000 µg $L^{-1}$) and in lakes (up to 6,500 µg $L^{-1}$). In surface waters, oxytetracycline was detected in extremely high concentrations of 361 µg $L^{-1}$ and 56 µg $L^{-1}$ in northern China and Colorado, USA, respectively. Furthermore, the relatively high concentration of tetracycline (15 µg $L^{-1}$) was reported in Portugal. In general, the concentrations of ABs in Asian developing countries tend to be higher compared to pollution levels reported in European and North American countries [24].

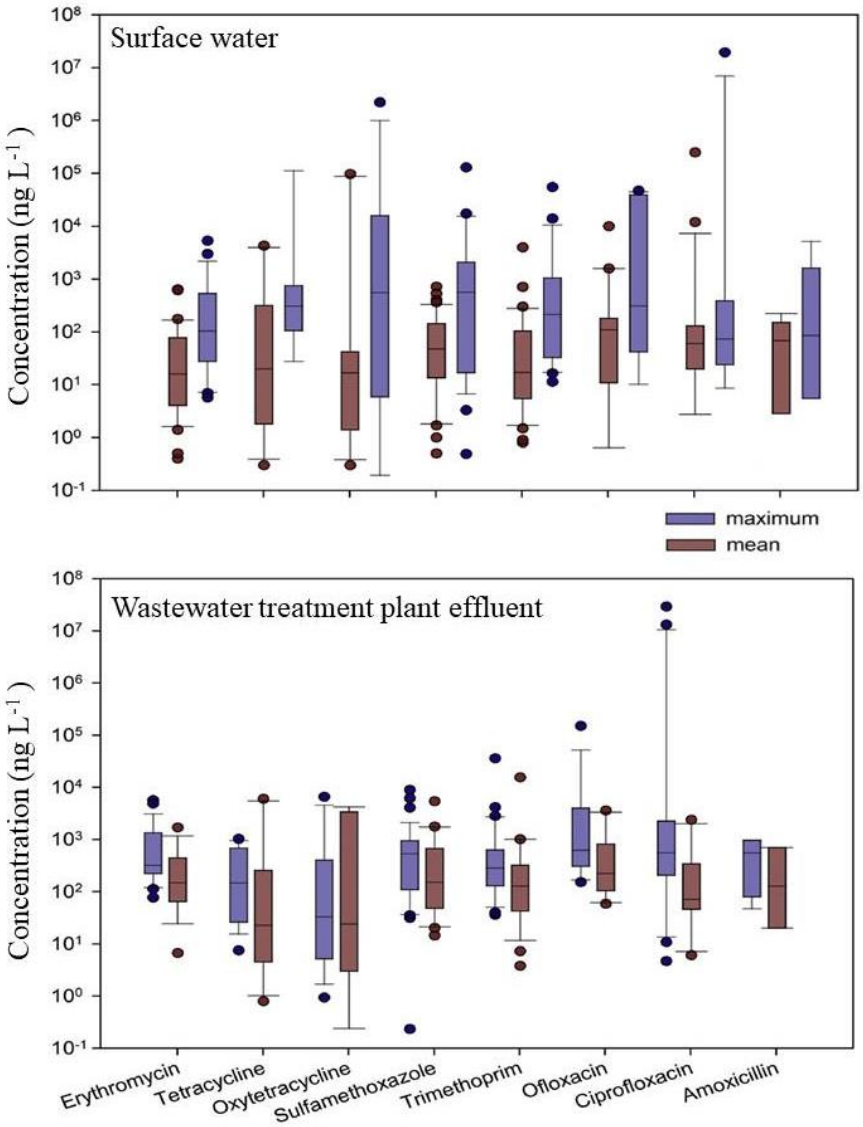



Figure 1.3. Global mean and maximum concentrations of selected ABs in surface waters and WWTP effluents [24]

## 1.4. Fate of antibiotics in soil-plant systems

### Adsorption of antibiotics in soil

ABs interact with the soil solid phase in sorption and desorption processes (Figure 1.4). Sorption and desorption controls not only their mobility and uptake by plants but also their biotransformation and biological effects [25].

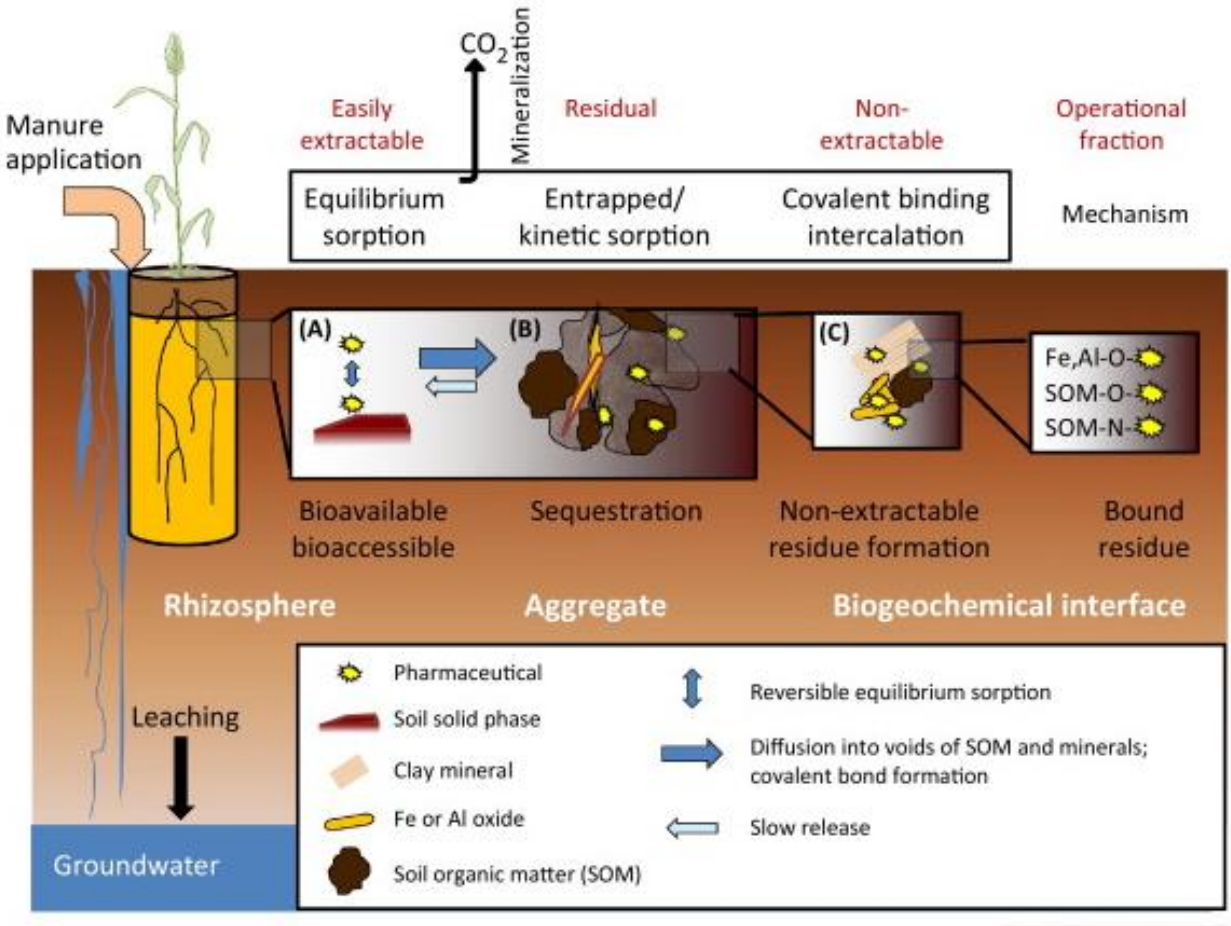



Figure 1.4. Fate of ABs in soil and its compartments [25]

The extent of AB adsorption to soils depends on the AB species present and soil properties including pH, clay content, organic matter content, and cation exchange capacity [26,27]. Sorption, binding, and fixation of the ABs in the soil matrix are indirectly influenced by temperature, moisture, and the soil solution chemistry [28].

The degree to which ABs may adsorb to soil varies widely with reported sorption coefficients ($K_d$, L $kg^{-1}$) spanning in some instances by more than four orders of magnitude (Table 1.3), depending on the soil type [29]. The $K_d$ value is the ratio between the concentration of the

compound in soil ($C_s$, mg $kg^{-1}$) to the concentration of the dissolved compound in water ($C_w$, mg $L^{-1}$) under equilibrium conditions:

$$K_d = \frac{C_S}{C_W} \quad (1)$$

Because sorption coefficients are not always the same at all aqueous concentrations, and since adsorption affinity is site dependant, linear relationships are not always fulfilled. Accordingly, equilibrium isotherms are widely used to represent the relationship between the adsorbed concentration in the adsorbent phase and the dissolved concentration at equilibrium. Such isotherms are a characteristic feature for a specific system at particular environmental conditions. There are several adsorption isotherm models such as the Langmuir, Freundlich, Brunauer–Emmett–Teller, Temkin, Frumkin, Harkins–Jura, Smith, and Dubinin–Radushkevich isotherms. Among them, the Freundlich sorption coefficient ($K_f$, $mg^{1-n}$ $L^n$ $g^{-1}$) and Langmuir fitting parameter ($K_l$, L $mg^{-1}$) provide a better estimate of partitioning, and can be calculated from the following equations:

Freundlich model: $$C_s = K_f \times {C_w}^{1/n} \quad (2)$$

Langmuir model: $$C_s = \frac{Q_{max} \times K_l \times C_w}{1 + K_l \times C_w} \quad (3)$$

where n, the Freundlich exponent, is a measure of the isotherm nonlinearity, and $Q_{max}$ is the maximum adsorption capacity (mg $g^{-1}$). The Freundlich isotherm is an empirical model suitable for heterogeneous surface adsorption, whereas the Langmuir model assumes monolayer adsorption on adsorbents which have homogeneous energy distribution [30].

It is believed that sorption of organic contaminants in soil is mainly via interactions with soil organic matter and thus, the adsorption coefficient $K_{OC}$ ($K_d$ normalized by soil organic carbon content) also serves as a measure of sorption [28]. $K_{OC}$ is a very important parameter for estimating environmental distribution and environmental exposure level of ABs. ABs with values of $K_{OC}$> 4,000 L $kg^{-1}$ are non-mobile and refractory to degradation in soils (very persistent) and the time span needed for the degradation of 50% of an initial dose is > 60 days. In contrast, ABs characterized by $K_{OC}$< 15 L $kg^{-1}$ values are highly mobile and are easily degraded. These can be classified as compounds with low persistence in soils [12]. Although $K_{OC}$ may help reduce variation between soil samples, it cannot be universally applied to all antimicrobials, particularly those that have ionizable functional groups [31]. For hydrophilic,

ionizable ABs, hydrophobic partitioning may not be dominant. Instead, physiochemical interactions with soil minerals become more important, suggesting that clay minerals play an important role in binding of ABs [28].

## Transport and mobility of antibiotics in soil

Soil adsorption is the main physicochemical mechanism that prevents the ABs from "subsurface transport" to some extent. For example, fluoroquinolones and sulfonamides adsorb better to manure with a high organic matter content [32]. Due to their sorption affinity to organic matter, fluoroquinolones are often detected at higher concentration in soil in comparison to the aquatic environment. For instance, 21 months after sludge application residual amounts of fluoroquinolones could still be detected in soil, demonstrating their reduced mobility and recalcitrance to biological degradation. Trimethoprim (TMP) and sulfonamides adsorb weakly to sediments or sludge, and therefore its mobility among environmental compartments is slightly restricted [33,34].

Mobility and transport of accumulated ABs in soil are important processes involved in the environmental fate, which may evoke when irrigation and precipitation occur [35]. AB transport within soils and to groundwater and surface water can occur by both leaching and runoff (Fig. 1.4). The degree of mobility of ABs in the environment can depend on several factors including chemical properties (water solubility, dissociation constants, sorption–desorption processes, and partitioning coefficients at various pH), temperature, moisture content of the soil, as well as prevailing weather conditions [26]. Chemicals weakly bound to soil materials (small $K_d$, Table 1.3) are likely to migrate out of the field in runoff water or be leached down in the soil vertical profile by percolation water, whereas those strongly sorbed by soil solids (high $K_d$, Table 1.3) can move to other locations together with associated soil particles eroded by runoff water. The mobility of AB in soils is controlled by their sorptivity, life time and influenced by soil solution pH and ionic strength [28].

## Abiotic and biotic degradation processes

The degradability and degradation pathways vary significantly among different veterinary chemicals and the transformation rate is influenced by a number of environmental factors including soil type, soil conditions (temperature, moisture, and oxygen status), pH, and light. Degradation of veterinary pharmaceuticals in agricultural soils is the combined result of microbial decomposition, organic transformation, oxidation, photolysis, and hydrolysis. ABs

can be transformed by biotic and abiotic processes. The latter includes photochemical- and chemical-mediated transformations such as oxidation, reduction, and hydrolysis [27]. Hydrolysis is generally considered one of the most important pathways for abiotic degradation of ABs. β-lactams are especially susceptible to hydrolytic degradation, whereas macrolides and sulfonamides are known to be less susceptible to hydrolysis [12,28]. Photo-degradation contributes to degradation of ABs (e.g., quinolones and tetracyclines) spread on the soil surface, however it is limited by poor light penetration [12,36].

Biodegradation is the major pathway for ABs transformation in soil. Many veterinary chemicals are susceptible to enzymatic degradation reactions such as oxidative decarboxylation and hydroxylation [28]. The central role of microorganisms in AB degradation or transformation in soil has been confirmed by results of many studies carried out in sterile and non-sterile soils [12,27]. Bioaccessibility and bioavailability of the AB decreases with increasing contact time in soil due to the 'sequestration'. Sequestration involves secondary sorption reactions and diffusion into micro- and nano-pores that are too small for microorganisms and enzymes. It is important to note that sequestration is a reversible process that allows subsequent slow release of sequestered ABs back into a bioaccessible form (Fig. 1.4). As a result, small sub-inhibitory concentrations of ABs may be detectable for extended periods of time, as shown for sulfonamides [25]. It has been previously reported that the uptake of ABs by crop plants is largely dependent on their bioavailability/bioaccessibility in soil pore water near the rhizosphere [37]. The accurate prediction or determination of bioavailable concentrations at soil−water interfaces is still impeded by a number of factors [38]. Persistence is an another important factor which can vary a lot and dominates the plant uptake of ABs in soil [27]. For instance, amoxicillin was easily degradable, with a half-life of 0.43–0.57 day. Whereas long-term persistence was observed for azithromycin, OFL, and tetracycline in soils with half-lives of 408−3466, 866–1733, and 578 days, respectively [12]. Ambient temperature, wind speed and air humidity may affect the uptake of ABs, as well, as it will be discussed in the next Section. Crops grown in hot and dry agricultural sites may show increased rates of ABs uptake. Therefore, the transpiration rate of plants, determined by climatic and plant specific values may be a good indicator for the ability of crop plants to uptake and accumulate ABs. The physiology of plants greatly determines their uptake potential [37]. Thus we may conclude that the uptake and accumulation differ according to plant species and structure of pharmaceutical [39]. More specifically, plant species within the same genus have been reported to have different uptake patterns, even more, differences were reported at the subspecies level [37,40].

## 1.5. Uptake, translocation and metabolization of antibiotics in plants

### Uptake and translocation mechanisms

Roots are the most important plant organ for uptake of chemicals from soil. This uptake process has been shown to be diffusive transport and generally involves passive transport. In this way, chemicals are carried into the plant during the natural transpiration cycle, emphasizing the importance of the uptake of soil pore water [41]. In addition, uptake may be an active process by which a carrier protein transfers chemicals.

Once taken up, compounds will either be translocated or stored in roots cells. In order to move up to other plant compartments, ABs have to reach the stele (xylem and phloem) [42]. Since edible parts of many vegetables include fruits, leaves, tubers, and stems, any chemical accumulating in any of these parts may constitute potential risk to human health [41]. There are two parallel pathways of movement of solutes and water across the cortex towards the stele: one passing through the apoplast (cell walls and intercellular spaces) and another passing from cell to cell in the symplast through the plasmodesmata (Figure 1.5).

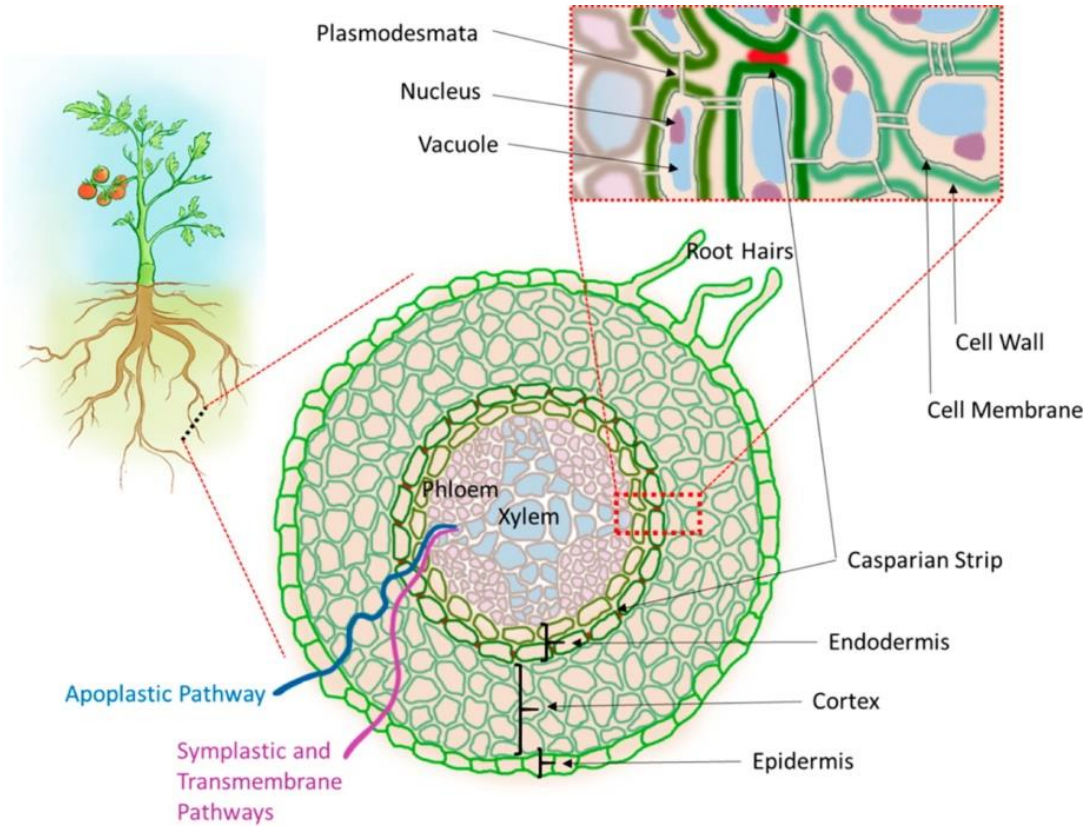



Figure 1.5. Cross-sectional diagram of a young dicot root with apoplastical and symplastical pathways [43]

In most of the root, the apoplastical movement to the stele is restricted by the Casparian band in the walls of endodermal cells, blocking the contaminants translocation. Thus, the symplastical pathway is considered as a predominant in delivering most pollutants to the xylem, although apoplastical movement of water and solutes to the stele still can occur at the root apex, where the Casparian band is not yet fully developed and/or in basal root zones where the structural continuity of the endodermis is disrupted. In addition, the volume of the free space accessible for apoplastical solute movement, represents only a small fraction of the total root volume [42]. Accordingly, AB entering to the symplastic pathway either at the exodermis (root hairs) or at the endodermis will reach the stele. In the cases where ABs and/or their metabolites are not able to pass through a lipid membrane, they must be transported by means of specific transport proteins called drug transporters [39]. Translocation of pharmaceuticals in lettuce depending on their physicochemical properties is presented in Figure 1.6.

As mentioned, water flow is believed to be the primary driver for uptake and transport of pharmaceuticals in plants. The poor relationship between accumulation and log pH-adjusted octanol-water partition coefficient ($D_{OW}$) indicates that the accumulation and transport of most pharmaceuticals in lettuce is not governed by their lipophilic characteristics, instead by water movement because of the relatively high water solubility of pharmaceuticals [44].

Generally, plant uptake of neutral pharmaceuticals is greater than for ionic species, because anionic pharmaceuticals are repelled by cell membranes with negative electrical potential, and cationic species are attracted to the cell membranes thus limiting their movement into plants [44]. In addition to charge speciation and hydrophobicity, the uptake and translocation of ABs could also be affected by MW, plant physiology, metabolism, exposure time, and plant growth rates. For instance, studies on diffusion have indicated that the compounds with MW> 500 g $mol^{-1}$ have the restricted membrane permeability, while the compounds with MW> 1000 g $mol^{-1}$ are unlikely to be absorbed by cell membranes [45].

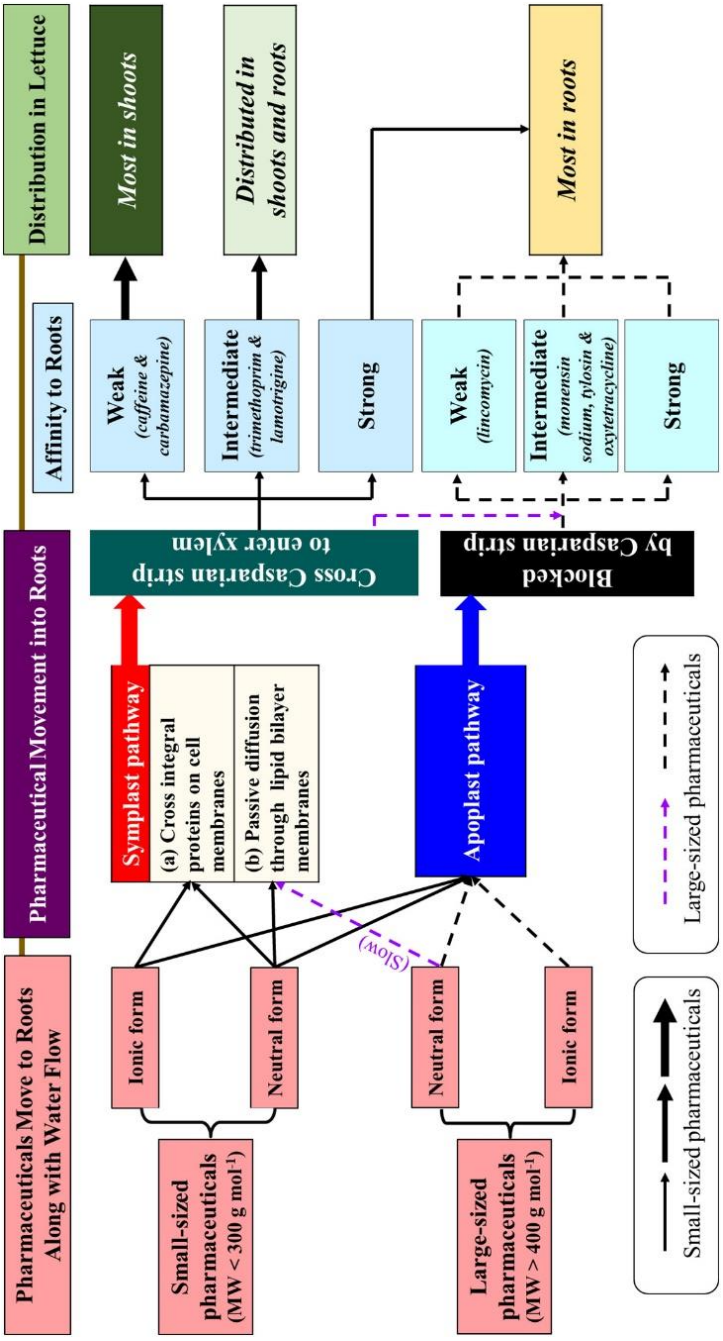



Figure 1.6. Pathways of pharmaceutical movements in lettuce [44]

## Plant metabolization of antibiotics

In phytoremediation technologies, plants have been considered as a “green liver”, since the participating enzymes have numerous similarities to those of xenobiotic metabolism in mammalian liver [46,47]. For ABs, just as for other xenobiotics, metabolism in plants can be divided into three phases. More specifically, biotransformation of ABs passes during the first two phases. Oxidation, reduction or hydrolysis of ABs represent phase I of biotransformation. In this step, reactive and hydrophilic groups are inserted or uncovered in the structures of AB moiety. In phase II, ABs or their phase I metabolites undergo conjugation reactions with endogenous compounds [39]. ABs having hydroxyl, carboxyl, amine, or other functional groups as a constituent part of their molecules are directly subjected to glycosylation [48]. Amino acids effectively participate in the detoxification (conjugation) of a broad spectrum of xenobiotics. One of the most important pathways for maintaining the health of plants via detoxification is the conjugation with the tripeptide–reduced glutathione [48]. Thus, glutathione and saccharides represent the main conjugation agents in plants [39]. Phase III of plant metabolism includes internal compartmentation and storage processes since plants do not have effective excretion pathways [47]. Soluble conjugates of drugs (coupled with peptides, sugars, amino acids etc.) are accumulated in vacuoles, while insoluble conjugates (coupled with protein, lignin, starch, pectin, cellulose, xylan and other polysaccharides) are moved out of the cell via exocytosis and are accumulated in the apoplast or cell wall [48].

There are some reports on the specific metabolic pathways for different ABs. For example, clindamycin (CLI) (a lincosamide) follows four major metabolic pathways in lettuce, including cladinose hydrolysis, demethylation, methylation, and oxidation [49]. Sulphonamides constitute the group for which more information about their metabolization in plants is available. In *A. thaliana* glycosylation is the main transformation pathway reported for sulfamethazine (SMZ) and sulfamethoxazole (SMX). Additionally, N4-glycosyl-glycoside, pterin and methylsalicylate conjugates were reported. While hydroxylation and desulfation have been discovered to occur during the phase I of the biotransformation process [50,51]. Another study revealed different TPs of SMX in *A. thaliana* cells. Namely, the phase I metabolism involved the oxidation, while phase II involved conjugation with glutathione, glucuronic acid and leucine [52]. Interestingly, sulfadiazine (SDI) appears to follow two phase II metabolic pathways in lettuce unusual for other sulfonamides, i.e., formylation and acetylation [49].

Conjugation does not lead to complete detoxification and inactivation of the AB, like full mineralization, but preserves its basic molecular structure and hence only partially and

temporary loses its toxicity and/or activity [48]. Moreover, some drug conjugates (e.g., glycosides) are unstable and they easily undergo hydrolysis via action of microorganisms or enzymes in the digestive tract of warm-blooded animals [39]. Even if not digested, degradation of plant materials reintroduces them as organic contaminants into the environment (e.g., soil) [51]. Additionally, it is necessary to keep in mind that some drug metabolites can be even more toxic than parent compound [39]. When ABs are degraded, the degree that the degraded products remain bioactive is dependent on the presence or absence of functional groups, and this varies depending on the mechanism of degradation [32]. For that reason, knowledge of biotransformation pathways of each drug in plants and testing of biological activity of drug metabolites is of paramount importance [39].

## 1.6. Ecotoxicological effects

Ranking of pharmaceuticals in terms of their occurrence, persistence, bioaccumulation, and toxicity shows that ABs, anti-inflammatories and antilipidemic are considered to have the highest levels of risk in the aquatic environments in China [53]. Still the knowledge about environmental impacts and ecotoxicological effects of ABs is scarce.

According to Du and Liu [35] environmental impacts of residual ABs include:

- resistance evolution of pathogens and bacteria through long-time exposure, genetic variation, and transfer of antibiotic-resistant genes (ARGs), e.g., transferring resistance from non-pathogenic to pathogenic bacteria,

- human health impacts of AB ingestion via animal- or plant- based food products and drinking water with AB residues,

- ecotoxicological effects on non-target organisms in aquatic environment and terrestrial environments, especially environmental microorganisms,

- ecological impacts on agro-ecosystems of introduced ABs through amending with manure and irrigation water.

The assessment on whether or not a chemical substance causes a threat to the environment is usually carried out on the basis of tiered toxicity tests using established standard organisms like bacteria, fish, algae or other appropriate sensitive species. The effects of ABs in standard

acute ecotoxicological bioassays towards different groups of organisms are shown in Figure 1.7. Among included, cyanobacteria are the most sensitive organisms to the selected ABs (OFL, ciprofloxacin, and amoxicillin), followed by aquatic plants and algae. However, the assessment of the environmental risk of ABs is hindered by their very low concentrations and specific modes of action and thus sensitive endpoints are needed.

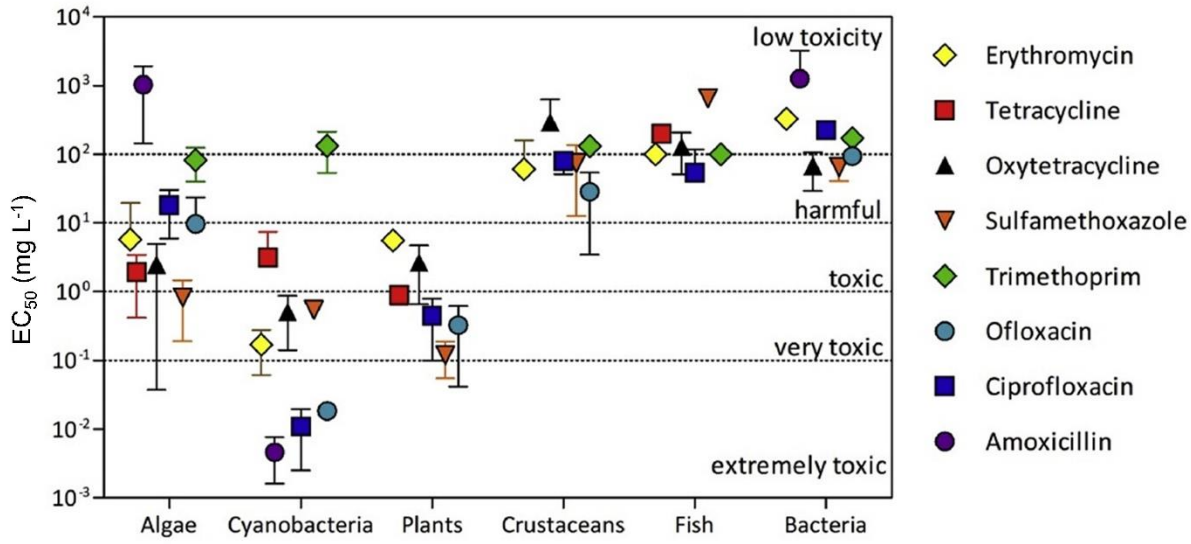



Figure 1.7. Acute ecotoxicity of selected ABs towards different groups of organisms [24]

Freshwater organisms show different sensitivity to antimicrobials depending on the type, class and species [11]. It has been demonstrated that doses of ABs legally allowed in aquaculture may cause severe systemic issues in non-target organisms such as prawns [54] or fish [55]. In addition, besides a priori environmental risk assessment of ABs including toxicological test on very sensitive species, very few studies report their ecotoxicological impact on microbial communities [56].

The main concern related to the ABs occurrence in the environment is that their selection pressure may accelerate the evolution and dissemination of antibiotic-resistant bacteria (ARB). As an example, fruits and vegetables are declared to be safe for consumption if AB residues are below certain limits. However, it is not the AB residue that should be the only concern but the process by which the vegetal is obtained. If AB was used during its growth, it will contribute to the AB resistance pool even though all residues are later washed away from the vegetal. The resistant bacteria that are selected for due to the ABs can then transfer the resistance to other bacteria including those that infect humans. It was shown that adding low dose oxytetracycline in chicken feed resulted in the appearance of tetracycline-

resistant *E. coli* in the intestinal microbiota of not only the chickens but also the farm workers who routinely handle the poultry farms [8].

The effectiveness of an AB is determined by its minimum inhibitory concentration (MIC), which is the minimum concentration of the AB that can stop growth of a particular microorganism. The lowest antimicrobial concentration needed to choose for the resistant mutant over the wild type is called as the minimal selective concentration (MSC). Selection for the resistant mutants also occurs at concentrations of the sub-MIC selective window (between the MSC and the MIC of the susceptible strain). Sub-MIC concentrations of AB can be chosen for a low-level of resistance, which eventually serve as a stepping stones paving the way for high-level resistance [57]. To experimentally determine MSC in complex microbial ecosystems for all ABs would involve a considerable effort. Thus, compound-specific estimates for MSCs and predicted no-effect concentrations based on MIC data were calculated for ABs in water [58]. In any case, understanding how the anthropogenic input of ABs in the environment influences antimicrobial resistance is of the greatest importance.

## 1.7. Human exposure and risk assessment

Concerns regarding the human health risks of ABs resulted in developing different tools for its assessment. The most important are the estimation of the daily or annual exposure of humans to ABs and its conversion to medical dose equivalent; the threshold of toxicological concern; and the hazard quotient (HQ) approach [59]. However, from both human and environmental health perspectives, it is important that risk assessment frameworks incorporate the risk of AB resistance selection [60].

There is the concern that the environmental risk assessment for ABs is biased towards testing on metazoan species (invertebrates and fish in this instance), and does not consider fully the possible impacts of ABs on microbial community structure, function and resilience [60]. Concerns over the human health threats of AB resistance associated with AB residues in the environment are mainly (1) the potential hazard of ingested AB residues altering the human microbiome and promoting emergence and selection for resistant bacteria, inhabiting the human body namely as human AB resistance; and (2) the potential hazard of creating a selection pressure on the environmental microbiome and leading to reservoirs of ARGs and ARB referred as environmental AB resistance [16]. So far, however, there is no generally accepted approach to assess the risk of development or dissemination of AB resistance in the environment [61]. Major obstacles in risk assessment of a mixture ABs is the high variability

of environmental mixtures and the complexity of their interactions (antagonistic or synergistic) between themselves and/or with other contaminants, i.e., heavy elements [62–65].

Moreover, risk assessment tools underestimate the hazard associate to AB TPs, which are present in environmental matrices (e.g., crops), and they may accumulate in higher concentrations and/or even exert higher toxic effects than the parent compounds [59]. Therefore, significance of metabolites should be carefully evaluated for monitoring strategy, priority setting, and scoping of the environmental risk assessment of active pharmaceutical ingredients [66].

The unintended intake of a certain AB and its TPs may elicit potentially unfavorable responses. These responses may vary according to the exposure concentration and the potential simultaneous exposure (synergistic effect) to a mixture of pharmaceuticals and other pollutants [59]. The veterinary ABs and preferred as veterinary ABs detected in urines might be primarily derived from long-term ingestion of contaminated foods [67]. For instance, Wang et al. [68] found that some adults in Shanghai were at health risk related to the disturbance of gut microbiota as a results of an extensive exposure to low-dose multiple ABs. In fact, main exposure to ABs is through contaminated food consumption instead of medication. Therefore, the primary exposure mode in adults should be the long-term and low-dose exposure mode caused by AB residues in food or drinking water, rather than the short-term and high-dose exposure mode caused by AB clinical use [68,69].

## 1.8. Overview of the methodologies

### Brief overview of the selected antibiotics

- Sulfonamides**.** ABs of this class differ in the heterocyclic group, and are presently most commonly used for veterinary purposes and as growth promoters in animal husbandry. In human medicine, these ABs, specifically SMX, are still highly relevant when used in combination with TMP [33].

- TMP, a diaminopyrimidine, despite being structurally different, it shares most of the antibacterial spectrum and mechanism of action with sulfonamides [33].

- Fluoroquinolones**.** First-generation quinolones (nalidixic acid, flumequine and oxolinic acid) are scarcely used, whereas fluoroquinolones (second generation onward) are

still highly relevant to human therapy but less frequently used in veterinary medicine [34].

- Lincosamides (CLI) are used as an alternative to penicillin G in human medicine. Although some lincosamides have been authorized for use in food producing animals, CLI is used only for human medication [70].

Quinolones are considered by the WHO as 'critically important antimicrobials' in medicine, while sulfonamides, TMP and lincosomides as 'highly important antimicrobials' [71]. Tetracyclines, which are widely used in veterinary applications, are not considered in this Thesis since they exhibit high $K_d$ values in soils (Table 1.3) and consequently, it is not expected to be up taken by plants as they are poorly bioavailable.

## Sampling campaign strategy

The sampling campaign strategy is a key step in order to achieve desirable and reliable results. Six farm plots were selected based on different types of anthropogenic impact and considering the complexity of real, field-scale agricultural environments. These plots differ on their irrigation water sources (i.e., ground water, surface water, harvested water, and RWW) and fertilizer type (i.e., chemical fertilizer, pigeon and horse manure). In this way, obtained results will show influence of different agricultural practices on the AB pollution loads in vegetables. From selected farms, different types of crops (i.e., lettuce, tomato, cauliflower, and broad bean) were collected during 2016 and 2017, according to their availability. All vegetables were grown for human consumption, hence providing realistic data for a human health risk assessment. In such way, sampling campaign strategy provides foundation for the evaluation of distinct predictors, related to AB pollution loads in edible vegetables. Moreover, predictor interactions and their relative importance in the specific time-space boundaries of given field-scale conditions.

## Target screening

As presented in Table 1.4, liquid chromatography tandem mass spectrometry (LC-MS/MS) has emerged as the primary analytical tool for quantifying ABs in crops. Advances in LC-MS/MS have allowed detection of ABs in biological matrices due to the high sensitivity and selectivity of mass spectrometers [72]. Because of different physicochemical properties of ABs (Tables 1.2 and 1.3), their low concentrations in crops, and the complexity of biological

matrices, sample preparation is considered to be a critical step in analytical method development. Exhaustive extraction techniques are required for the simultaneous multi-residue analytical procedures that can be applied to more than one type of vegetable matrices. Among published reports, the most common extraction techniques are ultrasound-assisted solvent extraction (UAE) and accelerated solvent extraction (ASE) due to reduced solvent usage in comparison to Soxhlet extraction methods (Table 1.4). However, because these extraction techniques are not analyte selective, the obtained extracts using these techniques will, alongside target ABs, contain compounds that interfere following LC-MS/MS analysis. These co-eluting, interfering compounds may cause suppression or enhancement of ionization in the electrospray ion source, leading to quantification errors. In order to minimize errors caused by this phenomenon, known as matrix effect, clean-up steps are needed. However, extensive clean-up techniques should be selected carefully, since they may decrease sample preparation throughput, increase costs of analysis and the sample losses. As shown in Table 1.4, extraction techniques are usually followed by solid-phase extraction (SPE) as a clean-up step. Wide spectrum sorbents with both lipophilic and hydrophilic retention characteristics are frequently used in the SPE clean-up procedures (e.g., hydrophilic-lipophilic balance (HLB) polymer).

For several years now, high resolution mass spectrometry instruments (HRMS) have begun to be more accessible and seem a suitable alternative to MS/MS. Namely, HRMS (e.g., Time of Flight, Orbitrap) selectivity is increased due to the higher resolving power, which allows them to make the difference between a target compound and a co-eluting, interfering compound with the decimal difference. Although, thus far target HRMS methodology were not reported for the analysis of ABs in vegetables, promising results were obtained analyzing another complex matrices, e.g., manure [73].

### Suspect and non-target screening

Targeted approaches can quantify specific classes or groups of metabolites that are initially included in the target list, while suspect and non-targeted approaches aim to acquire as much metabolic information as possible [74]. We have come a long way in the last ten years in solving the issue of metabolite annotation. Significant developments related to instrumentation (e.g., increased mass resolving power) and the data treatment (software and databases) have increased the number of metabolites detected and annotated in non-targeted approaches [75]. However, structural identification and quantification of unknown chemical compounds is a constant question in analytical science [76].

In suspect screening approaches, analysis is carried out against a database of potentially present TPs and metabolites. The database is compiled based on the estimations of m/z values by knowing transformation, breakdown, and metabolization reactions of ABs and include previously reported compounds as well [77,78]. In addition, *in-silico* transformation and metabolization prediction tools (e.g., EAWAG-BBD Pathway Prediction System) may enrich the list. These databases should contain accurate mass of suspected compounds, and if possible information on their retention time (RT) and MS/MS fragmentation patterns. Even though, these databases contain relatively large number of compounds, still the ones that are not included in the list will remain overlooked. Thus, comprehensive non-target determination is needed since the potential TPs and metabolites of ABs that can occur in plants are basically not limited in their number.

One of the most common strategies in non-target screening for identification of AB TPs and metabolites in complex matrices is comparison of exposed vs. control samples [51,52,73,79,80]. This strategy is based on finding the exact monoisotopic MW of compounds that are higher in the treated sample than in the control (non-exposed) sample.

In both cases, suspect and non-target screening, after a preliminary identification, the possible product structures are further confirmed in $MS^2$ scan mode, in order to get product ion spectrum. Additionally, the structures of possible TPs and metabolites are ascertained by analyzing the main fragmentation patterns, isotope patterns and comparing the data with database and existing study reports [49]. Once identified and annotated, metabolites are further compared with literature reporting common reactions and enzymes in the metabolism of other xenobiotics in order to determine the most likely biotransformation pathways [81].

Table 1.4. Overview of the analytical methodologies for target screening of ABs in vegetables

| Matrix | Extraction | Clean-up | Target compounds | Instrumentation | Reference |
|---|---|---|---|---|---|
| Lettuce | Freeze-and-thaw lysing and liquid extraction | HLB SPE | Lincomycin, oxytetracycline, SMX, doxycycline, roxithromycin, demeclocycline | LC-MS/MS | [83] |
| Chinese white cabbage, water spinach, Chinese radish, corn, rice | UAE | HLB SPE | Tetracycline, SMZ, norfloxacin, erythromycin, chloramphenicol | LC-MS/MS | [84] |
| Wheat | ASE | HLB SPE | SMX, TMP, OFL | LC-MS/MS | [85] |
| Radish, rape, celery, coriander | UAE | HLB SPE | SMX, sulfadoxine, sulfachloropyridazine, chloramphenicol, oxytetracycline, tetracycline, chlortetracycline, lincomycin, OFL, ciprofloxacin, pefloxacin | LC-MS/MS | [86] |
| Lettuce, spinach, cucumber, pepper | UAE | HLB SPE | SMX, TMP | LC-MS/MS | [87] |
| Tomatoes, carrots, potatoes, sweet corn | UAE | HLB SPE | Ciprofloxacin, norfloxacin, OFL, tetracycline, azithromycin, erythromycin, clarithromycin, doxycycline, SMX, TMP | LC-MS/MS | [88] |
| Green bell-pepper, bermuda grass, cantaloupe, carrot, lettuce, spinach | ASE | Liquid extraction (hexane) | Clarithromycin, azithromycin, CLI, roxithromycin | LC-MS/MS | [89] |
| Lettuce ,carrot | ASE | HLB SPE | Tetracycline, amoxicillin | LC-MS/MS | [90] |
| Cucumber, tomato, carrot, sweet potato | ASE | In-cell florisil | Sulfapyridine, SMX | LC-MS/MS | [40] [91] |
| Tomato, eggplant, zucchini, pepper, cabbage, lettuce, parsley, arugula, potato, carrot | UAE | | Ciprofloxacine, SMX, | LC-MS/MS | [92] |

(continuation) Table 1.4

| Matrix | Extraction | Clean-up | Target compounds | Instrumentation | Reference |
|---|---|---|---|---|---|
| Lettuce | Pressurized liquid extraction | | SMZ | LC-MS/MS | [93] |
| Corn, arugula | UAE | HLB SPE | Lincomycin, OFL | LC-MS/MS | [94] |
| Tomato | UAE | HLB SPE | SMX, TMP | LC-MS/MS | [95] |
| Pak choi | QuEChERS | | Sulfamethoxypyridazine, tetracycline, OFL, norfloxacin, difloxacin | LC-MS/MS | [96] |
| Spinach, lettuce, cabbage, carrot, radish, onion, garlic, tomato, green bell pepper, sweet corn, potato | Liquid extraction | | Chlortetracycline, monensin, SMZ, tylosin, virginiamycin | enzyme-linked immunosorbent assay (ELISA) | [97] |
| Radish | QuEChERS | | Chlortetracycline, enrofloxacin, sulfathiazole | LC-MS/MS | [98] |
| Pumpkin, potato, cabbage, lettuce, tomato, carrot, radish, soybeans, zucchini, cucumber, sweet corn, green bell pepper, white onion, green onion, garlic, green beans | ASE | HLB SPE | SMZ, ciprofloxacin, enrofloxacin, levofloxacin, norfloxacin, chloramphenicol, florfenicol, doxycycline, metronidazole | LC-MS/MS | [99] |
| Eggplant, long bean, wheat, cucumber | Soxhlet extraction | | Sulfamonomethoxine, sulfathiazole, sulfisoxazole, sulfachlorpyridazine, SMZ, sulfamerazine, TMP, chloramphenicol, SMX | LC-MS/MS | [100] |

## 1.9. Hypothesis and objectives

### Hypothesis

**Hypothesis I:** Different agricultural practices determine pollution loads of ABs in vegetables.

**Hypothesis II:** Through vegetable consumption, humans are exposed to ABs TPs alongside with the parent AB.

### Objectives

The overall objective of this Thesis is to study occurrence and metabolization of ABs in vegetables. In order to achieve this general objective, the following specific objectives have been set:

1. Develop an analytical methodology for target screening of ABs and their metabolites in edible parts of different vegetables

2. Investigate human health risk of AB compounds occurrence in vegetables intended for human consumption

3. Study different agricultural factors related to the AB occurrence in agricultural systems

4. Develop a workflow for annotation and identification of ABs TPs

5. Elucidate biotransformation pathways of OFL in lettuce as model plant

# Chapter II: Validation of method for determination of antibiotics in vegetables

This Chapter is based on the article:

Đorđe Tadić, Víctor Matamoros, Josep Maria Bayona. Simultaneous determination of multiclass antibiotics and their metabolites in four types of field-grown vegetables, Analytical and Bioanalytical Chemistry (2019) 411:5209–5222

***In brief:***

An analytical procedure was developed and evaluated for the determination of 10 ABs belonging to four chemical classes (fluoroquinolones, sulfonamides, lincosamides, and diaminopyrimidine) and 6 of their metabolites in 4 vegetable matrices (lettuce, tomato, cauliflower, and broad bean). The reported method detection limits were sufficiently low (0.1–5.8 ng $g^{-1}$ dry weight) to detect target compounds in vegetables under real agricultural practices. Absolute and relative recovery values ranged from 40 to 118% and from 70 to 118%, respectively for all targeted compounds at the spike level of 100 ng $g^{-1}$ dry weight. Regarding method precision, the highest relative standard deviation (RSD) was obtained for enrofloxacin in lettuce (20%), while for the rest of the compounds in all matrices, the RSD values were below 20% for the same spike level. Matrix effects, due to electrospray ionization ranged from -26 to 29% for 85% of all estimated values. In a field study, 4 of the 10 targeted ABs were detected in tested vegetables. For the first time AB metabolites were quantified in vegetables grown under real field conditions. More specifically, decarboxyl OFL and keto TMP were detected in tomato fruits (1.5 ng $g^{-1}$ dry weight) and lettuce leaves (21.0–23.1 ng $g^{-1}$ dry weight), respectively. It is important to remark that the concentration of keto TMP was five times higher than that from the parental compound, emphasising the importance of the metabolite analysis in monitoring studies. Therefore, the method provided a robust, reliable, and simple-to-use tool for routine multiclass analysis of ABs and their metabolites in vegetable samples.

## 2.1. Introduction

Currently, ABs are used for the treatment of infections in humans, but they are also increasingly used in animal husbandry and fish farms for disease prevention, prophylactic treatment, and, in some countries, to promote animal growth [82]. However, there is growing public concern about the environmental impact of the widespread use of veterinary antimicrobials, since population growth, increasing prosperity, and inappropriate use have stimulated the production of thousands of tons of ABs [15,83].

ABs enter the environment via various human activities, including direct disposal of unused or expired medication, human waste, release from pharmaceutical manufacturing plants and hospitals, and veterinary drug use [84–86]. Large amounts of human and veterinary ABs are excreted unchanged or as metabolites and, because they are only partially removed at WWTPs, lead to notably widespread contamination of water and soil resources [19,87–89]. In this regard, there is major public concern regarding agricultural applications of RWW and manure due to the introduction of ABs into crops via plant uptake. Although the risk that AB residues may pose to humans through the consumption of the edible portions of crops is not yet well documented [83,90], continuous consumption of AB-contaminated vegetables may result in detrimental health effects due to the development and spread of AB resistance and/or chronic toxic effects as a result of prolonged low-level exposure to ABs themselves and to their metabolites [83,91–93].

Consequently, the monitoring of AB residues in food has become essential to assess human exposure. The main difficulties in the analysis of ABs and their metabolites in crops are associated with the low concentration at which these compounds occur in vegetable tissues and the presence of matrix components containing high concentrations of endogenous components, such as carbohydrates, pigments, and fatty or waxy materials, that can interfere in their determination [15].

In recent years, various analytical methodologies have been developed for the determination of AB residues in different vegetable matrices [40,90,94–103]. However, these analytical methodologies are still limited to the determination of parent compounds while little attention has been paid to the identification of the AB phytometabolites. The presence of AB metabolites in plant tissues should be monitored in studies evaluating the uptake of ABs, since their occurrence and toxicity may be compelling [89,104]. Moreover, the problem of AB metabolites is not restricted to their toxicity due to the fact that they may retain antimicrobial activity and promote AB resistance. Prominent findings from Majewsky et al. [105] showed that two

metabolites of SMX inhibit growth of *Vibrio Fischeri* to a clearly greater extent than the parent compound. Similar phenomenon was observed in case of TPs of fluoroquinolones [106]. Accordingly, the main goal of this paper was to overcome the aforementioned analytical limitations and to develop and validate a quantitative, sensitive, reliable, and practical methodology for the simultaneous determination of 10 ABs belonging to four chemical classes (i.e., fluoroquinolones, sulfonamides, lincosamides, and diaminopyrimidines) and, for the first time, 6 of their metabolites (Table 2.1) in four different vegetables matrices. Metabolites were selected according to their commercial availability and reported occurrence in the environment i.e., N-Acetyl SMX has become evident in digested sludge, agricultural soil and reclaim water [107,108]. Moreover, it has been highlighted as predominant metabolite in the study published by Dudley et al. [52] who investigated the metabolism of SMX in *Arabidopsis thaliana* cells and cucumber seedlings. In the experiments conducted by Jewell et al. [109], TMP306 which is formed by hydroxylation of TMP, was one of two identified metabolites during biological WW treatment with high TMP concentration.

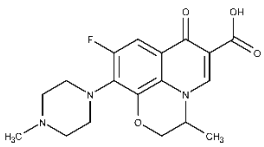
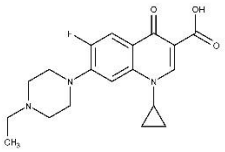
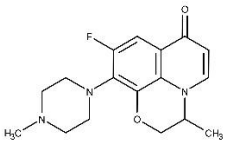
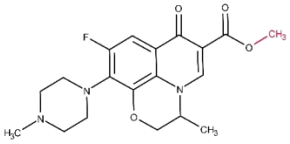
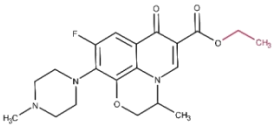
Among several extraction techniques, such as solid-liquid extraction [99], UAE [110,111], pressurized liquid extraction [40,97,112] and QuEChERS [83], that have been reported for pharmaceutical determination in vegetables, in the present work, UAE was selected. UAE was a technique of choice since it is robust, simple and does not require specialized and expensive extraction equipment. Although UAE combined with SPE may require considerable processing time compared to ASE or QuEChERS, Wu et al. [113] and Yu et al. [94] found that UAE in combination with SPE had better recovery results than ASE and QuEChERS, for several pharmaceutical compounds, including ABs. Finally, in most of the published methodologies, the ABs were separated by liquid chromatography and detected by mass spectrometry [114]. Although TPs and metabolites were reported in RWW [108,115] and animal tissue [116,117], respectively, to the best of the authors' knowledge, this is the first study dealing with simultaneous determination of AB metabolites in vegetable matrices. Finally, the robustness of the method is demonstrated through the analysis of harvested vegetables irrigated with RWW under real field conditions.

## 2.2. Material and methods

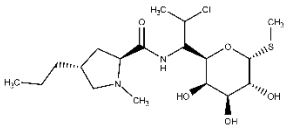
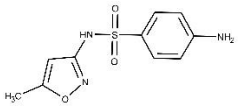
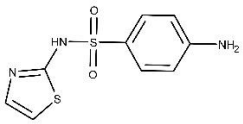
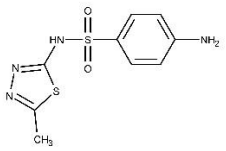
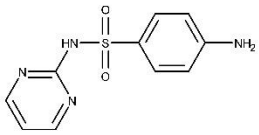
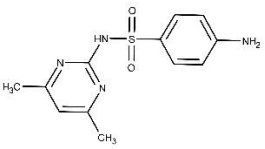
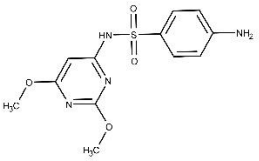
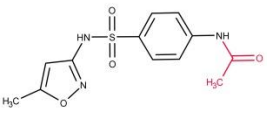
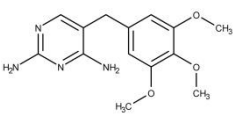
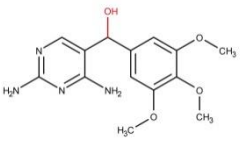
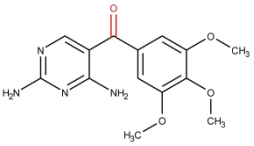
### 2.2.1. Chemicals and materials

Sulfathiazole (STZ), sulfamethizole (SMT), SDI, SMZ, sulfadimethoxine (SDM), SMX, OFL, enrofloxacin (ENR), ofloxacin-d3 (OFL-d3), enrofloxacin-d5 hydrochloride (ENR-d5), CLI hydrochloride, and TMP were purchased from Sigma Aldrich (St. Louis, MO, USA) whereas OFL Methyl Ester, OFL Ethyl Ester, Decarboxyl OFL, keto TMP (2,4-diaminopyrimidin-5-yl)-(3,4,5-trimethoxyphenyl) methanone, TMP306 (2,4-diaminopyrimidin-5-yl)-(3,4,5-trimethoxyphenyl) methanol and N-Acetyl SMX were purchased from LGC standards S.L.U. (Barcelona, Spain). Clindamycin-d3 hydrochloride (CLI-d3) was purchased from Toronto Research Chemicals (Toronto, Canada), trimethoprim-d3 (TMP-d3) was purchased from Dr. Ehrenstorfer (Augsburg, Germany), and sulfamethoxazole-d4 (SMX-d4) was purchased from Analytical Standard Solutions – A2S (Saint Jean d'Illac, France), respectively. All standards were high-purity (95% or higher). The structure and physicochemical properties of the studied compounds are summarized in Table 2.1.

Table 2.1. List of target compounds along with their molecular structure, CAS number, molecular formula, and Log $K_{OW}$

| Compound | Molecular structure | CAS Number | Molecular formula | Log $K_{OW}$ [a] |
|---|---|---|---|---|
| OFL |  | 82419-36-1 | $C_{18}H_{20}FN_3O_4$ | -0.39 |
| ENR |  | 93106-60-6 | $C_{19}H_{22}FN_3O_3$ | 0.70 |
| Decarboxyl OFL |  | 123155-82-8 | $C_{17}H_{20}FN_3O_2$ | 2.19 |
| OFL methyl ester |  | 108224-82-4 | $C_{19}H_{22}FN_3O_4$ | 1.79 |
| OFL ethyl ester |  | 177472-30-9 | $C_{20}H_{24}FN_3O_4$ | 2.28 |

(continuation) Table 2.1

| Compound | Molecular structure | CAS Number | Molecular formula | Log $K_{OW}$ [a] |
|---|---|---|---|---|
| CLI |  | 18323-44-9 | $C_{18}H_{33}ClN_2O_5S$ | 2.16 |
| SMX |  | 723-46-6 | $C_{10}H_{11}N_3O_3S$ | 0.89 |
| STZ |  | 72-14-0 | $C_9H_9N_3O_2S_2$ | 0.05 |
| SMT |  | 144-82-1 | $C_9H_{10}N_4O_2S_2$ | 0.54 |
| SDI |  | 68-35-9 | $C_{10}H_{10}N_4O_2S$ | -0.09 |
| SMZ |  | 57-68-1 | $C_{12}H_{14}N_4O_2S$ | 0.19 |
| SDM |  | 122-11-2 | $C_{12}H_{14}N_4O_4S$ | 1.63 |
| N-Acetyl SMX |  | 21312-10-7 | $C_{12}H_{13}N_3O_4S$ | 1.21 |
| TMP |  | 738-70-5 | $C_{14}H_{18}N_4O_3$ | 0.91 |
| TMP306 |  | 29606-06-2 | $C_{14}H_{18}N_4O_4$ | -0.81 |
| Keto TMP |  | 30806-86-1 | $C_{14}H_{16}N_4O_4$ | 0.44 |

[a] Episuite v4.11 (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm)

Individual stock solutions were prepared at a concentration of 200 µg $mL^{-1}$ in methanol. The stock solutions were stored in the freezer at −20ºC. Spiking solutions made of mixed working standards were prepared by appropriately diluting the stock solutions on the day of the experiment. Methanol and water (both LC-MS grade), ethyl acetate (GC-ECD/FID grade), and formic acid (98-100%, pro analysis) were obtained from Merck (Darmstadt, Germany), whereas acetonitrile (LC-MS grade) was obtained from Fisher Scientific UK (Loughborough, UK). SPE Strata-X cartridge (100 mg, 6 mL) were obtained from Phenomenex (Torrance, CA, USA) and 0.22 µm pore nylon filter were purchased from Sigma Aldrich (St. Louis, MO, USA).

### 2.2.2. Sample collection and preparation

Four types of vegetable matrices, namely, lettuce leaves (*Lactuca sativa* L.), tomato fruits (*Solanum lycopersic*um L.), cauliflower inflorescence (*Brassica oleracea* L.), and broad bean seeds (*Vicia faba* L.) were harvested from a farm located in the peri-urban area of Barcelona, Spain. The farm plot was irrigated with RWW. Further details on the sampling site and irrigation water quality are provided elsewhere [118]. All samples were comminuted with liquid nitrogen and stored at −20°C until analysis. Frozen samples were ground under liquid nitrogen using mortar and pestle.

### 2.2.3. Extraction and SPE clean-up

In order to enable equilibration, OFL-d3, TMP-d3, CLI-d3, and SMX-d4 were spiked one hour prior to extraction into the weighted and homogenized samples as surrogate standards at a concentration of 100 ng $g^{-1}$ dry weight (dw). Lettuce leaves were chosen as the matrix for the optimization studies since it has been mostly investigated matrix among tested vegetables, with proven complexity. One gram of fresh weight (fw) vegetable sample was extracted with 10 mL of methanol in an ultrasonic bath for 15 min. The extracts were centrifuged for 15 min at 3000 g. Two extraction cycles were required. The supernatants were decanted and combined in a glass vial and evaporated under a gentle nitrogen stream at a temperature of 40ºC until they had been reduced to 1 mL. The concentrated extracts were then diluted with 20 mL of water to perform the SPE clean-up step. The SPE cartridges (Strata-X cartridge, 100 mg, 6 mL) were firstly preconditioned with 6 mL of methanol and 6 mL of water. After sample loading, the polymeric cartridge was washed with 1 mL of water with 5% methanol. The cartridges were then dried under a nitrogen stream, followed by elution with 2 mL of a mixture of methanol and ethyl acetate (1:1, v:v).The eluted fraction was evaporated to dryness

and reconstituted in water. The final extracts were filtered through a 0.22 µm pore nylon filter and fortified with ENR-d5 (50 ng $g^{-1}$ dw) as an internal standard prior to injection.

### 2.2.4. Chromatographic conditions

The present study used a Waters Acquity Ultra-Performance Liquid Chromatography™ System (Milford, MA, USA) equipped with a binary pump, degasser, autosampler and column oven. The chromatographic separation was achieved on a core-shell Ascentis® Express RP-Amide column (10 cm x 2.1 mm, 2.7 µm particle size) (Supelco, Bellefonte, PA, USA) with a guard column (0.5 cm x 2.1 mm) containing the same packing material. The flow rate was 0.25 mL $min^{-1}$, and the injection volume was 10 µL. A binary gradient elution program using mobile phases A (acetonitrile with 0.1% formic acid) and B (water with 0.1% formic acid) was set as follows: from 0 to 2 min, isocratic, 10% A; from 2 to 7 min, 10-60% A; from 7 to 9 min, 60-95% A; from 9 to 15 min, isocratic, 95% A; from 15 to 17 min, 95-10% A; from 17 to 23 min, isocratic, 10% A. The column oven and autosampler temperatures were set at 25ºC and 15ºC, respectively.

### 2.2.5. Mass spectrometry conditions

The mass spectrometry equipment consisted of a Waters TQ-Detector (Manchester, UK). The target compounds were determined with tandem mass spectrometry in multiple reactions monitoring (MRM) mode of at least two transitions for the selected compounds. Ions were generated using electrospray ionization in positive mode (ESI+). Source and desolvation temperatures were set at 150ºC and 350ºC, respectively. Evaluation of cone voltage, collision energy, and transitions was performed by direct infusion of standard solutions of 1 mg $L^{-1}$ of each compound in a water:methanol (50:50, v:v) mixture. By injecting standards containing target compounds in full-scan mode, using ESI+ mode, protonated molecular ions $[M + H]^+$ were observed and selected as precursor ions. Cone voltage and collision energy were optimized to obtain the best transition of the precursor ion to the most abundant fragments. The fragment with the highest intensity was selected as the quantifier (ion used for quantification), and the second specific fragment was used as the qualifier (ion used for identification).

### 2.2.6. Method validation

Owing to the lack of vegetable reference materials with certified values for ABs and their metabolites, the precision and accuracy of the method were investigated by spiking samples at two concentration levels (10 and 100 ng $g^{-1}$ dw). The method was validated for four vegetable matrices (lettuce leaves, tomato fruits, cauliflower inflorescences, and broad bean seeds). The instrumental limits of detection (IDLs) and quantification (IQLs) were determined based on a signal-to-noise ratio of 3 and 10, respectively, when the standard solution was injected. The method detection limits (MDLs) and method quantification limits (MQLs) were calculated as the mean background noise in a blank triplicate plus three or ten times, respectively, the standard deviation of the background noise from three blanks. For the inter-day precision, five replicates at two spike concentrations were analyzed every day for three consecutive days. Precision was expressed as the RSD estimated by dividing the standard deviation by the mean value obtained for each set of concentrations and multiplying it by 100. Intra-day precision was also estimated for two spike concentrations in five replicates in one day. Calibration curves were prepared in real matrix extracts and in standard solution, the slopes of the calibration curves in the matrix and standard solutions were compared for each analyte to evaluate the matrix effect. As proposed by Paíga et al. [119], the matrix effect was calculated according to the following equation:

$$\text{Matrix effect (\%)} = \left({}^{\text{slope matrix\_matched}}/_{\text{slope standard solution}} - 1\right) \times 100 \qquad (4)$$

A negative value indicates ion suppression, while a positive value indicates ion enhancement. All the AB concentrations in this Chapter are expressed on dry weight basis to better compare with published data.

### 2.2.7. Data analysis

The data were analyzed using MassLynx 4.1 software and IBM SPSS Statistics v.23. A paired t-test was used to compare recoveries between spiking concentration levels and vegetable matrices. Statistical significance was established at $\alpha = 0.05$.

## 2.3. Results and discussion

### 2.3.1. Optimization of the extraction procedure

Since the 16 studied compounds (10 parent ABs and 6 metabolites) exhibit different physicochemical properties (Table 2.1) and have been reported in vegetables in the low ng $g^{-1}$ range, an exhaustive sample extraction technique was selected, namely, UAE. As previously reported by Wu et al. [113] comparison of recoveries showed that UAE consistently resulted in better recoveries than ASE for most of the target pharmaceuticals. More specifically, Yu et al. [94] showed that UAE combined with SPE is slightly better than QuEChERS method, for determination of ABs in vegetable matrices, whereas QuEChERS method is more efficient for honey, meat and aquatic products. During the UAE optimization, the efficiency of three different extraction media was tested, i.e., aqueous phosphate buffer (APB) at pH 7.5, APB at pH 3, and methanol. Two cycles of UAE with 10 mL of each solvent for 15 min were performed. Table S2.1 shows the effect of solvent on the extraction of ABs from vegetable material. The recovery values obtained using APB at pH 7.5 were better than those obtained using APB at pH 3, but still unsatisfactory since for 60% of the studied ABs recovery values were lower than 20%. APB at pH 7.5 yielded poor absolute recovery values compared to the methanol solvent (p value< 0.05) which ranged from 48 to 111%. Furthermore, the matrix effect for both extraction solvents (methanol and APB at pH 7.5) ranged from -35 to 23%. In view of these results, methanol was selected as the extraction solvent. In fact, previous studies have proven that methanol can efficiently extract ABs and that it performs poorly in protein and fat extraction – which is an important advantage since both protein and fats are considered to be interfering matrix compounds [120] – yielding recovery values similar to those presented in the present study [103,121].

### 2.3.2. Optimization of SPE clean-up

A clean-up step is crucial to concentrate analytes and remove interfering co-eluting matrix components from complex matrices such as vegetable material. Due to the exhaustive nature of UAE and the lack of selectivity, the amount of co-extracted matrix material is greater [122]. Two elution solvents in the SPE clean-up procedure – methanol and a mixture of methanol and ethyl acetate (1:1, v:v) – were tested at different volumes (1, 2, and 3 mL). Table S2.2 shows the effect of clean-up on recovery values after the SPE cartridge has been eluted with different solvent mixtures (methanol or methanol/ethyl acetate). Recoveries ranged from 7 to 89% for methanol and from 49 to 96% for the methanol/ethyl acetate mixture. Furthermore,

the methanol-ethyl acetate mixture yielded significantly higher recovery values than methanol (paired *t*-test, $p < 0.05$). The first 2 mL of elution solvent proved to be sufficient, as increasing the volume up to 3 mL did not increase the recovery values (Table S2.2) but could promote the elution of impurities, which may interfere with ESI-MS. Subsequently, in order to optimize the washing of the SPE cartridge, three different volumes (1, 2, and 3 mL) of 5% and 10% methanol were evaluated. Among the tested combinations of SPE washing solvents, 1 mL of 5% methanol was shown to be efficient in removing matrix compounds, which can potentially offset improvements in sensitivity, yielding the lowest AB elution. Increasing the volume of washing solvent and the methanol content led to higher elution of the targeted compounds (Table S2.3). This in agreement with previous studies reported by Huber et al. [123] in which 5% methanol was selected as a washing solvent to remove unwanted plant matrix components for the determination of diclofenac and its metabolites in barley.

### 2.3.3. Optimization of chromatographic and mass spectrometry conditions

The chromatographic conditions were optimized by evaluating mobile phase composition, flow rate, and column temperature in order to achieve the best resolution and sensitivity and to minimize the analysis time. The best separation was obtained using 0.1% formic acid in both acetonitrile and water as mobile phases, which is in accordance with previously published data [83,119,124–126]. Several gradient elution profiles were tested. The appropriate change of the mobile phase composition during the separation process (described in Section 2.4) improved the retention of later eluting components and peak shapes and resulted in an optimum separation in the minimum time. Several column temperatures (20ºC, 25ºC, 30ºC, 35ºC, and 40ºC) and flow rates (0.2 mL $min^{-1}$, 0.25 mL $min^{-1}$, 0.3 mL $min^{-1}$, 0.35 mL $min^{-1}$, and 0.4 mL $min^{-1}$) were also tested. Increasing of column temperature leaded to lower column backpressure but poorer chromatographic resolution. Similarly, with the increase of the mobile phase flow rate insufficient chromatographic separation was observed. Setting the column temperature to 25ºC and the flow rate to 0.25 mL $min^{-1}$ proved to be the best separation condition and yielded good peak shape (Figure 2.1). MRM was applied to detect and quantify the targeted compounds. A full-scan mass mode was applied, as an initial step, to identify the precursor ions. Furthermore, product ions, the most abundant fragments of the precursor ion, were monitored at different collision energies (10, 15, 20, 25, 30, 35, 40, 45, and 50 eV). To maximize sensitivity, various cone voltage values (10, 20, 30, 40, 50, and 60 V) were also tested. Table S2.4 summarizes the optimum values for the MRM acquisition mode, as well as the precursor and product ions. Both precursor ion fragments and RT were used to ensure correct peak assignment.

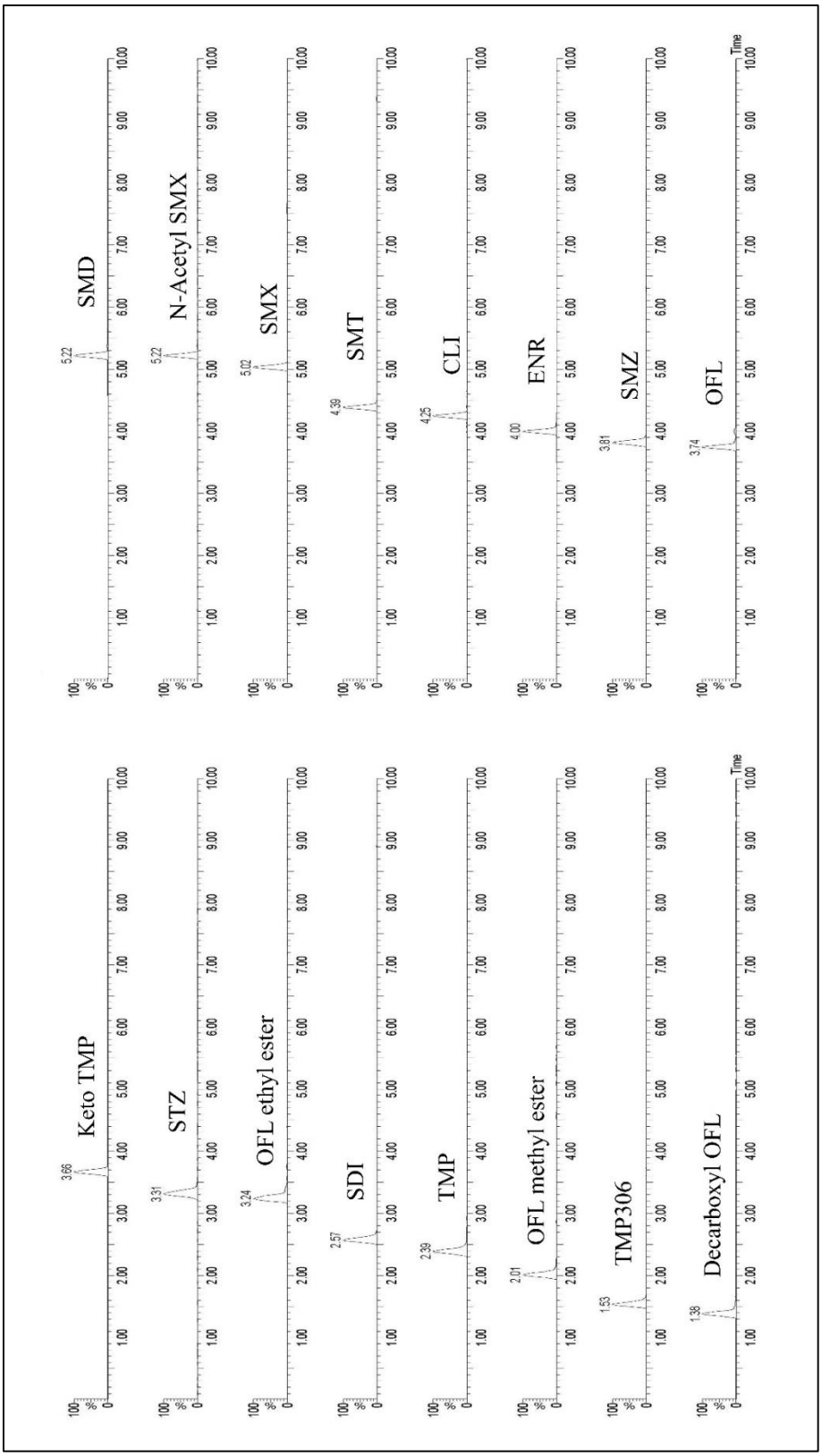



Figure 2.1. LC-MS/MS chromatograms obtained from a spiked (100 ng $g^{-1}$ dw) lettuce sample

**Method validation**

### 2.3.3.1. Instrumental figures of merit

A nine-point (0.5, 1, 2.5, 5, 10, 25, 50, 100, and 200 µg $L^{-1}$) calibration curve was prepared for all tested compounds. Good linearity was observed with correlation coefficients (R) higher than 0.99 for all tested compounds. The IDLs ranged from 0.1 to 0.88 ng, and the IQLs, from 0.35 to 2.55 ng (Table 2.2). Good results were obtained for the intra-day precision, with values less than 14% for all tested compounds. The inter-day precision was less than 18% for all tested compounds. Response variability was generally higher at lower spike levels. For most of the tested compounds, the intra- and inter-day precision confirmed the good method repeatability. The instrumental figures of merit were in the same range as those previously reported in LC-MS/MS devices [127–129].

Table 2.2. Validation parameters (IDLs; IQLs; the intra- and inter-day precision)

| Compound | IDL (ng) | IQL (ng) | Intra-day (%) | | Inter-day (%) | |
|---|---|---|---|---|---|---|
| | | | 10 ng | 100 ng | 10 ng | 100 ng |
| OFL | 0.11 | 0.35 | 0.10 | 0.08 | 0.14 | 0.09 |
| ENR | 0.50 | 1.70 | 0.05 | 0.02 | 0.06 | 0.06 |
| Decarboxyl OFL | 0.62 | 1.78 | 0.09 | 0.04 | 0.11 | 0.08 |
| OFL methyl ester | 0.71 | 2.34 | 0.14 | 0.11 | 0.18 | 0.15 |
| OFL ethyl ester | 0.88 | 2.55 | 0.08 | 0.05 | 0.13 | 0.11 |
| CLI | 0.15 | 0.47 | 0.04 | 0.03 | 0.07 | 0.04 |
| SMX | 0.40 | 1.30 | 0.03 | 0.03 | 0.09 | 0.04 |
| STZ | 0.28 | 0.90 | 0.07 | 0.05 | 0.11 | 0.05 |
| SMT | 0.50 | 1.70 | 0.12 | 0.08 | 0.09 | 0.07 |
| SDI | 0.38 | 1.30 | 0.09 | 0.07 | 0.13 | 0.07 |
| SMZ | 0.17 | 0.60 | 0.06 | 0.03 | 0.08 | 0.04 |
| SDM | 0.20 | 0.72 | 0.05 | 0.04 | 0.10 | 0.05 |
| N-Acetyl SMX | 0.35 | 1.24 | 0.04 | 0.04 | 0.06 | 0.05 |
| TMP | 0.10 | 0.37 | 0.05 | 0.03 | 0.08 | 0.04 |
| TMP306 | 0.42 | 1.42 | 0.04 | 0.03 | 0.07 | 0.05 |
| Keto TMP | 0.31 | 0.99 | 0.07 | 0.05 | 0.10 | 0.07 |

### 2.3.3.2. Recoveries and precision of the method

The recovery values and precision of the developed method are shown in Table 2.3. The absolute recovery values for the selected compounds in all investigated matrices (lettuce, tomato, broad bean, and cauliflower) ranged from 30 (broad bean) to 125% (cauliflower) for the lower concentration level and from 41 (broad bean) to 118% (tomato) for the higher. Method precision, expressed as a RSD of the recoveries (n=3) at higher spike level, ranged from 1 to 20%. The highest RSD (20%) was obtained for ENR in lettuce, whereas for the rest of the compounds in all matrix types, the RSD values were below 20% for the higher spike level. While for sulfonamides, more specifically STZ, SMT, SDI and SDM, RSDs at lower spiking level were relatively higher (up to 27%) in lettuce and broad beans. These RSD values are comparable to those (10–26%) obtained when ABs are determined in lettuce using pressurized liquid extraction [112]. According to guideline SANTE/11813/2017 lower recovery values (30–70%) are acceptable in case of their proven consistency (RSD< 20%), in that regard for CLI in tomato fruits and broad beans requirements were not fulfilled.

For all investigated compounds and matrices, absolute recovery values ranged from 40 to 118% at the highest spiking level, while those values ranged from 30 to 125% at the lowest spiking level. After correcting the recoveries using surrogate standards, relative recoveries for both, lower and higher spiking level, were in the range from 47 to 118% with RSDs less than 20%. Explicitly, relative recoveries were determined using the following equation:

$$\text{relative recovery } (\%) = \left({}^{\text{AAR}}/_{\text{ASR}}\right) \times 100 \quad (5)$$

where AAR is absolute analyte recovery and ASR is absolute surrogate recovery. Absolute recoveries for tomato ranged from 40 to 118% (higher spiking level) and from 35 to 90% (lower spiking level), including 10 and 7 compounds in the range from 70 to 120% at the higher and lower concentration level, respectively. Relative recovery values for the tomato fruits were between 60 and 109% for the higher concentration level and between 40 and 93% for the lower concentration level. The recovery results obtained are in the similar range as those reported in the literature, but are compound-dependent. For instance, Wu et al. [113] and Riemenschneider et al. [129] reported lower recovery values (10 and 50%, respectively) for SMX, while two other studies [40,98] reported higher recovery values (74%). For fluoroquinolones in tomato fruits, Li et al. [130] reported recoveries higher than 78% for ENR, applying an analytical method specific to fluoroquinolones. With regard to TMP, Wu et al. [113] reported poor recovery value (18%), while Christou et al. [98] reported the highest one (91%).

Table 2.3. Absolute and relative (in parenthesis) recovery values and standard deviation (n=3) of target compounds in different vegetable matrices at two spike levels of 10 ng $g^{-1}$ dw (L) and 100 ng $g^{-1}$ dw (H), MDLs and MQLs

| Compound | Lettuce | | Tomato | | Broad bean | | Cauliflower | |
|---|---|---|---|---|---|---|---|---|
| | L | H | L | H | L | H | L | H |
| OFL | 40±6 | 51±5 | 56±10 | 118±1 | 36±7 | 41±5 | 65±12 | 87±8 |
| | (66±9) | (81±6) | (59±7) | (117±1) | (117±15) | (118±3) | (109±4) | (100±1) |
| ENR | 39±7 | 50±10 | 35±2 | 57±9 | 30±4 | 54±6 | 125±19 | 118±18 |
| | (64±8) | (80±6) | | | | | | |
| Decarboxyl OFL | 54±6 | 63±7 | 65±7 | 73±5 | 38±7 | 48±7 | 65±12 | 70±13 |
| | (89±5) | (99±6) | | | | | | |
| OFL methyl ester | 51±6 | 66±5 | 72±11 | 80±4 | 44±9 | 52±3 | 61±7 | 74±6 |
| | (84±11) | (104±9) | | | | | | |
| OFL ethyl ester | 44±6 | 56±5 | 63±6 | 79±3 | 42±8 | 51±5 | 55±8 | 60±3 |
| | (72±10) | (88±4) | | | | | | |
| CLI | 68±14 | 88±16 | | | | | 68±9 | 76±10 |
| | (70±13) | (97±9) | | | | | (96±3) | (103±3) |
| SMX | 65±11 | 70±8 | 59±9 | 65±4 | 44±8 | 49±7 | 65±10 | 68±5 |
| | (85±13) | (92±8) | (72±7) | (99±1) | (82±12) | (82±1) | (94±8) | (94±3) |
| STZ | 45±11 | 68±8 | 70±11 | 72±10 | 60±14 | 66±8 | 51±3 | 53±2 |
| | (60±8) | (92±2) | (85±9) | (109±4) | | | (73±4) | (75±3) |
| SMT | 42±9 | 65±8 | 55±7 | 60±4 | 42±11 | 43±7 | 46±6 | 66±4 |
| | (55±6) | (87±2) | (67±3) | (91±2) | (78±4) | (78±3) | (67±5) | (91±3) |
| SDI | 122±31 | 73±12 | 81±5 | 91±11 | 69±15 | 80±6 | 64±2 | 66±2 |
| | | | | | | | (91±5) | (95±3) |
| SMZ | 75±13 | 84±8 | 75±7 | 90±11 | 65±12 | 76±7 | 63±7 | 64±2 |
| | (99±13) | (110±5) | | | | | (89±5) | (91±4) |
| SDM | 38±8 | 48±6 | 38±1 | 40±2 | 49±13 | 52±10 | 49±8 | 53±6 |
| | (49±9) | (63±8) | (47±2) | (60±1) | (91±7) | (93±6) | (74±6) | (74±4) |
| N-Acetyl SMX | 61±5 | 67±6 | 57±9 | 65±2 | 61±10 | 71±9 | 62±11 | 73±14 |
| | (81±7) | (87±4) | (69±7) | (97±2) | | | (89±10) | (101±9) |
| TMP | 118±23 | 111±16 | 90±14 | 96±13 | 53±10 | 60±11 | 58±7 | 74±4 |
| | (92±4) | (94±2) | (93±5) | (98±3) | (76±3) | (84±2) | (93±2) | (98±1) |
| TMP306 | 62±9 | 71±6 | 77±9 | 84±7 | 55±9 | 63±6 | 54±7 | 65±4 |
| | | | (80±11) | (86±3) | (79±7) | (82±3) | (86±9) | (86±6) |
| Keto TMP | 44±8 | 59±10 | 71±4 | 76±2 | 41±4 | 49±5 | 46±8 | 56±7 |
| | | | (73±5) | (78±1) | (59±4) | (69±4) | (73±10) | (74±7) |

(continuation) Table 2.3

| Compound | Lettuce | | Tomato | | Broad bean | | Cauliflower | |
|---|---|---|---|---|---|---|---|---|
| | L | H | L | H | | L | H | L |
| | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) |
| OFL | 4.2 | 6.3 | 0.2 | 0.4 | 1.9 | 3.5 | 5.1 | 9.2 |
| Decarboxyl OFL | 0.9 | 1.4 | 0.2 | 0.7 | 0.8 | 1.7 | 3.3 | 6.3 |
| OFL methyl ester | 3.8 | 6.2 | 1.4 | 2.9 | 0.7 | 1.6 | 2.2 | 4.6 |
| OFL ethyl ester | 5.5 | 8.2 | 2.2 | 4.1 | 1.1 | 2.5 | 2.4 | 5.5 |
| ENR | 5.8 | 7.6 | 0.5 | 0.9 | 2.6 | 3.2 | 4.0 | 7.4 |
| CLI | 1.0 | 1.5 | | | | | 1.5 | 4.4 |
| SMX | 1.6 | 2.3 | 0.4 | 0.5 | 1.4 | 2.8 | 1.3 | 3.9 |
| STZ | 2.7 | 3.6 | 0.4 | 0.6 | 1.9 | 2.7 | 1.2 | 2.3 |
| SMT | 0.7 | 1.3 | 0.2 | 0.2 | 3.3 | 5.5 | 0.6 | 1.4 |
| SDI | 1.7 | 6.6 | 0.2 | 0.4 | 1.9 | 3.5 | 1.1 | 3.4 |
| SMZ | 2.5 | 3.9 | 0.4 | 0.8 | 0.5 | 1.0 | 2.9 | 5.8 |
| SDM | 3.4 | 5.7 | 0.7 | 1.1 | 0.8 | 0.9 | 1.2 | 3.5 |
| N-Acetyl SMX | 2.3 | 3.3 | 1.8 | 2.4 | 1.1 | 2.5 | 2.7 | 7.1 |
| TMP | 0.6 | 1.1 | 0.1 | 0.2 | 1.8 | 3.4 | 2.6 | 6.4 |
| TMP306 | 1.4 | 2.6 | 0.4 | 0.8 | 1.3 | 2.4 | 2.1 | 5.4 |
| Keto TMP | 1.1 | 2.1 | 0.5 | 0.9 | 0.7 | 1.5 | 1.7 | 3.6 |

For the lettuce matrix, absolute recoveries were in the range of 48–111% and 38–122% at the higher and lower spiking concentration levels, respectively. Relative recovery values for lettuce leaves ranged from 49 to 99% at the lowest concentration level and from 63 to 110% at the highest. Pan et al. [110], combining acidified acetonitrile (pH 3) and acetone as an extraction solvent for UAE and the SPE clean-up step, obtained higher recoveries for SMZ (98%) than those presented here. The pressurized liquid extraction technique was applied by Jones-Lepp et al. [112], but reported value for CLI (30%) was lower than the one obtained in the present study. For TMP, both Holmes et al. [131] and Wu et al. [113] obtained 57%, which is lower than in the present study.

For 10 compounds in broad beans, recoveries ranged from 50 to 80% at the higher concentration level. In contrast, at the lower concentration level, recovery values were lower than 50% for 9 studied compounds. However, in the case of relative recoveries, obtained

values were more satisfactory, ranging from 69 to 118% and from 59 to 106% for higher and lower spike levels, respectively.
Absolute recoveries for cauliflower were slightly better than for broad bean. Namely, for 7 tested compounds, at higher spiking level, recoveries were between 70 and 116%, while at lower spiking level recovery values were below 50% for 3 compounds. Relative recovery values ranged from 67 to 96% and from 74 to 103% for lower and higher spiking levels, respectively.

Additionally, effect of particular vegetable type on AB group was evaluated. More specifically, for sulfonamide group, 3 out of 7 targeted compounds (consisting of 6 parent ABs and N-Acetyl SMX) recovery values were reported in the range of 70–120% with satisfactory RSDs (< 20%) in tomato, while relatively poorer recoveries (cauliflower) or RSDs (lettuce and broad bean) were observed in other types of studies matrices. For fluoroquinolone group consisting of OFL, ENR and three OFL's metabolites both, cauliflower and tomato showed the best results with 4 out of 5 compounds detected in 70–120% range, while for lettuce and broad bean lower recovery values were observed. Finally, for TMP and it's 2 metabolites, since RSD values were in acceptable range for all matrix-compound combinations, nuanced, descending order based on recoveries was: tomato>lettuce>cauliflower>broad bean. Due to the aforementioned, we could conclude that the composition of broad bean seeds affected extraction greatly for the given groups of compounds while tomato fruits, among tested vegetables, did slightly.

#### 2.3.3.3. Matrix effect

Co-extracted components from the matrix usually affect ESI by suppressing or enhancing it in an unpredictable fashion compromising quantitative data. Since it is both matrix- and compound-dependent, it has to be estimated [132]. The results obtained for all vegetable matrices are presented in Figure 2.2. Negative matrix effect values indicate signal suppression, while positive ones indicate signal enhancement. As shown in Fig. 2.2, except for OFL (-26%), OFL methyl ester (27%), OFL ethyl ester (30%), SDM (23%) and keto TMP (32%), the matrix effect in lettuce was lower than 15%. Similar trend was observed for tomato. For the majority of compounds, matrix effect of tomato was lower than 18%, except for OFL methyl ester (35%), OFL ethyl ester (29%), SDM (34%) and keto TMP (38%). In the case of broad beans, matrix effect for ENR (21%), OFL methyl ester (33%), OFL ethyl ester (34%), N-Acetyl SMX (-25%) and keto TMP (26%) was higher than 20%, whereas for the rest of the compounds it was lower than 18%. Among tested vegetables, cauliflower matrix affected the

biggest number of selected compounds, such as ENR (47%), OFL methyl ester (20%), OFL ethyl ester (34%), CLI (22%), SDM (23%) and keto TMP (37%) more than 20%. The lowest matrix effect values (< 10%) in all types of vegetable matrices were estimated for TMP.

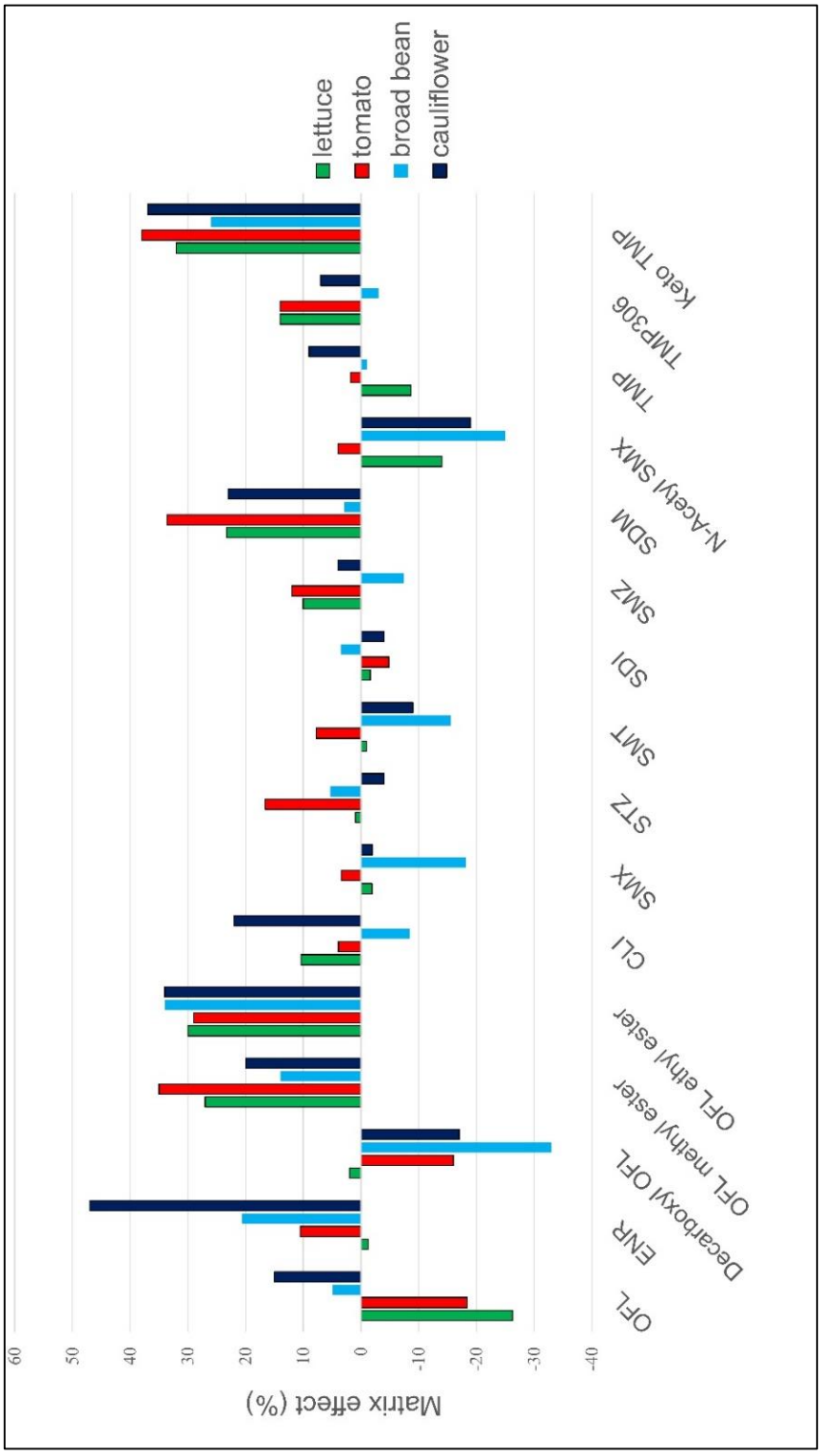



Figure 2.2. Matrix effect of 4 vegetable matrices (lettuce, tomato, broad bean and cauliflower)

For the sulfonamides class, estimated values were lower than 19%, except for SDM in tomato fruits, lettuce leaves, and cauliflower inflorescences and N-Acetyl SMX in broad beans. Fluoroquinolones in the presented data set showed diverse interaction in compound – matrix relation ranging from low (-1%) to high (47%) influence of the matrix, whereas CLI appears to be moderately affected compound only by cauliflower matrix. Only few publications studied matrix effects in vegetable matrices on the LC-MS/MS antibiotic response. Namely, He et al. [95] reported matrix effect in cabbage for 49 ABs (i.e., around 50% for SDI and STZ, more than 25% for SMT, SMX and OFL and around 10% for ENR). Hu et al. [83] who studied veterinary antimicrobials in vegetables by a modified QuEChERS procedure reported higher matrix effect for SMZ (> 50%) and CLI (~ 20%) and similar matrix effect for ENR (< 5%) in lettuce compared to the values obtained in the presented study. Both aforementioned studies included various types of vegetables (i.e., cabbage, white radish, Chinese cabbage, cucumber, string bean and green pepper) and targeted ABs that differ from the ones that were included in this study. Thus, comparison of each individual analyte/matrix combinations is feasible just in a small number of combinations. Finally, if matrix effects are less than 20%, it is defined as soft, between 20 and 50% as medium and above 50% as strong. It can be seen that presented method successfully reduce matrix effect in a way that strong suppression or enhancement (> 50%) was not observed for any of the targeted analytes.

#### 2.3.3.4. Method detection and quantification limits

The MDLs for the targeted compounds were in the range of 0.6–5.8 ng $g^{-1}$ dw, 0.1–2.2 ng $g^{-1}$ dw, 0.5–3.3 ng $g^{-1}$ dw, and 0.6–5.1 ng $g^{-1}$ dw for lettuce, tomato, broad beans, and cauliflower, respectively (Table 2.3). The MQLs varied between 1.1 and 8.2 ng $g^{-1}$ dw for lettuce, 0.2 and 4.1 ng $g^{-1}$ dw for tomato, 0.9 and 5.5 ng $g^{-1}$ dw for broad beans, and 1.4 and 9.2 ng $g^{-1}$ dw for cauliflower. The highest MDLs were obtained for ENR (5.8 ng $g^{-1}$ dw) and OFL ethyl ester (5.5 ng $g^{-1}$ dw) in lettuce and OFL (5.1 ng $g^{-1}$ dw) in cauliflower, while for the rest of the tested compounds, the MDLs were less than 5 ng $g^{-1}$ dw. The MDLs differed from class to class, but also from compound to compound within a single AB class. For instance, in lettuce, OFL ethyl ester and ENR among fluoroquinolone class exhibited the highest values, of which ENR was the highest of all tested compounds, while on contrast MDL for Decarboxyl OFL was 0.9 ng $g^{-1}$ dw. Among sulfonamides, SDM showed the highest value in lettuce matrix, while for the rest of the class, together with TMP, its metabolites and CLI, the MDLs were less than 2.7 ng $g^{-1}$ dw. In another study comparing different methods, Sallach et al. [132] obtained the highest MDL values for ciprofloxacin (15 ng $g^{-1}$ fw or ca. 300 ng $g^{-1}$ dw) which belong to the class of fluoroquinolones when mechanical maceration was applied to lettuce leaves and

for SMX (24 ng $g^{-1}$ fw or ca. 480 ng $g^{-1}$ dw) when tissue sonication was applied. Other authors reported lower MDL values for SMX in lettuce leaves, namely 0.5 ng $g^{-1}$ dw [113] and < 0.2 ng $g^{-1}$ dw [129]. The MDLs for TMP presented in this study are in the same low ng $g^{-1}$ range as published by Holmes et al. [131] (0.7 ng $g^{-1}$ dw) and Wu et al. [113] (0.3 ng $g^{-1}$ dw). In this regard, the analytical methodology developed in the present study achieved an MDL for SDI (1.7 ng $g^{-1}$ dw) 10 times lower than that reported by Holmes et al. [131]. However, in the same study, the MDL for ENR was lower (1.6 ng $g^{-1}$ dw) than in the present study (5.8 ng $g^{-1}$ dw). Low ng $g^{-1}$ level of MDL values was achieved for tomato fruits in case of all studied compounds, with the highest value for OFL ethyl ester of 2.2 ng $g^{-1}$ dw. With regard to the MDL for SMX in tomato fruits, 0.44 ng $g^{-1}$ dw has been shown to be sufficiently low to determine SMX in real samples, even though some previously published reports [40,129] have achieved lower values (< 0.2 ng $g^{-1}$ dw and 0.1 ng $g^{-1}$ dw). The MDL of ENR (0.49 ng $g^{-1}$ dw) in tomato was higher than the values published by Li et al. [130] (< 0.1 ng $g^{-1}$ dw). In the event of broad bean seeds for only two compounds, SMT (3.3 ng $g^{-1}$ dw) and ENR (2.6 ng $g^{-1}$ dw), MDLs were higher than 2 ng $g^{-1}$ dw. In case of cauliflower, MDLs except for fluoroquinolone class of compounds (2.2–5.1 ng $g^{-1}$ dw) were lower than 3 ng $g^{-1}$ dw. In summary, the sensitivity of the developed multi-residue method was consistent with that reported for other methods in the literature, but for the first time we included the determination of different AB metabolites achieving similar low MDL values.

## 2.4. Application to incurred AB in crops

The developed method was successfully applied to the analysis of vegetable samples grown in the peri-urban area of the city of Barcelona, irrigated with RWW. Three samples of four kinds of vegetables (lettuce, tomato, broad bean, and cauliflower) were analysed using the method described above. Among 16 target compounds OFL and SMX were detected in all tested vegetable matrices (Table 2.4).

OFL was the compound detected at the highest concentration level in lettuce leaves (25.2–37.8 ng $g^{-1}$ dw), while in cauliflower, tomato fruits and broad beans, OFL was detected in the ranges of 8.8–14.4 ng $g^{-1}$ dw, 2.1–8.0 ng $g^{-1}$ dw and 2.4–8.5 ng $g^{-1}$ dw, respectively. Likewise, the highest contamination levels of SMX were found in lettuce leaves (11.0 ng $g^{-1}$ dw), compared to the ones in tomato fruits (1.7–3.0 ng $g^{-1}$ dw), cauliflower (2.6 ng $g^{-1}$ dw), and broad bean seeds (1.7 ng $g^{-1}$ dw). ENR was detected in all vegetable types except cauliflower, at the highest concentration in lettuce leaves (12.7–17.3 ng $g^{-1}$ dw) followed by tomato fruits (10.4–11.3 ng $g^{-1}$ dw) and broad bean seeds (7.0 ng $g^{-1}$ dw). Decarboxyl OFL, this metabolite

was detected above MQL only in tomato fruits (1.5 ng $g^{-1}$ dw), while in broad bean and cauliflower the levels were below MQLs. TMP was detected in the 3.5–8.0 ng $g^{-1}$ dw range in cauliflower, 1.9–4.5 ng $g^{-1}$ dw range in lettuce leaves, and 0.5–0.7 ng $g^{-1}$ dw range in tomato fruits. Christou et al. [98] reported similar results for TMP (0.1–2.0 ng $g^{-1}$ dw) and slightly lower concentrations for SMX (0.2–1.0 ng $g^{-1}$ dw) in tomato fruits. Detected concentrations of OFL were higher than the ones reported by Hussain et al. [133] and Hu et al. [134] who analysed different types of vegetables than the ones in the presented study. Finally, it is important to remark that keto TMP was detected in lettuce leaves at concentrations (21.0–23.1 ng $g^{-1}$ dw) which were more than five times greater than those found for the parent compound (3.1–4.5 ng $g^{-1}$ dw). These results allow confirming that both ABs and their metabolites are occurring in commercially available vegetables and demonstrates the need for metabolites quantification in food.

Table 2.4. Concentration of detected compounds, in ng $g^{-1}$ dw, detected in lettuce, tomato, broad bean and cauliflower

| Compound | TMP | OFL | ENR | SMX | Keto TMP | Decarboxyl OFL |
|---|---|---|---|---|---|---|
| Sample | Lettuce leaves | | | | | |
| 1 | 3.1 | 32.0 | <MQL | nd | 21.0 | nd |
| 2 | 1.9 | 25.2 | 12.7 | 11.0 | <MQL | nd |
| 3 | 4.5 | 37.8 | 17.3 | nd | 23.1 | nd |
| | Tomato fruits | | | | | |
| 1 | 0.6 | 8.0 | 10.7 | 3.0 | nd | 1.5 |
| 2 | 0.7 | 2.1 | 11.3 | 1.7 | nd | nd |
| 3 | 0.5 | 7.1 | 10.4 | 2.1 | nd | nd |
| | Broad bean seeds | | | | | |
| 1 | nd | 2.5 | <MQL | <MQL | nd | <MQL |
| 2 | nd | 2.4 | <MQL | <MQL | nd | <MQL |
| 3 | nd | 8.5 | 7.0 | 1.7 | nd | <MQL |
| | Cauliflower inflorescences | | | | | |
| 1 | 8.0 | 13.7 | nd | nd | nd | <MQL |
| 2 | 4.4 | 14.4 | nd | nd | nd | nd |
| 3 | 3.5 | 8.8 | nd | 2.6 | nd | nd |

nd – not detected

## 2.5. Conclusions

A specific, rapid, sensitive, and simple analytical method based on UAE, SPE clean-up, and LC-MS/MS has been successfully developed for the quantitative determination of 10 ABs belonging to 4 classes and 6 of their metabolites in crop samples. The developed method was validated for 4 matrices, namely, lettuce leaves, tomato fruits, cauliflower inflorescence, and broad bean seeds. The method yielded MDLs in the low ng $g^{-1}$ range for all studied compounds, providing a robust, reliable, simple-to-use tool that could prove useful for the routine analysis of incurred AB compounds in vegetable samples. The reported MDLs were sufficiently low to detect target compounds in vegetables, enabling the monitoring of ABs and their metabolites in real samples.

The preliminary analysis of vegetables irrigated with RWW shows that the occurrence of AB metabolites might exceed their parent concentration. Having in mind that usually parent compounds are included in environmental and human health risk assessments, we may conclude that underestimation is feasible. Therefore, presented analytical method responds to a need to reveal qualitative and quantitative occurrence of ABs and their metabolites, in order to better understand their fate and risks due to the reuse of WW or manure application in agriculture. Furthermore, additional pharmaceuticals can be detected in the same samples if suspect screening approach by using data independent acquisition HRMS is used instead of target screening method based on MS/MS.

## 2.6. Supplementary information

Supplementary Table S2.1. UAE – comparison two different solvent used for extraction (APB pH 7.5 and methanol) at 100 ng $g^{-1}$ dw spike level

| Compound | Absolute recovery (%) | |
|---|---|---|
| | APB pH 7.5 | methanol |
| OFL | 19 | 51 |
| ENR | 117 | 50 |
| CLI | 18 | 88 |
| SMX | 17 | 70 |
| STZ | 29 | 68 |
| SMT | 8 | 65 |
| SDI | 12 | 73 |
| SMZ | 16 | 84 |
| SDM | 27 | 48 |
| TMP | 85 | 111 |

Supplementary Table S2.2. Optimization of the elution solvent (SPE clean-up). Three collected fractions of eluent (E1, E2 and E3) and three different combinations.

| Compound | Recovery (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | E1 | E2 | E3 | E1 | E2 | E3 | E1 | E2 | E3 |
| | 2 ml MeOH | 1ml MeOH | 1 ml MeOH | 2 ml MeOH | 1 ml MeOH/ EtAc | 1 ml MeOH/ EtAc | 2 ml MeOH/ EtAc | 1 ml MeOH/ EtAc | 1 ml MeOH/ EtAc |
| OFL | 87 | 3 | 1 | 89 | nd | nd | 89 | nd | nd |
| ENR | 7 | 1 | 4 | 10 | 1 | 2 | 69 | 1 | 1 |
| CLI | 89 | 1 | 1 | 91 | nd | nd | 95 | 1 | 1 |
| SMX | 69 | 1 | 1 | 73 | 1 | 1 | 81 | 1 | 1 |
| STZ | 77 | 1 | 1 | 74 | 1 | 1 | 78 | 1 | 1 |
| SMT | 42 | 1 | 1 | 38 | 1 | 1 | 69 | 1 | 1 |
| SDI | 61 | nd | nd | 60 | nd | nd | 87 | nd | nd |
| SMZ | 84 | 1 | 1 | 86 | 1 | 1 | 96 | 1 | 1 |
| SDM | 73 | 1 | 1 | 68 | 1 | 1 | 75 | 1 | 1 |
| TMP | 75 | 1 | 1 | 79 | 1 | 1 | 91 | 1 | 1 |

nd – not detected

Supplementary Table S2.3. Optimization of the washing solvent (SPE clean-up). Two different washing solvent mixture ratios were tested (5% and 10% MeOH in water), since 5% showed better results, three different volumes (1, 2, and 3 ml) were tested

| Compound | Recovery (%) | | | |
|---|---|---|---|---|
| | W1 | W2 | W3 | W4 |
| | 1 ml 5% MeOH | 2 ml 5% MeOH | 3 ml 5% MeOH | 1 ml 10% MeOH |
| OFL | 84 | 76 | 69 | 65 |
| ENR | 57 | 41 | 34 | 39 |
| CLI | 94 | 87 | 84 | 84 |
| SMX | 78 | 71 | 64 | 69 |
| STZ | 77 | 71 | 68 | 72 |
| SMT | 69 | 60 | 57 | 59 |
| SDI | 89 | 78 | 71 | 62 |
| SMZ | 91 | 87 | 81 | 85 |
| SDM | 75 | 65 | 59 | 60 |
| TMP | 90 | 87 | 79 | 82 |

Supplementary Table S2.4. Optimized MS/MS parameters (retention time; cone voltage and collision energy) and precursor, quantification and qualification ions used for the MRM acquisition mode

| Compound | RT (min) | CV[a] (V) | Precursor ion | Quantification (CE[b]) | Qualification (CE[b]) |
|---|---|---|---|---|---|
| OFL | 3.75 | 30 | 362 | 261 (15) | 318 (25) |
| OFL-d3 | 3.75 | 40 | 365 | 261 (30) | 221 (20) |
| ENR | 4.00 | 30 | 360 | 316 (20) | 342 (20) |
| ENR-d5 | 4.00 | 30 | 365 | 245 (25) | 347 (25) |
| Decarboxyl OFL | 1.39 | 45 | 318 | 261 (25) | 221 (35) |
| OFL methyl ester | 2.01 | 45 | 376 | 287 (30) | 344 (25) |
| OFL ethyl ester | 3.25 | 45 | 390 | 287 (25) | 261 (30) |
| CLI | 4.25 | 40 | 425 | 126 (30) | 377 (20) |
| CLI-d3 | 4.25 | 50 | 428 | 129 (30) | 380 (20) |
| SMX | 5.04 | 30 | 254 | 156 (20) | 92 (25) |
| SMX-d4 | 5.04 | 30 | 258 | 160 (15) | 112 (25) |
| STZ | 3.31 | 30 | 256 | 156 (15) | 108 (20) |
| SMT | 4.39 | 30 | 271 | 156 (15) | 108 (20) |
| SDI | 2.57 | 30 | 251 | 156 (15) | 108 (20) |
| SMZ | 3.83 | 20 | 279 | 156 (20) | 124 (20) |
| SDM | 5.52 | 30 | 311 | 156 (20) | 108 (25) |
| N-Acetyl SMX | 5.22 | 35 | 296 | 93 (25) | 134 (15) |
| TMP | 2.39 | 40 | 291 | 261 (25) | 230 (30) |
| TMP-d3 | 2.39 | 50 | 294 | 364 (30) | 233 (35) |
| TMP306 | 1.54 | 40 | 307 | 289 (30) | 318 (25) |
| Keto TMP | 3.66 | 45 | 305 | 275 (25) | 244 (20) |

[a] Cone voltage

[b] Collision energy (eV)

## 2.7. Appendix to Chapter II

### Non-target screening of vegetable samples

#### Sample Analysis

Vegetable extracts, described in the Section 2.4, in addition of LC-MS/MS were analyzed with HRMS. Namely, samples collected from farm irrigated with RWW were injected in Orbitrap Fusion Lumos mass spectrometer. Detailed description of the instrumentation and chromatographic conditions are provided below in Section 4.2.2; spectrometry conditions were slightly different than the one described in Section 4.2.3 since for this study data independent acquisition was performed as well. Full scan acquisition was performed at a resolution of 60,000, in the mass range from 50 to 1000 Da. The data independent acquisition was performed using the detector at the same resolution and mass range, employing dynamic exclusion: if precursor with the same m/z was fragmented 4 times within 30 s, it is excluded for next 60 s. Isolation window was set to 2,000, applying higher energy collisional dissociation at 20, 30, and 60 eV.

#### Data treatment

Samples were screened for 2098 suspected emerging substances. Suspect list “S1 MASSBANK” was used and can be found in website of NORMAN Suspect list exchange (NORMAN Suspect list exchange: a central website to access various lists of substances for suspect screening. https://www.norman-network.com/?q=node/236). Data independent chromatograms were converted to mzML using msconvert module of Proteowizard software (https://www.nature.com/articles/nbt.2377) and the collision energy channels were separated using an in-house script (https://pubs.acs.org/doi/abs/10.1021/acs.est.8b00365). Peak picking using centWave algorithm (https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-9-504) with optimized parameters (https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-015-0562-8) for chromatography and mass spectrometry (ppm 5.45, min peakwidth 14, max peakwidth 60, snthresh 10, fitgauss TRUE) was used to find peaks in the data. Isotopic peaks and adduct peaks of $MS^1$ full-scan data were grouped (componentization) using non-target R-package (M. Loos (2016). Non-target: Detection isotope, adduct and homologue relations in LC-MS data. R package version 1.9. https://cran.r-project.org/web/packages/nontarget/index.html). Suspect list used included the major fragments of the suspected compounds. Tentative identification achieved in cases

which molecular ion was detected with mass accuracy below 2 ppm and at least 2 qualifier fragment ions were detected.

**Results and discussion**

Thirteen emerging substances mainly belonging to pesticides and pharmaceuticals were detected in at least one sample type (Table A2.1). As presented none of ABs or their metabolites quantified with LC-MS/MS was detected with HRMS. Under given conditions, data independent acquisition showed lower sensitivity than conventional triple quadrupole but allowed the detection of major components not detected in MS/MS. In this regard, this suspect screening HRMS is complementary to targeted MS/MS analysis.

Table A2.1. Detected emerging substances and their class in the investigated vegetables irrigated with reclaimed water (cauliflower, lettuce, tomato, and broad bean)

| Compound | Class | Cauliflower | Lettuce | Tomato | Broad bean |
|---|---|---|---|---|---|
| Paracetamol | Analgesic drug | + | + | + | + |
| Ibuprofen | NSAIDs | + | + | + | + |
| Varenicline | Drug for nicotine addiction | | + | + | + |
| 8-Hydroxyquinoline | Disinfectant | | + | + | + |
| Penciclovir | Antiviral drug | + | + | | |
| Metolachlor OXA | Herbicide metabolite | | + | | |
| Aciclovir | Antiviral drug | | + | | |
| Antipyrine | Analgesic drug | | | + | |
| Atenolol | Antihypertensive drug | | | + | |
| Propranolol | Antihypertensive drug | | | | + |
| Dimethyl phthalate | Insect repellent | | | | + |
| O-Desmethyl venlafaxine | Antidepressant metabolite | | | | + |
| Codeine | Analgesic drug | | | | + |

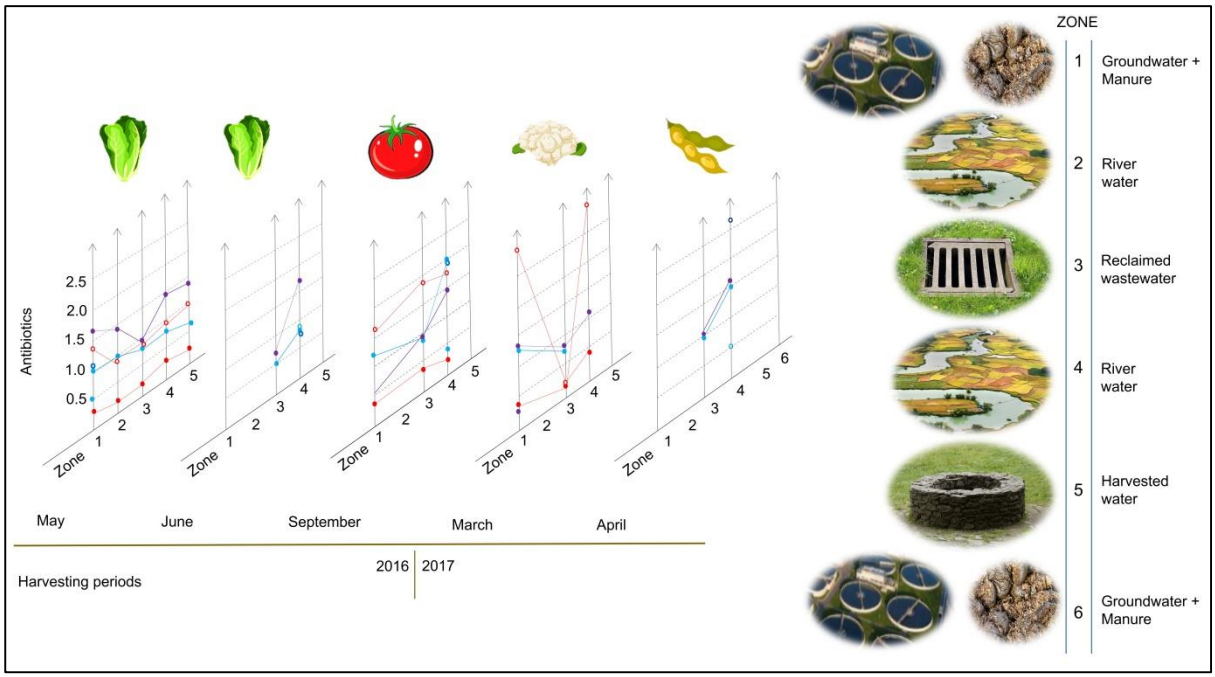
The analgesic paracetamol and the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen were detected in all collected vegetables, which make their occurrence of concern. Moreover, the drugs varenicline used as replacement of nicotine and the disinfectant 8-Hydroxyquinoline were detected in lettuce, tomato and broad bean while remained undetected in cauliflower. The antiviral drug penciclovir was detected in lettuce and cauliflower. The rest eight compounds were detected sporadically. The herbicide Metolachlor OXA was detected in

lettuce and the insect repellent dimethyl phthalate was detected in broad bean. Occurrence of these pesticides in vegetables may originate from RWW and from the used agricultural practice. The analgesic drugs antipyrine and codeine were detected in tomato and cauliflower respectively. Two antihypertensive pharmaceuticals atenolol and propranolol were detected in tomato and cauliflower respectively. In total, eight compounds were detected in broad bean, seven in lettuce, six in tomato and three is cauliflower. Additional 46 naturally occurring compounds were detected in vegetables, e.g., amino acids, vitamins, plant hormones, neurotransmitters, and nucleoside (data not presented) demonstrating that developed method is suitable for other target compounds as well.

# Chapter III: Monitoring of antibiotics and their metabolites in field grown vegetables

This Chapter is based on the article under review:

Đorđe Tadić, Maria José Bleda Hernandez, Francisco Cerqueira, Víctor Matamoros, Benjamin Piña, Josep Maria Bayona. Occurrence and human health risk assessment of antibiotics and their metabolites in vegetables grown in field-scale agricultural systems. Journal of Hazardous Material



***In brief:***

The occurrence of ABs in four types of commercially grown vegetables (lettuce leaves, tomato fruits, cauliflower inflorescences, and broad bean seeds) was analyzed to assess the human exposure and health risks associated with different agronomical practices. Out of 16 targeted AB residues, seven ABs belonging to three groups (i.e., fluoroquinolones, sulfonamides, and diaminopyrimidine) were above the method detection limits in vegetable samples ranging from 0.09 ng $g^{-1}$ fw to 3.61 ng $g^{-1}$ fw. Data analysis (quantile regression models, principal component and hierarchical cluster analysis) showed manure application, irrigation with river water (indirect WW reuse), and vegetable type to be the most significant factors for AB occurrence in the targeted crops. Metabolites were detected in 70 of the 80 vegetable samples analyzed, and their occurrence was both plant- and compound-specific. In 73% of the total samples, the concentration of AB metabolites was higher than the concentration of their parent compound. Finally, the potential human health risk estimated using the HQ approach, based on the acceptable daily intake and the estimated daily intake, showed a negligible risk for human health from vegetable consumption. Nevertheless, further studies are needed to assess the risks of AB resistance promotion in vegetables and the significance of the occurrence of their metabolites.

## 3.1. Introduction

The occurrence and potential effects of ABs in different environmental compartments is an emerging issue worldwide [13,14,19]. ABs enter aquatic and terrestrial ecosystems by different pathways [25]. The partial removal of ABs in conventional WWTPs leads to the occurrence of significantly high concentrations in treated effluents and sewage sludge [85,135,136]. Moreover, the use of ABs in veterinary applications leads to their occurrence in manure and slurries [137]. Accordingly, the application of manure, biosolids, or RWW in agriculture leads to the introduction of ABs in agricultural soil. Indeed, the expanding market of organic food and the circular economy promoted by the EU give rise to an increase in the application of manure-based organic fertilizers [138]. Additionally, agriculture is the first sector in RWW reuse by volume, and this reuse is expected to increase in the coming decades [20,22].

Plant uptake and translocation of ABs have been proven in studies conducted in controlled conditions [45,90,139–145]. These studies provide fundamental knowledge for further understanding the key factors affecting AB uptake and translocation. Moreover, assessments conducted in real, non-controlled conditions with manure and/or biosolid amendments in soil have demonstrated AB uptake in those agricultural practices as well [134,146–148]. Similarly, several authors have confirmed AB uptake as a consequence of RWW application, under real-field or simulated real-field conditions [98,110,149–151]. However, specific studies focusing on the occurrence of ABs in agroecosystems according to different agricultural practices, which are essential for realistic human health risk assessments, are lacking. Furthermore, ABs may exert a continuous selective pressure on soil microbiomes for extended periods, selecting those microorganisms with ARGs [152–154]. ARGs may be transmitted to potential pathogens through horizontal gene transfer mechanisms, perpetuating the dissemination of AB resistance in the environment and exacerbating this public health concern [155]. Additionally, fresh edible vegetable parts ultimately intended for human consumption could potentially act as reservoirs of AB resistance [156–158].

The occurrence of AB TPs and AB metabolites has been reported in WW effluents [108,115] and animal-related matrices [116,117], but similar knowledge in vegetables is scarce. The occurrence of AB TPs has drawn attention because of their potential toxicity [159], residual antimicrobial activity [105], and promotion of AB resistance. Recent findings on the extensive metabolization of SMX [50,52] in *Arabidopsis thaliana*, provide an additional reason for widening the range of commonly analyzed ABs.

ABs not only affect bacteria but can also elicit hitherto unknown effects in non-targeted organisms [160]. The human health effects of ABs are generally manifested in two ways: first, through adverse drug reactions (e.g., hypersensitive reactions, protracted toxic effects due to long-term exposure to ABs, or abnormal digestion); and, second, through the potential prevalence of AB resistance as a result of the exertion of selective pressure on bacteria of clinical importance [89,150]. Owing to their low concentrations in vegetables, ABs adverse effects are inconsequential in comparison to the possibility of AB resistance promotion. Andersson and Hughes [161] highlight that bacteria are often exposed to sub-lethal (subinhibitory) concentrations of ABs in humans, animals, and the environment. Consequently, it is essential to establish the subinhibitory concentrations (minimum selective concentration) of ABs that can select for resistant bacteria.

With regard to the human health risk, a holistic approach is needed to evaluate environmental exposure to ABs for quantitative risk assessment, as the occurrence of ABs in vegetables is one of the most important daily intake pathways [16]. Another approach to evaluate midterm exposure is to analyze ABs in human urine. Several studies suggest that, in adults, long-term (chronic) exposure to low doses, rather than short-term high-dose exposure (acute exposure) is prevalent [67–69,125]. Therefore, the primary exposure is though AB residues in food and/or drinking water, especially in the case of veterinary ABs.

To investigate the concentration level of ABs in vegetables and the human health risk, four types of vegetables (i.e., lettuce leaves, tomato fruits, cauliflower inflorescences, and broad bean seeds) were collected from six different commercial farm plots. For the sake of comprehensiveness, the farm plots were carefully selected to ensure that the main sources of ABs (irrigation water and manure application) varied among them. To the best of the authors' knowledge, this is the first survey to include AB metabolite determination in vegetables grown in real agricultural systems and miscellaneous environmental factors, including irrigation water quality, manure application, and crop types. It thus offers a realistic assessment of the occurrence and potential effects of ABs in commercially available vegetables.

## 3.2. Material and methods

### 3.2.1. Chemicals

Information about chemicals is provided in Section 2.2.1.

### 3.2.2. Environmental setting

Four types of vegetable matrices, namely, lettuce leaves (*Lactuca sativa* L.), tomato fruits (*Solanum lycopersicum* L.), cauliflower inflorescence (*Brassica oleracea* L.), and broad bean seeds (*Vicia faba* L.), were harvested from six locations (NE Spain). As presented in Figure 3.1, four zones (Z2-Z5) were in the Llobregat River Delta and its lower valley. Zone 1 (Z1) was located in the littoral mountains west of the Llobregat Delta, and Zone 6 (Z6) was located northeast of the Barcelona metropolitan area (Cabrera de Mar).

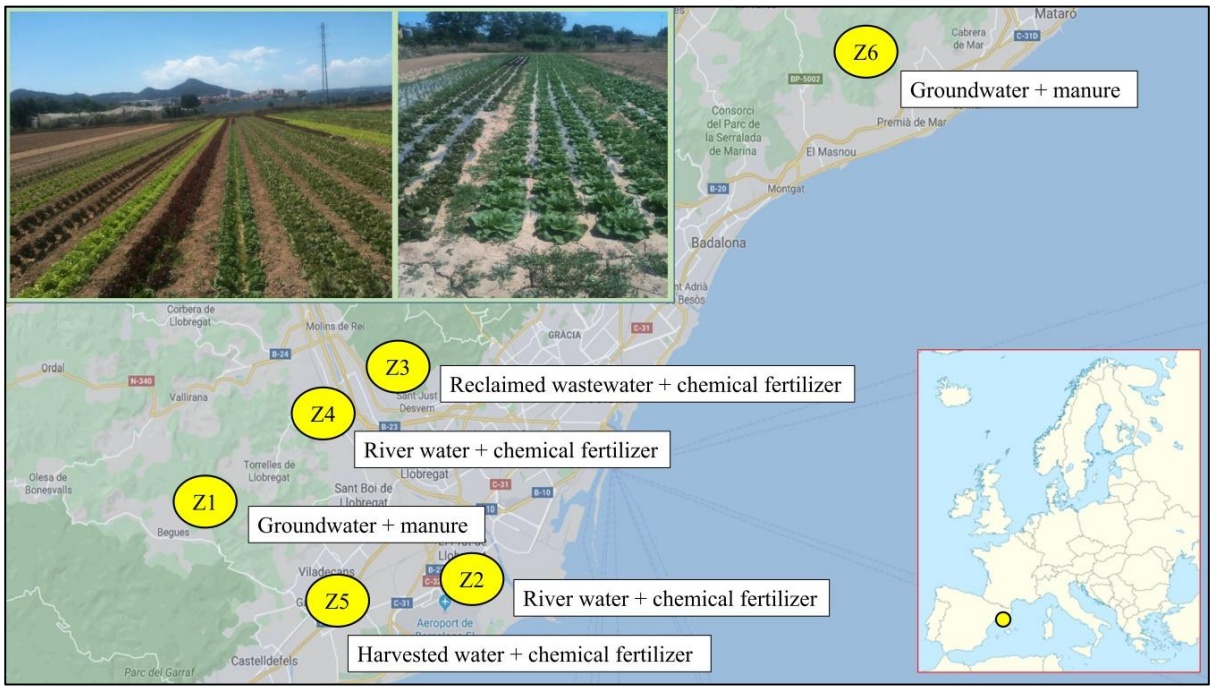



Figure 3.1. Locations of the six farm plots where crops were collected in NE Spain and types of irrigation water and fertilizer used

The sampling sites were primarily selected based on their irrigation sources. Z1 and Z6, both rural organic farming plots, were irrigated with groundwater and/or harvested rainwater. Due to the pristine water source, these two sites were selected as a reference in terms of water quality. The irrigation water for site 3 was RWW originating from the Infanta Channel, which is mostly made up of WWTP effluents (Rubí Creek). Z2 and Z4 were irrigated with river water, which is an indirect WW reuse, since the Llobregat River and its tributaries receive discharges from 60 urban and industrial WWTPs [23]. Z5 was irrigated with well water impacted by industrial and agricultural runoff. Z2-Z5 were chemically fertilized when needed, while Z1 and Z6 were fertilized with horse and pigeon manure, respectively. Detailed information regarding the irrigation water and soil characteristics of the sampling zones can be found elsewhere [118,162]. More information about the sampling strategy is provided in the Sampling information of the Chapter. Briefly, each farm zone was divided into 5 sections, and 5 to 15 vegetable specimens were collected per section. For each section, a composite sample was obtained; thus, 5 composite subsamples were obtained per zone (Table S3.1). All analyzed samples (n=80) represent real-field, commercially available vegetables. All the vegetable types were grown and harvested in Z3 and Z4. Thus, the comparison of AB content in the different vegetable types was performed in these 2 zones. In addition, lettuce samples were collected twice in Z3 and Z4, representing spring and summer samples, so Z3s and Z4s denote samples grown during summer months.

### 3.2.3. Analytical procedures

The occurrence of ABs and their metabolites in vegetable matrices at the required trace level was investigated by applying analytical method described in Sections 2.2.2., 2.2.4., and 2.2.5. The method MDLs and MQLs ranged from 0.02 ng $g^{-1}$ fw to 0.73 ng $g^{-1}$ fw and from 0.04 ng $g^{-1}$ fw to 1.22 ng $g^{-1}$ fw, respectively (Table S3.2). For non-detected cases (<MDL) and values between the MDL and MQL (<MQL), MDL/2 and MQL/2 censored values were used, respectively. All data reported in this Chapter are expressed on a fw basis as human health risk assessment of ingested vegetables through their daily intake was attempted in this Chapter.

### 3.2.4. Estimated daily intake and risk assessment

The estimated daily intake (EDI) (µg kg $bw^{-1}$ $day^{-1}$) was calculated according to Equation 6, where DI (g $day^{-1}$) and BW (kg) are the daily intake of edible parts of vegetables and body weight, respectively. The DI for fresh vegetables in Spain was taken from the European Food Safety Authority's Comprehensive Food Consumption Database (Table S3.3). The average BWs of a Catalan male adult (20–65 years) and child (4–9 years) were used, i.e. 70 and 24 kg, respectively. Ce (µg $g^{-1}$ fw) indicates the average AB concentration in vegetables.

$$\mathrm{EDI} = \frac{\mathrm{DI} \times \mathrm{Ce}}{\mathrm{BW}} \quad (6)$$

The potential risk was estimated using the HQ approach [163,164]. The HQ is the ratio between the EDI and threshold levels defined as acceptable daily intakes (ADIs). ADI (µg kg $bw^{-1}$ $day^{-1}$) values from a dataset based on microbiological and toxicological endpoints previously compiled by Wang et al. [69] (Table S3.4) were used. The hazard index (HI) is estimated as the sum of individual HQs. If HI is less than 1, the risk is generally deemed to be acceptable.

### 3.2.5. Data analysis

To analyze the relationship between each OFL, ENR, decarboxyl OFL, OFL methyl ester, SMX, TMP, and keto TMP concentration and the independent variables, namely, sample type, area, production cycle duration, planting quarter, irrigation water type, fertilizer, and soil texture, quantile regression models (univariate and multivariate) using the $75^{th}$ percentile as a measure of interest were performed using the statistical software STATA 15.1 (StataCorp. 2017. *Stata: Release 15*. Statistical Software. College Station, TX: StataCorp LLC) (Table S3.7). Principal component analysis (PCA) was used to visualize the differences among AB concentrations in different vegetables and zones. The factors with eigenvalues greater than 1 were selected as the principal components (PCs), explaining most of the variance in the dataset. We performed Hierarchical Agglomerative Clustering Analysis (HCA) using the Euclidean distance as metric and Ward's minimum variance (minimal total intra-cluster variance) as linkage criteria. Data were log-transformed and normalized prior to the PCA and HCA. PCA, HCA, and stack bar charts were performed in the R environment (version 3.6.2; http://www.r-project.org/), using the pca3d (https://CRAN.R-project.org/package=pca3d), factoextra (https://cran.r-project.org/web/packages/factoextra/index.html) and ggplot2 (https://CRAN.R-project.org/package=ggplot2) packages, respectively.

The ARG loads in the edible part of the vegetables for three different crops were taken from previously published data in this area [156–158]. Namely, selected ARGs of clinical relevance (*sul*1, $bla_{TEM}$, $bla_{CTX\text{-}M\text{-}32}$, *mec*A, *qnrS*1, *tet*M, $bla_{OXA\text{-}58}$) and *intI*1 as a proxy for anthropogenic pressure were quantified by quantitative real-time polymerase chain reaction. In order to investigate possible relationships between detected ABs and ARGs, canonical-correspondence analysis (CCA) was used. CCA was performed with lettuce leaves from Z1, Z3, Z4, and Z5, tomato fruits from Z1 and Z4, and broad bean seeds from Z3, Z4, and Z6. CCA was computed in the R environment (version 3.6.0; http://www.r-project.org/) using the vegan package (https://cran.rproject.org/package=vegan). The axes for the reduced model were chosen based on the permutation tests. Y and X axes have been scaled independently for each dataset to fit the graph. The axes that were significant, due to the variability that they explain, is not due to chance (Table S3.9).

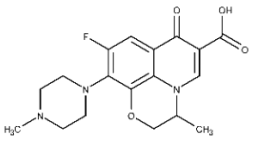
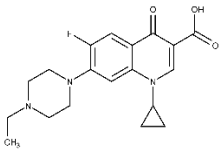
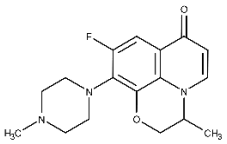
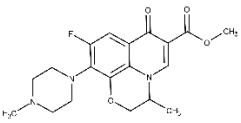
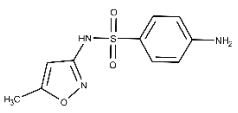
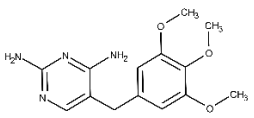
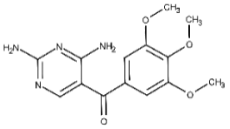
Significance level was established at $\alpha = 0.05$.

## 3.3. Results and discussion

### 3.3.1. Distribution of ABs and their biotransformation products per zones

A total of 10 ABs belonging to four chemical classes and six of their major metabolites were analyzed. The ABs detected above the MDLs belonged to three groups, namely, benzyl pyrimidines, sulfonamides, and fluoroquinolones (Table 3.1). The lincosamide group was not detected in any vegetable matrix. The concentration of ABs in the vegetables ranged from non-detectable to 3.61 ng $g^{-1}$ (for keto TMP in cauliflower from Z4). The minimum, maximum, and mean concentrations of the detected ABs, as well as the frequency of detection (FOD) per vegetable type, are presented in Table S3.5.

Table 3.1. Physicochemical properties of the detected compounds

| Compound | Molecular structure | MW[a] (g mol$^{-1}$) | solubility[a] (mg L$^{-1}$) | p$K_a$[b] | log $K_{OW}$[a] | $f_n$[c] |
|---|---|---|---|---|---|---|
| OFL |  | 361 | 28260 | 5.3 [+/0±]<br>6.7 [0±/-] | -0.39 | 0.3-0.5% |
| ENR |  | 359 | 3397 | 5.6 [+/0±]<br>7.2 [0±/-] | 0.70 | 0.5-0.6% |
| Decarboxyl OFL |  | 317 | 1334 | 6.7[+/0] | 2.12 | 89-93% |
| OFL methyl ester |  | 375 | 1318 | 6.7 [+/0] | 1.79 | 89-93% |
| SMX |  | 253 | 3942 | 2.0 [+/0]<br>6.2 [0/-] | 0.89 | 2-4% |
| TMP |  | 290 | 2334 | 7.2 [+/0] | 0.91 | 64-74% |
| Keto TMP |  | 304 | 4870 | 6.3 [+/0] | 0.44 | 95-97% |

[a] MW, solubility and log $K_{OW}$ were calculated using Episuite v4.11 (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm)

[b] dissociation constants were estimated using MarvinSkech (https://chemaxon.com/products/marvin). Dissociation reactions, [0]: neutral; [+]: cationic; [-]: anionic; [±]: zwitterionic

[c] neutral fraction in range of soil pH (7.6-7.8) was estimated using MarvinSkech (https://chemaxon.com/products/marvin).

Figure 3.2 shows the cumulative distribution of mean concentrations of ABs and their TPs in the different studied zones. Broad bean and tomato samples from Z4 showed the highest cumulative average concentrations of ABs. For cauliflower, Z1 showed the highest pollutant loads, followed by Z4. Similarly, for lettuce samples, the two zones with the highest loads were Z4 and Z1.

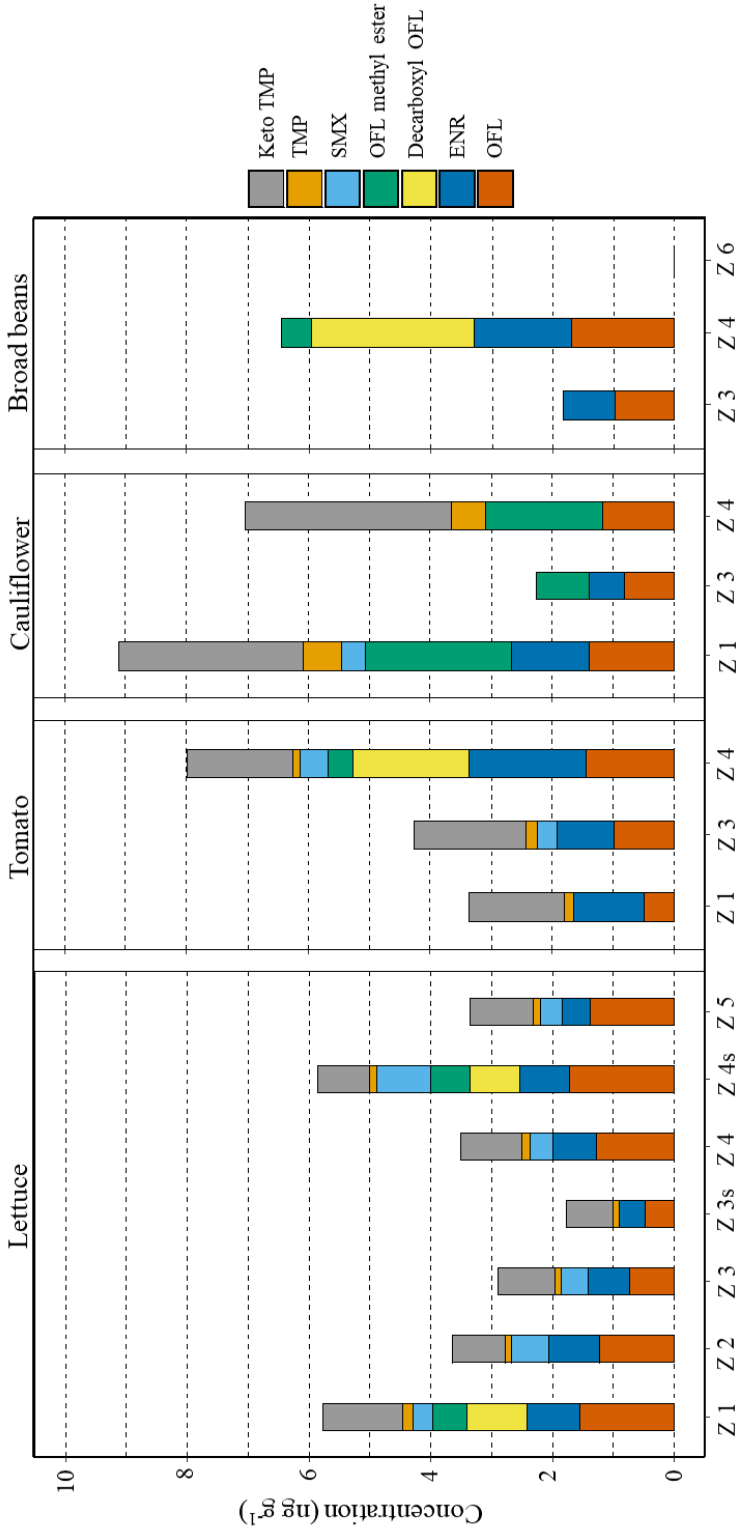



Figure 3.2. Cumulative mean concentrations (ng $g^{-1}$ fw) of the detected ABs and their metabolites per sampling zone and vegetables. Relative standard deviation was less than 10%

Fluoroquinolones, OFL, and ENR were the most frequently detected ABs, above their MQLs in 94 and 81% of total samples, respectively. The results of the multivariate 75$^{th}$ percentile regression models including zones, vegetables, and the interaction between them showed that both OFL (range of predicted 75$^{th}$ percentile (75$^{th}$ pp: 1.79 ng g$^{-1}$) and ENR (75$^{th}$ pp: 1.53 ng g$^{-1}$) values were significantly higher in the broad bean sample in Z4 than in Z3 and Z6. In tomato fruits, significant differences were observed for OFL and ENR levels between all zones, in the following decreasing orders: Z4>Z3>Z1 and Z4>Z1>Z3, respectively. The highest loadings of fluoroquinolones were observed in Z4 for broad beans and tomatoes and in Z1 for cauliflower (OFL 75$^{th}$ pp: 1.49 ng g$^{-1}$; ENR 75$^{th}$ pp: 1.38 ng g$^{-1}$). Unlike in other vegetables, where OFL and ENR showed similar occurrence patterns, the concentration levels in lettuce varied between the two fluoroquinolones within the same zone. Specifically, OFL levels in lettuce during the summer campaign were highest in Z4 (75$^{th}$ pp: 1.78 ng g$^{-1}$) and Z1 (75$^{th}$ pp: 1.71 ng g$^{-1}$), with no significant difference between the two zones; in contrast, ENR levels were higher in lettuce collected from Z1 (75$^{th}$ pp: 0.96 ng g$^{-1}$) and Z2 (75$^{th}$ pp: 0.88 ng g$^{-1}$).

The detection of OFL methyl ester and decarboxyl OFL in different samples reflected the distribution pattern of their parent compound. In fact, both OFL metabolites were detected in lettuce, tomato, and broad bean samples from zones in which OFL levels were relatively high. A similar trend was observed in cauliflower samples for OFL methyl ester, although in this case decarboxyl OFL was not detected in any of the analyzed samples.

TMP and keto TMP were detected above the MQLs in 75% of samples, namely, in all sample types except the broad beans. Very uniform concentration levels were observed in lettuce for both of them. However, TMP exhibited the highest concentrations in Z1 (75$^{th}$ pp: 0.19 ng g$^{-1}$), significantly higher than in the other zones, except Z5 (75$^{th}$ pp: 0.16 ng g$^{-1}$). Similarly, like its parent compound, keto TMP concentrations were highest in Z1 (75$^{th}$ pp: 1.37 ng g$^{-1}$). For tomatoes, Z3 was the area with the highest pollutant loads, since the concentrations of TMP (75$^{th}$ pp: 0.20 ng g$^{-1}$) and keto TMP (75$^{th}$ pp: 1.92 ng g$^{-1}$) were significantly higher in Z3 than in the other zones. The only case in which the metabolite did not reflect the occurrence pattern of the parent compound was observed in the cauliflower samples. Namely, TMP was significantly higher in Z1 than Z4, while the opposite was true for keto TMP (Z4>Z1; p= 0.052).

SMX was detected above the MQLs in 56% of all the vegetable samples. SMX was significantly higher in lettuce samples harvested during summer from Z4 (75$^{th}$ pp: 1.00 ng g$^{-1}$), followed by Z2 (75$^{th}$ pp: 0.65 ng g$^{-1}$). In contrast, SMX was not detected in the lettuce samples

collected during summer from Z3. In tomatoes, SMX levels decreased in the following order Z4>Z3>>Z1, ranging from below the MQL to 75$^{th}$ pp: 0.50 ng g$^{-1}$. However, in cauliflower, SMX was detected above the MQL only in samples from Z1 (75$^{th}$ pp: 0.43 ng g$^{-1}$). SMX concentrations in broad beans were below the MQL.

### 3.3.2. Distribution of ABs and their biotransformation products per crop

Pairwise vegetable comparisons of the predicted 75$^{th}$ percentiles of the multivariate regression models in Z3 and Z4 are shown in Figure S3.1 and Table S3.6. Comparison of the ABs and their TPs showed lower levels of pollutants in lettuce relative to the other vegetables. Only SMX was significantly higher in lettuce than in tomato samples harvested from Z3, while TMP and keto TMP were significantly higher in tomato fruits than in lettuce samples. No significant differences were found between tomato and lettuce for SMX and TMP in Z4. TMP content in cauliflower in Z4 was significantly higher than in lettuce and tomato. Keto TMP was detected in significantly higher concentrations in the cauliflower samples, followed by tomato and lettuce in Z4. Although there were no significant differences in TMP content between tomato and lettuce in Z4, significant differences were observed for its metabolite keto TMP, which may be attributable to the high metabolization rate of TMP in tomato (discussed below).

ENR and OFL were generally higher in tomato and broad bean than in lettuce and cauliflower. Statistically significant differences were observed in Z4 for ENR in the following descending order: tomato, broad beans, lettuce, and cauliflower (<MQL). The same pattern was observed in Z3, but with significant differences only between tomato and broad bean versus cauliflower and tomato versus lettuce. As mentioned earlier, in Z3 and Z4, the highest concentrations of OFL were detected in broad bean followed by tomato. The difference in the concentration between lettuce and broad bean was only significant in Z3, whereas broad bean from Z4 showed higher OFL levels than lettuce and cauliflower from the same zone. Similarly, broad bean showed the highest levels of decarboxyl OFL, followed by tomato and lettuce samples, a result that might be attributed to the possible intensive metabolization of OFL in broad beans. Cauliflower samples showed relatively low concentrations of OFL combined with high levels of its metabolite OFL methyl ester, suggesting that OFL was rapidly metabolized into OFL methyl ester in cauliflower.

These results confirm the uptake and translocation of ABs to different plant compartments, in line with previous studies. As reported by Christou et al. [15], fluoroquinolones and sulfonamides exhibited higher AB bioconcentration factors in crops. Li et al. [130] analyzed ciprofloxacin, norfloxacin, and ENR in 10 different vegetable types grown in greenhouses in

northern China coupled with the record of manure application over several years. The mean reported concentrations of ciprofloxacin, norfloxacin, and ENR were 104.4, 55.7, and 18.6 ng $g^{-1}$, respectively. Their results revealed that solanaceous fruits had higher fluoroquinolone bioaccumulation potential than leafy vegetables, with tomato having the highest among the tested vegetables (eggplant, pepper, cucumber, spinach, and crown daisy), which is consistent with our observations. Nevertheless, neither metabolites nor TPs of fluoroquinolones were reported in these previous studies.

The reported values for TMP in tomato obtained in the present study are slightly higher than those reported by Sabourin et al. [146] in tomato grown on the premises of the University of Western Ontario, fertilized with manure (0.4 ng $g^{-1}$ dw; approximately 0.08 ng $g^{-1}$ fw). Christou et al. [98] reported higher values for both SMX (0.4–3.2 ng $g^{-1}$) and TMP (0.5–5.2 ng $g^{-1}$) in tomato irrigated with RWW over three years in Cyprus. Azanu et al. [150] reported lower values of TMP (0.05 ng $g^{-1}$) and SMX (0.015 ng $g^{-1}$) in lettuce collected from farms in Ghana irrigated with low-quality water and local markets.

The uptake and translocation of the detected ABs by other types of plants as a consequence of RWW and/or manure application have also been confirmed. Hu et al. [134] investigated the occurrence of AB content in different plants (radish, rape, celery, and coriander) and their compartments as a consequence of manure application. The plants were harvested from organic farms in northern China. The highest concentration of SMX (2.7 ng $g^{-1}$) was detected in the radish root, while for OFL (3.6 ng $g^{-1}$) the maximum levels were reported in coriander leaves. Liu et al. [149] reported TMP and SMX in the range of 0.02–0.08 ng $g^{-1}$ dw (approximately: 0.0016–0.004 ng $g^{-1}$ fw), and 0.05–0.9 ng $g^{-1}$ dw (approximately: 0.004–0.045 ng $g^{-1}$ fw), respectively, in different vegetables (i.e., eggplant, long bean, wheat, and cucumber) grown in suburbs of Beijing, China, and irrigated with RWW. SMX and ENR were detected in peanut plants, grown in the Yangtze River Delta, in Eastern China, as a consequence of long-term manure application, with the highest concentrations being found in the stem (3.64 ng $g^{-1}$ dw) and leaf (4.26 ng $g^{-1}$ dw; approximately 0.43 ng $g^{-1}$ fw), respectively [147]. The low ng $g^{-1}$ levels of ABs and their TPs found in the present work are consistent with these earlier studies.

### 3.3.3. Hierarchical cluster analysis and principal component analysis

To investigate the predominant factors affecting AB occurrence in crops, HCA and PCA were performed. The HCA illustrates a sample dendrogram based on the content of ABs and some of their TPs (Figure 3.3).

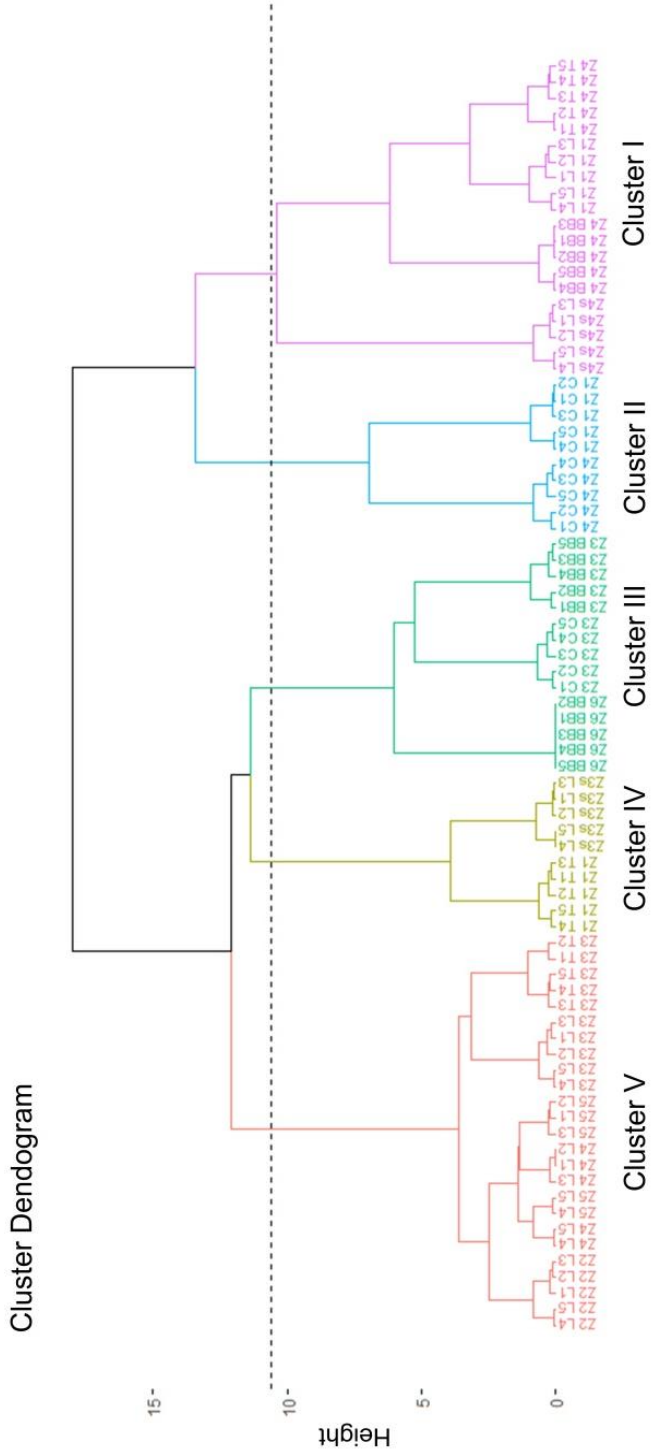



Figure 3.3. Hierarchical clustering of samples. Vegetable types were coded as L (lettuce), T (tomato), BB (broad beans), and C (cauliflower), as well as a digit (from 1 to 5) indicating the sample replicate

Five clusters can be observed, namely, Cluster I (lettuce: Z4s and Z1; broad beans: Z4; and tomato: Z4), Cluster II (cauliflower: Z1 and Z4), Cluster III (broad beans: Z6 and Z3; cauliflower: Z3), Cluster IV (tomato: Z1; lettuce: Z3s), and Cluster V (tomato: Z3; lettuce: Z2, Z3, Z4, Z5). The first two clusters represent the most polluted samples, highlighting the importance of the irrigation with water from the Llobregat River (Z4) and manure application (Z1). In addition, emphasizing the importance of the season for the lettuce samples from Z4, and the differentiation of cauliflower from the rest of the vegetables. Clusters III and IV represent samples with the lowest pollution loads, and show separation between broad bean and cauliflower on one side and tomato and lettuce on another. While Cluster V represent lettuce and tomato samples with moderate pollution loads. This outcome is mostly consistent with the observed cumulative concentrations (Fig. 3.2) and the following PCA.

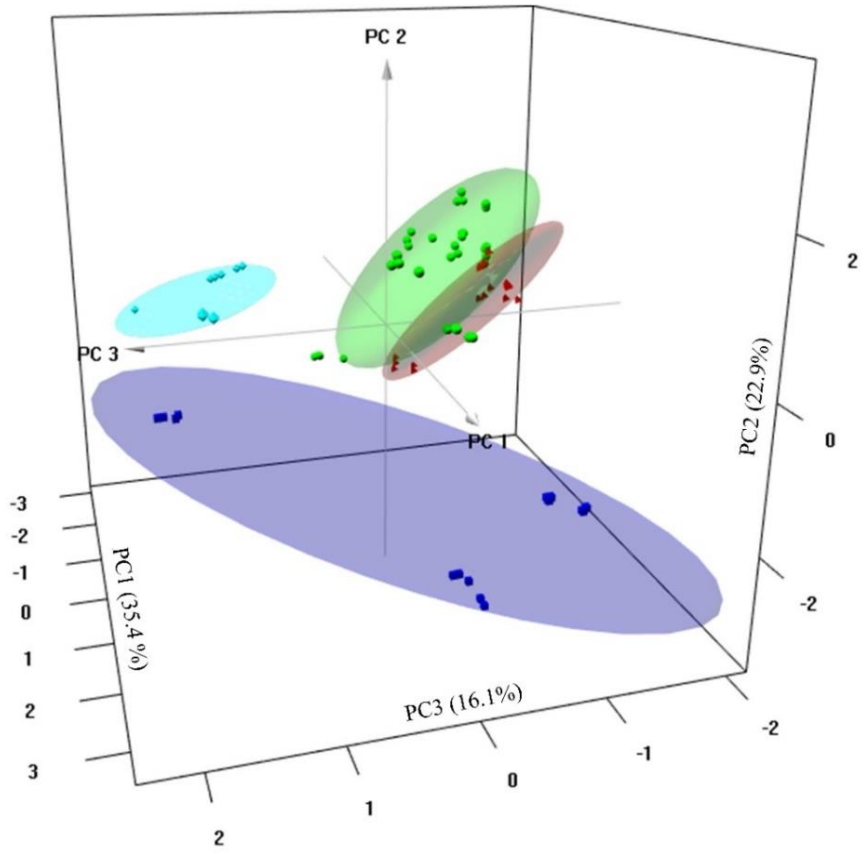



Figure 3.4. Distribution pattern of the ABs and their biotransformation products by PCA. Color indicates vegetable type (green: lettuce; red: tomato; darker blue: cauliflower; cyan: broad bean), and the color-shaded space indicates group samples by vegetable type.

A PCA was also performed to examine the distribution patterns of the ABs and their TPs in the vegetable samples (Figure 3.4). The first three components explained 74.4% of variance. PC1 (35.4% of total variance) showed positive loading values for OFL (0.5) and its two metabolites, OFL methyl ester (0.5) and decarboxyl OFL (0.5). PC2 (22.9% of total variance)

showed a positive loading for SMX (0.6) and a negative one for TMP (-0.5), while PC3 (16.1% of total variance) showed a positive loading for decarboxyl OFL (0.4) and a negative one for keto TMP (-0.8).

The three-dimensional PCA score plots presented in Fig. 3.4 did not show any separation between zones, but indicate moderate separation of broad bean and cauliflower samples, while the tomato and lettuce samples overlap. PC1 described a slightly clustered tomato and lettuce group and, more specifically, a subgroup consisting of lettuce from Z1 and Z4 (summer sample) and tomato samples from Z4. PC3 basically described the broad bean distribution. Cauliflower samples correlate with TMP, keto TMP, and OFL methyl ester and are separated by PC1 and, additionally, by PC2, which separates them from the tomato and lettuce samples. Two-dimensional plots of the first three components are presented in Figure S3.2 in the Supplementary information of this Chapter.

### 3.3.4. Factors affecting AB contamination

Since it was practically impossible to characterize and measure all known factors that might affect the crop uptake and translocation of ABs, multiple assumptions were made to draw conclusions using an inference procedure. The following discussion uses abductive reasoning to proceed to the likeliest possible explanation for each given set.

The occurrence of ABs in Z1 can be attributed to organic farming practices, including the use of manure as fertilizer, since this zone uses (presumably, pristine) groundwater for irrigation [118]. Manure can lead to significant AB contamination, especially in regions with (semi-) arid climates, where the AB residues contained in manure undergo little runoff or leaching [147]. Even the application of composted manure, as was the case in Z1, showed increased AB contamination levels. This is consistent with the fact that removal rates for different AB classes varied from complete removal for the macrolides to no removal for the fluoroquinolones during the manure composting process, when the maximum core pile temperature reached 68ºC [165].

River water is used as the irrigation source in Z2 and Z4, which can be associated with indirect WW reuse, given the high contribution of upstream WWTPs to the total flow of the Llobregat River. Among the large group of pharmaceuticals and personal care products monitored in the Llobregat River, SMX and OFL were the compounds detected at the highest concentrations, with SMX concentrations exceeding 10 µg $L^{-1}$ [166]. Thus, presumably, river water could be

the main source of the ABs present in the vegetables grown in these two zones. However, the concentrations vary a lot depending on the sampling time. Available data on the seasonal fluctuation of pollutants in the Llobregat River showed high pollutant concentrations in the summer season [166]. This may be related to the observation that summer lettuce exhibited higher concentrations than spring samples from Z4. At Z5, where no manure application was used, contaminated harvested water could be the possible source of the ABs. Out of all the selected zones, Z6 showed the lowest AB concentrations, most likely due to the nature of the applied agricultural practices (pristine irrigation water source and pigeon manure).

Conversely, Z3 was expected to be the area with the highest pollutant loads, due to the irrigation with RWW. However, lettuce samples collected from Z3 in the summer campaign showed the lowest observed concentration of all the samples. This may be attributable to the fact that it has the highest productivity rate for that season. Margenat at al. [118] reported the crop productivity of lettuces and tomatoes grown in the same sampling zones and found the highest vegetable yield in Z3. For instance, lettuce in Z3 had a total yield (96 kg $ha^{-1}$) more than twice as high as that reported for Z1, Z2, and Z5 (40–41 kg $ha^{-1}$). Growing time also varies a lot and may have a significant effect on the uptake. Despite the lower rainfall, higher transpiration and irrigation rates occur in summer, which may increase uptake. The growing period for lettuces during summer months was dramatically shorter than for lettuces grown in spring (25 versus 80 days, respectively), which may reduce the total level of pollution. Sallach et al. [143] observed a similar trend between antimicrobial uptake and plant growth rates and confirmed that the plant growth rate may exceed the antimicrobial uptake rate. Thus, a short growing period coupled with high biomass could be the reason for the low AB concentration levels despite the indirect use of RWW.

As discussed, the factors affecting the uptake and occurrence of ABs are complex and variable, depending on the crop type, water quality, growing period, productivity, etc. Presented results showed that productivity, crop type and growing season/period were one the prevalent factors alongside with manure application and irrigation with water from the River Llobregat. It is known that soil properties can affect AB uptake. In this research sampled soils were quite similar, hence further research is needed on how different soil properties in combination with other factors can mitigate AB contamination.

### 3.3.5. Uptake, translocation, and metabolization of ABs

OFL, ENR, and SMX occur either in their anionic or zwitterionic forms at the soil pH values which ranged from 7.6 to 7.8. Their uptake is expected to be reduced due to repulsion forces between negative charges at the cell wall and ionized analytes, but the acidic root exudates can displace the equilibrium to the neutral forms in the rhizosphere in the case of OFL and ENR. Thus, for OFL, ENR, and SMX, uptake into roots may happen by passive diffusion [44] and via integral transport proteins since the transporters catalyzing ion uptake are rarely specific [42]. TMP largely occurs (64–74%) in its neutral form at the usual soil pH (7.6–7.8) (Table 3.1) and may enter root cells simply by passive diffusion [44]. AB TPs can occur in the plant as a product of plant metabolism or by being transformed prior to the plant uptake. In the case of external transformation, detected TPs can be easily uptaken due to the occurrence of a high percentage in the neutral form in soil (Table 3.1).

The charge of ABs will change depending on the surrounding pH during the translocation process. At pH values above their $pK_a$ (6.7), OFL methyl ester and decarboxyl OFL are neutral, in contrast to OFL, which is negatively charged. Similarly, keto TMP has a lower $pK_a$ value (6.3) than its parent compound TMP (7.2), a significant difference related to the translocation processes. More specifically, at cytosolic pH (7.2), TMP (50%), keto TMP (95%), OFL methyl ester (75%), and decarboxyl OFL (75%) will still exhibit a high percentage in neutral forms, making them suitable for further translocation to upper parts of the plant via the symplasmic pathway. Accumulation of ABs in roots may be attributed to the vacuoles of root cells, which can remove potentially toxic compounds from the symplasmic pathway. In addition, ion trapping can occur when a compound is neutral in the apoplast but ionizes inside the cell, leading to accumulation within cells [43]. In the vacuole (pH 5.5), OFL methyl ester, decarboxyl OFL, and TMP occur in cationic form and, thus, could be trapped. In the cell vacuole, keto TMP predominantly occurs in the cationic form, but 15% persists in the neutral form, making it susceptible to translocation. This may explain the high concentration of keto TMP we found in plants. In addition to transport via integral transport protein, SMX, OFL, and ENR could be transported via apoplasmic water movement. At a given pH (5.5), SMX will occur in both anionic and neutral forms, with a predominance of the neutral form (82%), while OFL and ENR will occur in the zwitterionic form, 53% and 45%, respectively. A high translocation rate for another fluoroquinolone AB (norfloxacin) has also been reported [110]. Our findings are consistent with the results published by Miller et al. [43], who found that ionic compounds with log $K_{OW}$< 3 and $pK_a$< 7 can be translocated to fruits. All the detected compounds met these criteria, except TMP and ENR, which have a slightly higher $pK_a$ (7.2). Moreover, all the detected compounds have a relatively small MW, with OFL methyl ester having the highest

(375 g $mol^{-1}$). According to Chuang et al. [44], pharmaceuticals with an MW> 400 g $mol^{-1}$ are primarily accumulated in roots, while compounds with a smaller MW could move up to lettuce shoots.

TPs may exceed the concentration of the parent compound due to metabolization processes. For instance, the concentration of carbamazepine in spinach leaves plateaus after a period of time, but its primary metabolite continues to increase [167]. Evidence suggests that AB compounds are transformed inside the plant as well [40]. Our results show different ratios between the parent compound and its metabolite in different vegetables. In tomato, lettuce, and cauliflower, keto TMP was detected at concentrations 12, 7, and 5 times higher than TMP, respectively. The ratio between decarboxyl OFL and OFL was 1.3 and 1.9 for tomato and broad bean, respectively. Strikingly, OFL methyl ester was higher (1.5 times) than its parent only in cauliflower samples, while concentrations of all OFL metabolites in lettuce were lower than that of the parent compound. These results indicate that the occurrence of metabolites is both plant- and compound-dependent.

### 3.3.6. Estimated daily intake and human health risk assessment

EDI of ABs per vegetable is expressed as a sum of EDI values of all detected compounds in given vegetable type. Among the different vegetables assessed, the EDI through tomato consumption was the highest: 1.4E-02 µg kg $bw^{-1}$ $day^{-1}$ for adults and 2.0E-02 µg kg $bw^{-1}$ $day^{-1}$ for children. In comparison, the EDI via lettuce consumption was one order of magnitude lower: 6.1E-03 µg kg $bw^{-1}$ $day^{-1}$ for adults and 7.9E-03 µg kg $bw^{-1}$ $day^{-1}$ for children. Although ABs may be present in cauliflower at higher concentrations than in lettuce, the EDI values were lower, due to eating habits. In addition, and unlike other vegetables, exposure via cauliflower consumption for adults (2.8E-03 $µg^{-1}$ kg $bw^{-1}$ $day^{-1}$) was higher than for children (4.0E-04 $µg^{-1}$ kg $bw^{-1}$ $day^{-1}$). This fact exemplifies how consumption habits may be more important than the actual concentration of contaminants for calculating AB exposure values. Likewise, because of their relatively low ingestion rate, the EDI through broad beans was virtually negligible compared to other vegetables (1.4E-06 µg kg $bw^{-1}$ $day^{-1}$ for adults and 6.4E-05 $µg^{-}$ kg $bw^{-1}$ $day^{-1}$ for children). Taken together, the results suggest that children show higher exposure to ABs and their TPs than adults through consumption of lettuce and tomato. Finally, the highest EDIs were found for two metabolites (decarboxyl OFL and keto TMP), although their bioactivity and toxic effects are presently unknown. The EDI values of the individual detected compounds are shown in Figure S3.3. In summary, these values are

consistent with reports on AB contamination levels in crops irrigated with RWW, which have found AB concentrations to be relatively low [40,102].

Notwithstanding the aforementioned exposure data, estimated HQs showed that the risk posed by vegetable consumption is negligible, as the maximum estimated HQ values were still less than 0.01, as were the corresponding HIs (Table 3.2). These estimates are consistent with previous studies that have also shown a *de minimis* risk of AB occurrence in vegetables to human health [98,110,149–151].

Table 3.2. Minimum and maximum HQ and HI values

| HQ | TMP | OFL | ENR | SMX | HI |
|---|---|---|---|---|---|
| Min | 7.1E-06 | 1.7E-05 | 7.5E-06 | 1.4E-07 | 3.2E-05 |
| Max | 1.4E-04 | 1.2E-03 | 8.4E-04 | 1.1E-05 | 2.2E-03 |

However, the HI as a simple sum of individual HQs is rather questionable in the case of ABs, due to the synergetic and antagonistic effects exerted between ABs. Specifically, current approaches do not include the hazard associated with: 1) AB TPs; 2) possible synergistic effects (for instance, both SMX and TMP, which have a proven synergistic effect, were detected in 80% of lettuce and tomato samples); and 3) the occurrence of ARGs [158] and other chemical contaminants (e.g., heavy elements) [168] alongside ABs. Aga et al. [72] have pointed to similar concerns, stating that the presence of other common environmental contaminants should not be overlooked, as they contribute to the overall toxicity of AB mixtures [72]. There is also uncertainty as to whether or not low-dose exposure to a mixture of ABs over a prolonged period of time poses a health risk. Moreover, the highest-priority question is whether there is a risk of developing AB resistance in complex real field environments due to AB occurrence. Our results show that further research on this topic is necessary.

The potential relationship between the distribution of the ARGs and AB compounds was analyzed with CCA (Figure 3.5). The ARG distribution and abundance indicate that the ABs had a significant impact (selective pressure), as they explained 54% of the total variation (inertia proportion) in ARG abundance (Table S3.8) with a significant effect (Table S3.9) according to the permutational ANOVA ($p< 0.05$).

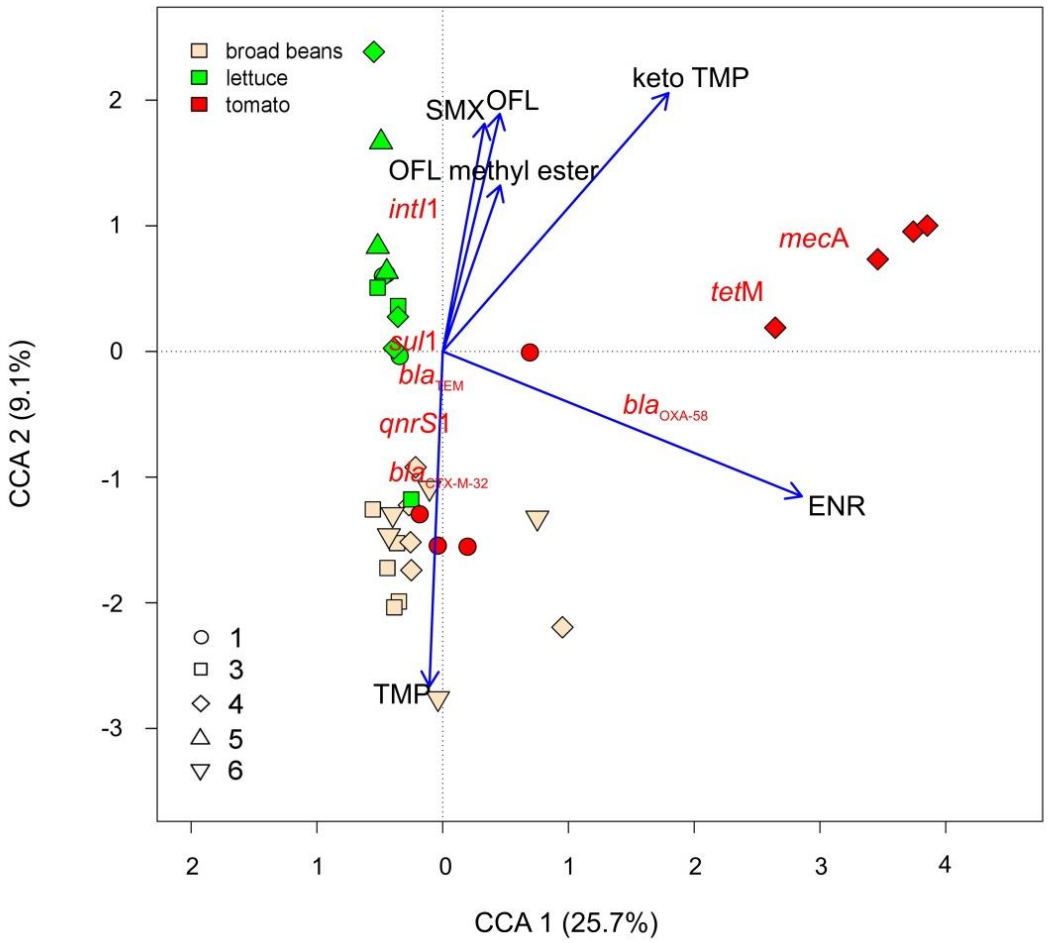



Figure 3.5. CCA compares the abundance of detected ARGs as a response to the concentration of ABs (arrows) as environmental variables. The ABs were selected for analysis based on variance inflation factors calculated during the CCA. The percentage of variation explained by each axis is shown

The samples were clustered mostly by vegetable type (Fig. 3.5). The first axis (CCA 1, 25.7% of variation) separated the tomato samples from Z4 from the other vegetables, while the second axis (CCA 2, 9.1% of variation) separated the lettuce samples. In the ranking of projection points (ARGs), the origin (0,0) indicates the global average of the variables (ABs). ARG points are weighted averages of the vegetable sample points, not only in the diagram as a whole, but also when projected on to any particular ABs arrow. In both the tomato and lettuce samples, the sulfonamide group of ABs was detected above the detection limits; this was not the case for broad beans. This could explain the relatively lower amount of *sul*1, expressed as log copy number/sample in broad bean compared to lettuce and tomato [158]. The correlation of broad beans with TMP (Fig. 3.5) appears due to the high MQL values used in the estimates, whereas only fluoroquinolones were detected in broad beans and could thus

affect the ARG loads. Therefore, the ABs (OFL, SMX, TMP, and ENR) were a significant factor driving ARG abundances in the different vegetable species (Table S3.8).

In light of the above, the present results support the need to readjust the risk assessments of AB intake as stated earlier [59].

## 3.4. Conclusions

The occurrence of ABs and their metabolites in crops was confirmed in 94% of all collected samples (n=80), confirming wide-scale pollution. Concentrations of detected AB metabolites, which ranged from 0.04 ng $g^{-1}$ to 3.61 ng $g^{-1}$, were higher than the concentrations of their parent compounds in 73% of all cases. This may be due to the metabolization process in the plant, in addition to the fact that their physicochemical properties are modified making them more susceptible to translocation. Among factors that drive the uptake and translocation processes, irrigation with water from the Llobregat River and fertilization with manure appeared to be the two most important sources of ABs in the evaluated agricultural ecosystem. Crop type, productivity, and growing time also appeared to play a significant role. PCA and HAC confirmed that vegetable type is a very important factor in the case of broad bean and cauliflower. Finally, eating habits will determine the level of human exposure through vegetable consumption. Up to date, this is the first study that quantifies AB and their metabolites in field grown vegetables and evaluates different agricultural practices, irrigation water sources, manure application, crop types and relationship between AB compounds and ARGs.

Comprehensive survey studies are a handy tool that could describe various factors and their influence on AB occurrence and exposure level. All these factors may greatly differ between the time and space boundaries of given cases and should thus be discussed accordingly. A case-specific, flagship approach to the investigation of factors that promote AB contamination is suggested. Basically, ranking the chief factors can help mitigate AB contamination in the given real field-scale case and prevent ABs from entering a food web. In this regard, further improvement in RWW and manure application schemes is essential. Although applied health risk assessment showed no risk of ABs through vegetable consumption, the authors support the idea of readjusting the health risk approach, as well as further research on synergistic effects on toxicity and antimicrobial activity between ABs, their metabolites, and other contaminants. Finally, because the ABs described more than 50% of the variation in the ARGs analyzed in the edible parts of vegetables, it is imperative to investigate the dose response for ARG promotion in agricultural environments.

## 3.5. Supplementary information

### 3.5.1. Sampling strategy

Sampling campaign was performed as reported by Margenat et al. [162,168]. Vegetables were selected on the basis of their availability at given sampling farms, agricultural practices including species with different seasonality. Only the edible parts of vegetables were analyzed, i.e., the leaf (lettuces), the fruit (tomatoes) the seed (broad beans), and the inflorescence (cauliflowers). The lettuces were planted in March and harvested during May 2016 on five sampling zones (1-5) representing spring season. In the zones 3 and 4 lettuce plants which were planted in June and harvested during the same month, representing summer season samples. Tomatoes were grown during summer 2017 (June-September) and collected from zones 1, 3 and 4. Cauliflowers were grown during winter months (November 2016 – March 2017) and harvested from zones 1, 3 and 4. Broad beans, planted in November 2016, were collected during April 2017 from zones 3, 4 and 6. Description of number of analyzed samples (n=80) is presented in Table S3.1.

Supplementary Table S3.1. Description of number of analyzed samples

| Zones | Lettuce | Tomato | Cauliflower | Broad bean | Total |
|---|---|---|---|---|---|
| Z 1 | 5 | 5 | 5 | 0 | 15 |
| Z 2 | 5 | 0 | 0 | 0 | 5 |
| Z 3 | 5 | 5 | 5 | 5 | 20 |
| Z 3s | 5 | 0 | 0 | 0 | 5 |
| Z 4 | 5 | 5 | 5 | 5 | 20 |
| Z 4s | 5 | 0 | 0 | 0 | 5 |
| Z 5 | 5 | 0 | 0 | 0 | 5 |
| Z 6 | 0 | 0 | 0 | 5 | 5 |
| Total | 35 | 15 | 15 | 15 | 80 |

Note: 3s means zone 3 summer campaign; 4s means zone 4 summer campaign

Supplementary Table S3.2. Method MDLs and MQLs estimated on fresh weight bases. Data modified from Table 2.3

| Compounds | Lettuce | | Tomato | | Cauliflower | | Broad bean | |
|---|---|---|---|---|---|---|---|---|
| | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) | MDLs (ng $g^{-1}$) | MQLs (ng $g^{-1}$) |
| OFL | 0.17 | 0.25 | 0.04 | 0.08 | 0.41 | 0.73 | 0.42 | 0.77 |
| Decarboxyl OFL | 0.04 | 0.06 | 0.04 | 0.14 | 0.26 | 0.50 | 0.18 | 0.38 |
| OFL methyl ester | 0.15 | 0.25 | 0.28 | 0.57 | 0.18 | 0.37 | 0.15 | 0.35 |
| OFL ethyl ester | 0.22 | 0.33 | 0.43 | 0.81 | 0.19 | 0.44 | 0.24 | 0.55 |
| ENR | 0.23 | 0.30 | 0.10 | 0.18 | 0.32 | 0.59 | 0.57 | 0.71 |
| SMX | 0.06 | 0.09 | 0.08 | 0.10 | 0.10 | 0.31 | 0.31 | 0.62 |
| STZ | 0.11 | 0.14 | 0.08 | 0.12 | 0.10 | 0.18 | 0.42 | 0.60 |
| SMT | 0.03 | 0.05 | 0.04 | 0.04 | 0.05 | 0.11 | 0.73 | 1.22 |
| SDI | 0.07 | 0.26 | 0.04 | 0.08 | 0.09 | 0.27 | 0.42 | 0.77 |
| SMZ | 0.10 | 0.16 | 0.08 | 0.16 | 0.23 | 0.46 | 0.11 | 0.22 |
| SDM | 0.14 | 0.23 | 0.14 | 0.22 | 0.10 | 0.28 | 0.18 | 0.20 |
| N-Acetyl SMX | 0.09 | 0.13 | 0.35 | 0.47 | 0.22 | 0.57 | 0.24 | 0.55 |
| TMP | 0.02 | 0.04 | 0.02 | 0.04 | 0.21 | 0.51 | 0.40 | 0.75 |
| TMP306 | 0.06 | 0.10 | 0.08 | 0.16 | 0.17 | 0.43 | 0.29 | 0.53 |
| Keto TMP | 0.04 | 0.08 | 0.10 | 0.18 | 0.14 | 0.29 | 0.15 | 0.33 |

Supplementary Table S3.3. Average DI and standard deviation of edible part of vegetables in Spain

| Vegetable | Population class | DI (g $day^{-1}$) |
|---|---|---|
| Lettuce | Adult | 86.7 ± 31.2 |
| | Children | 38.0 ± 18.1 |
| Tomato | Adult | 196.0 ± 66.1 |
| | Children | 94.3 ± 17.5 |
| Cauliflower | Adult | 24.4 ± 15.4 |
| | Children | 1.2 ± 0.9 |
| Broad beans | Adult | 0.02 ± 0.5 |
| | Children | 0.31 ± 1.9 |

Supplementary Table S3.4. Acceptable daily intake of detected ABs [69]

| Compounds | ADI (µg kg $bw^{-1}$ $day^{-1}$) |
|---|---|
| OFL | 3.2 |
| ENR | 6.2 |
| SMX | 130.0 |
| TMP | 4.2 |

Supplementary Table S3.5. Mean, minimum, and maximum concentration of ABs, FOD, and number of samples

| Compound | Lettuce (n=35) | | Tomato (n=15) | | Cauliflower (n=15) | | Broad beans (n=15) | |
|---|---|---|---|---|---|---|---|---|
| | Concentration (ng $g^{-1}$) | FOD (%) | Concentration (ng $g^{-1}$) | FOD (%) | Concentration (ng $g^{-1}$) | FOD (%) | Concentration (ng $g^{-1}$) | FOD (%) |
| OFL | 1.19 (0.44 – 1.8) | 100 | 0.98 (0.46 – 1.55) | 100 | 1.12 (0.73 – 1.51) | 100 | 1.34 (<0.77 – 1.80) | 67 |
| ENR | 0.66 (0.37 – 0.99) | 71 | 1.33 (0.82 – 2.09) | 100 | 0.93 (<0.59 – 1.39) | 67 | 1.23 (<0.71 – 1.79) | 67 |
| Decarboxyl OFL | 0.89 (<0.04 – 1.10) | 29 | 1.90 (<0.04 – 2.10) | 33 | <0.26 | nd | 2.65 (<0.24 – 2.89) | 33 |
| OFL methyl ester | 0.60 (<0.15 – 0.69) | 29 | 0.41 (<0.28 – 0.47) | 33 | 1.73 (<0.18 – 2.47) | 100 | 0.50 (<0.15 – 0.58) | 33 |
| SMX | 0.50 (<0.06 – 1.00) | 71 | 0.40 (<0.1 – 0.50) | 67 | 0.38 (0.34 – 0.44) | 33 | <0.62 | nd |
| TMP | 0.12 (0.09 – 0.20) | 100 | 0.15 (0.11 – 0.21) | 100 | 0.59 (<0.51 – 0.70) | 67 | <0.40 | nd |
| Keto TMP | 0.97 (0.71 – 1.39) | 100 | 1.71 (1.48 – 1.94) | 100 | 3.21 (<0.14 – 3.61) | 67 | <0.15 | nd |

nd – not detected

Supplementary Table S3.6. Pairwise vegetables comparisons of predicted 75$^{th}$ percentiles of multivariate regression models in zones 3 and 4

| Comparison | OFL | | ENR | | Decarboxyl OFL | | OFL methyl ester | | SMX | | TMP | | Keto TMP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Z 3 | Z 4 | Z 3 | Z 4 | Z 3 | Z 4 | Z 3 | Z 4 | Z 3 | Z 4 | Z 3 | Z 4 | Z 3 | Z 4 |
| Lettuce vs tomato | - | - | * | * | * | * | * | * | * | ° | * | - | * | * |
| Lettuce vs cauliflower | - | - | - | * | * | * | * | * | * | * | * | * | * | * |
| Lettuce vs broad beans | * | * | - | * | * | * | - | * | * | - | * | - | * | * |
| Tomato vs cauliflower | - | * | * | * | * | * | * | * | * | * | - | * | * | * |
| Tomato vs broad beans | - | ° | - | * | * | * | * | * | - | * | - | - | * | * |
| Cauliflower vs broad beans | - | * | * | * | * | * | * | * | * | * | - | * | * | * |

* $p \leq 0.05$; ° $0.05 < p \leq 0.10$

Supplementary Table S3.7. Results of univariate 75th percentile Regression Models

| | OFL | | ENR | | Decarboxyl OFL | | OFL methyl ester | | SMX | | TMP | | Keto TMP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) |
| **Sample type** | | | | | | | | | | | | | | |
| Lettuce (reference) | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | |
| Tomato | -0.10 | (-0.61 to 0.41) | 1.02* | (0.51 to 1.53) | 1.06* | (0.04 to 2.08) | -0.21 | (-0.60 to 0.18) | -0.15 | (-0.35 to 0.05) | 0.01 | (-0.03 to 0.05) | 0.75* | (0.53 to 0.97) |
| Cauliflower | -0.19 | (-0.70 to 0.32) | 0.43º | (-0.08 to 0.94) | -0.63 | (-1.65 to 0.39) | 1.79* | (1.40 to 2.18) | -0.22* | (-0.42 to -0.02) | 0.48* | (0.44 to 0.52) | 2.23* | (2.01 to 2.45) |
| Broad beans | 0.13 | (-0.38 to 0.64) | 0.71* | (0.20 to 1.22) | 1.75* | (0.73 to 2.77) | -0.12 | (-0.51 to 0.27) | -0.26* | (-0.46 to -0.06) | 0.05* | (0.01 to 0.08) | -1.02* | (-1.24 to -0.80) |
| _cons | 1.49* | (1.21 to 1.77) | 0.79* | (0.51 to 1.07) | 0.76* | (0.20 to 1.32) | 0.57* | (0.35 to 0.79) | 0.57* | (0.46 to 0.68) | 0.15 | (0.13 to 0.17) | 1.10* | (0.98 to 1.22) |
| **Area** | | | | | | | | | | | | | | |
| Zone 1 | 0.23 | (-0.06 to 0.52) | 0.35* | (0.01 to 0.69) | 0.88* | (0.11 to 1.65) | 2.28* | (2.06 to 2.50) | -0.29* | (-0.42 to -0.16) | 0.44* | (0.32 to 0.56) | 1.91* | (0.54 to 3.28) |
| Zone 2 (reference) | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | |
| Zone 3 | -0.34* | (-0.62 to -0.06) | -0.03 | (-0.36 to 0.30) | 0.07 | (-0.68 to 0.82) | 0.06 | (-0.15 to 0.27) | -0.27* | (-0.40 to -0.14) | 0.09 | (-0.02 to 0.20) | 0.03 | (-1.30 to 1.36) |
| Zone 3s | -0.76* | (-1.11 to -0.41) | -0.42* | (-0.84 to 0.00) | 0.00 | (-0.95 to 0.95) | 0.00 | (-0.27 to 0.27) | -0.62* | (-0.78 to -0.46) | 0.00 | (-0.15 to 0.15) | -0.09 | (-1.77 to 1.59) |
| Zone 4 | 0.27º | (-0.01 to 0.55) | 0.87* | (0.54 to 1.20) | 2.08* | (1.33 to 2.83) | 0.50* | (0.29 to 0.71) | -0.26* | (-0.39 to -0.13) | 0.08 | (-0.04 to 0.19) | 0.89 | (-0.44 to 2.22) |
| Zone 4s | 0.50* | (0.15 to 0.85) | -0.76* | (-1.18 to -0.34) | 0.87º | (-0.08 to 1.82) | 0.61* | (0.34 to 0.87) | 0.35* | (0.19 to 0.51) | 0.01 | (-0.14 to 0.16) | -0.01 | (-1.69 to 1.67) |
| Zone 5 | 0.18 | (-0.17 to 0.53) | -0.36º | (-0.78 to 0.06) | 0.00 | (-0.95 to 0.95) | 0.00 | (-0.27 to 0.27) | -0.27* | (-0.43 to -0.11) | 0.04 | (-0.11 to 0.19) | 0.18 | (-1.50 to 1.86) |
| Zone 6 | -0.89* | (-1.25 to -0.54) | -0.53* | (-0.95 to -0.11) | 0.07 | (-0.88 to 1.02) | 0.00 | (-0.27 to 0.26) | -0.34* | (-0.50 to -0.18) | 0.08 | (-0.07 to 0.22) | -0.88 | (-2.56 to 0.80) |
| _cons | 1.28* | (1.03 to 1.53) | 0.88* | (0.58 to 1.18) | 0.02 | (-0.65 to 0.69) | 0.08 | (-0.11 to 0.27) | 0.65* | (0.54 to 0.76) | 0.12* | (0.02 to 0.22) | 0.96 | (-0.23 to 2.15) |
| **Time of cultivation** | | | | | | | | | | | | | | |
| Time of cultivation (days) | -0.002 | (-0.007 to 0.003) | 0.01* | (0.005 to 0.013) | -0.007* | (-0.012 to -0.002) | 0.010* | (0.004 to 0.015) | -0.003* | (-0.005 to -0.002) | 0.001* | (0.001 to 0.002) | 0.013* | (0.004 to 0.023) |
| _cons | 1.616* | (1.103 to 2.128) | 0.150 | (-0.308 to 0.608) | 1.061 | (0.500 to 1.619) | -0.353 | (-0.939 to 0.233) | 0.792* | (0.600 to 0.983) | 0.055º | (-0.003 to 0.113) | 0.208 | (-0.853 to 1.269) |
| **Quarter of planting** | | | | | | | | | | | | | | |
| January to March | -0.12 | (-0.58 to 0.34) | -0.39 | (-0.89 to 0.11) | -0.87* | (-0.92 to -0.82) | -0.38 | (-0.89 to 0.14) | 0.00 | (-0.14 to 0.14) | 0.00 | (-0.14 to 0.14) | -0.66 | (-1.94 to 0.62) |
| April to June (reference) | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | |
| October to December | -0.21 | (-0.65 to 0.23) | 0.02 | (-0.45 to 0.49) | -0.76* | (-0.80 to -0.71) | 1.42* | (0.93 to 1.91) | -0.17* | (-0.30 to -0.04) | 0.40* | (0.27 to 0.53) | 1.13º | (-0.10 to 2.36) |
| _cons | 1.52* | (1.20 to 1.84) | 1.21* | (0.86 to 1.56) | 0.89* | (0.86 to 0.92) | 0.46* | (0.10 to 0.82) | 0.48* | (0.38 to 0.58) | 0.15* | (0.05 to 0.25) | 1.80* | (0.89 to 2.71) |

(continuation) Table S3.6

| | OFL | | ENR | | Decarboxyl OFL | | OFL methyl ester | | SMX | | TMP | | Keto TMP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) | Coef. | (95% CI) |
| **Type of irrigation water** | | | | | | | | | | | | | | |
| Well water and rainwater (reference) | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | |
| Well water impacted by industrial and road runoff | 0.01 | (-0.53 to 0.55) | -0.69 | (-1.60 to 0.22) | -0.11 | (-0.93 to 0.71) | -0.51° | (-1.08 to 0.07) | 0.03 | (-0.31 to 0.37) | -0.04 | (-0.15 to 0.07) | -0.49 | (-1.88 to 0.90) |
| Llobregat river | 0.17 | (-0.14 to 0.48) | 0.32 | (-0.21 to 0.85) | 1.70* | (1.22 to 2.17) | 0.03 | (-0.30 to 0.36) | 0.23* | (0.04 to 0.42) | 0.00 | (-0.07 to 0.07) | 0.07 | (-0.74 to 0.88) |
| Infanta channel - RWW | -0.53* | (-0.85 to -0.21) | -0.39 | (-0.94 to 0.16) | -0.04 | (-0.54 to 0.45) | -0.45* | (-0.80 to -0.11) | 0.02 | (-0.18 to 0.22) | 0.00 | (-0.07 to 0.07) | -0.65 | (-1.49 to 0.19) |
| _cons | 1.45* | (1.21 to 1.69) | 1.21* | (0.80 to 1.62) | 0.13 | (-0.23 to 0.50) | 0.59* | (0.33 to 0.85) | 0.35* | (0.20 to 0.50) | 0.20* | (0.15 to 0.25) | 1.63* | (1.01 to 2.25) |
| **Fertilizer** | | | | | | | | | | | | | | |
| Chemical fertilization (reference) | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | |
| Manure | 0.06 | (-0.35 to 0.47) | 0.33° | (-0.03 to 0.69) | 0.00 | (-0.30 to 0.30) | 0.01 | (-0.37 to 0.39) | -0.10 | (-0.27 to 0.07) | 0.00 | (-0.06 to 0.06) | 0.46 | (-0.25 to 1.17) |
| _cons | 1.39* | (1.18 to 1.60) | 0.88* | (0.70 to 1.06) | 0.13° | (-0.02 to 0.28) | 0.58* | (0.39 to 0.77) | 0.45* | (0.36 to 0.54) | 0.20* | (0.17 to 0.23) | 1.17* | (0.82 to 1.52) |
| **Soil texture** | | | | | | | | | | | | | | |
| Sandy loam (reference) | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.00 | |
| Sandy | -0.10 | (-0.66 to 0.46) | -0.34 | (-0.92 to 0.24) | -0.75* | (-1.48 to -0.02) | -0.54* | (-1.05 to -0.03) | 0.19° | (-0.03 to 0.41) | -0.06 | (-0.13 to 0.01) | -0.71 | (-1.86 to 0.44) |
| _cons | 1.45* | (1.25 to 1.65) | 1.14* | (0.93 to 1.35) | 0.77* | (0.51 to 1.03) | 0.62* | (0.44 to 0.80) | 0.39* | (0.31 to 0.47) | 0.20* | (0.17 to 0.23) | 1.70* | (1.29 to 2.11) |

* $p \leq 0.05$; ° $0.05 < p \leq 0.10$

Supplementary Table S3.8. Results of the final model

| | Inertia | Inertia proportion |
|---|---|---|
| Total | 0.7072 | 1.000 |
| Constrained | 0.380 | 0.537 |
| Unconstrained | 0.327 | 0.463 |

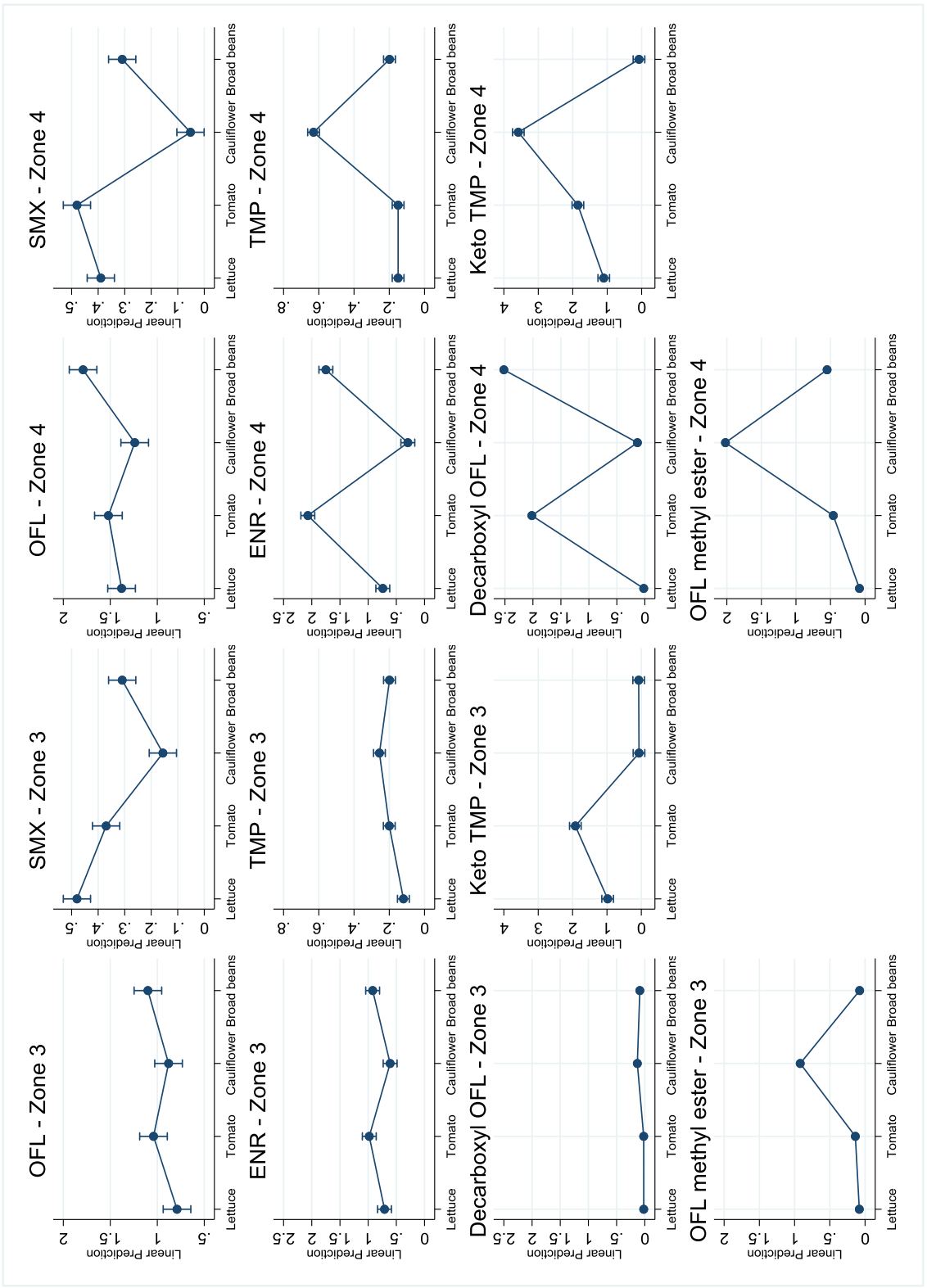
Supplementary Table S3.9. Permutation tests for constrains variables and axis for reduced model. Permutation: free. Number of permutations: 999

| | Df | Chi Square | F | p |
|---|---|---|---|---|
| Model | 6 | 0.3796 | 5.407 | 0.001*** |
| Residual | 28 | 0.3276 | | |
| Permutation tests for constrains variables | | | | |
| OFL | 1 | 0.0468 | 4.001 | 0.004** |
| ENR | 1 | 0.1997 | 17.068 | 0.001*** |
| OFL methyl ester | 1 | 0.0185 | 1.583 | 0.160 |
| SMX | 1 | 0.0494 | 4.219 | 0.004** |
| TMP | 1 | 0.0387 | 3.310 | 0.013* |
| keto TMP | 1 | 0.0264 | 2.260 | 0.049* |
| Residual | 28 | 0.3276 | | |
| Permutation tests for axes | | | | |
| CCA1 | 1 | 0.2568 | 21.952 | 0.001 *** |
| CCA2 | 1 | 0.0910 | 7.779 | 0.002 ** |
| CCA3 | 1 | 0.0173 | 1.477 | 0.808 |
| CCA4 | 1 | 0.0110 | 0.944 | 0.910 |
| CCA5 | 1 | 0.0025 | 0.215 | 1.000 |
| CCA6 | 1 | 0.0009 | 0.076 | 1.000 |
| Residual | 28 | 0.3276 | | |

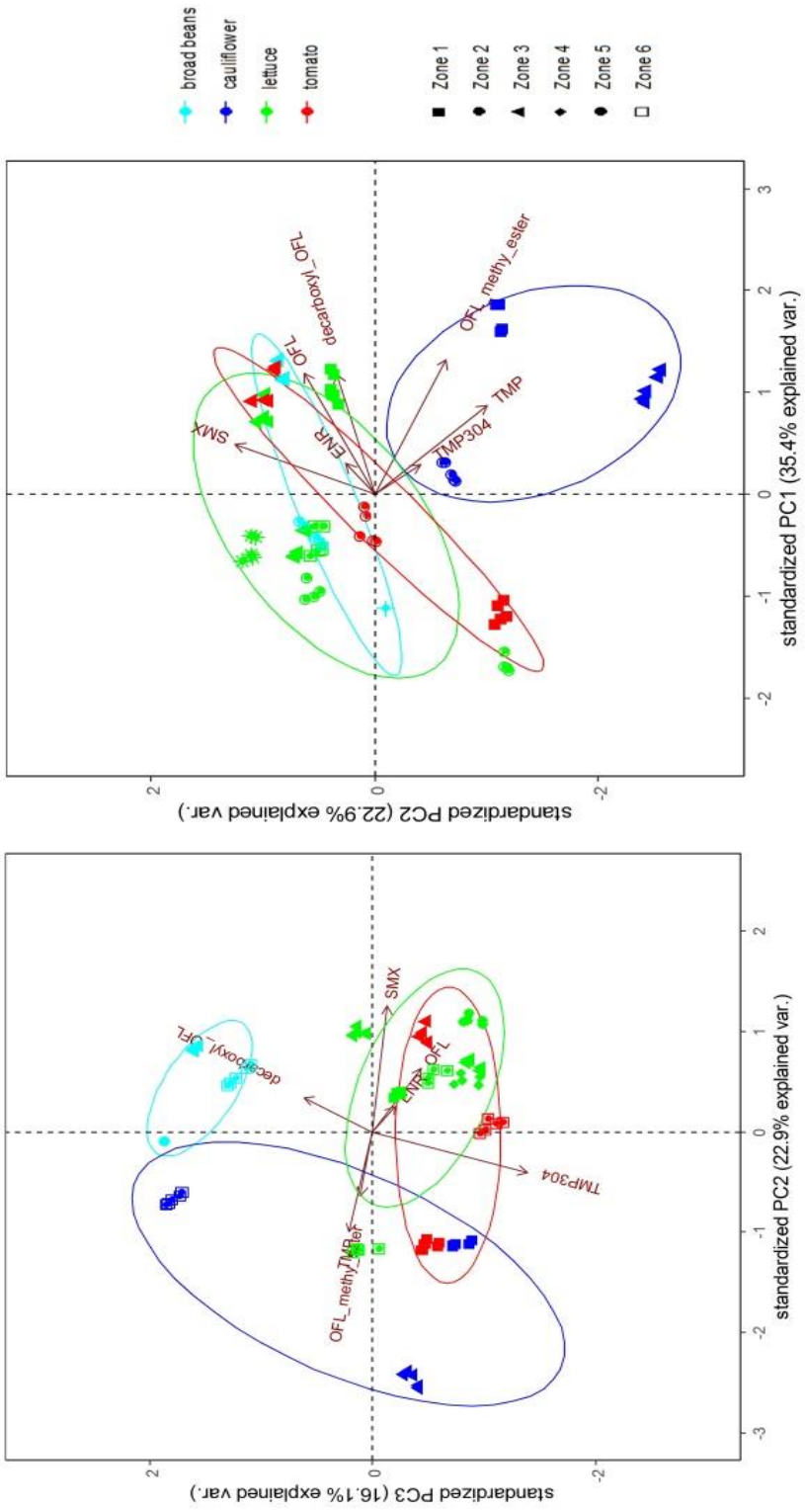
*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$.

Df: Degrees of freedom

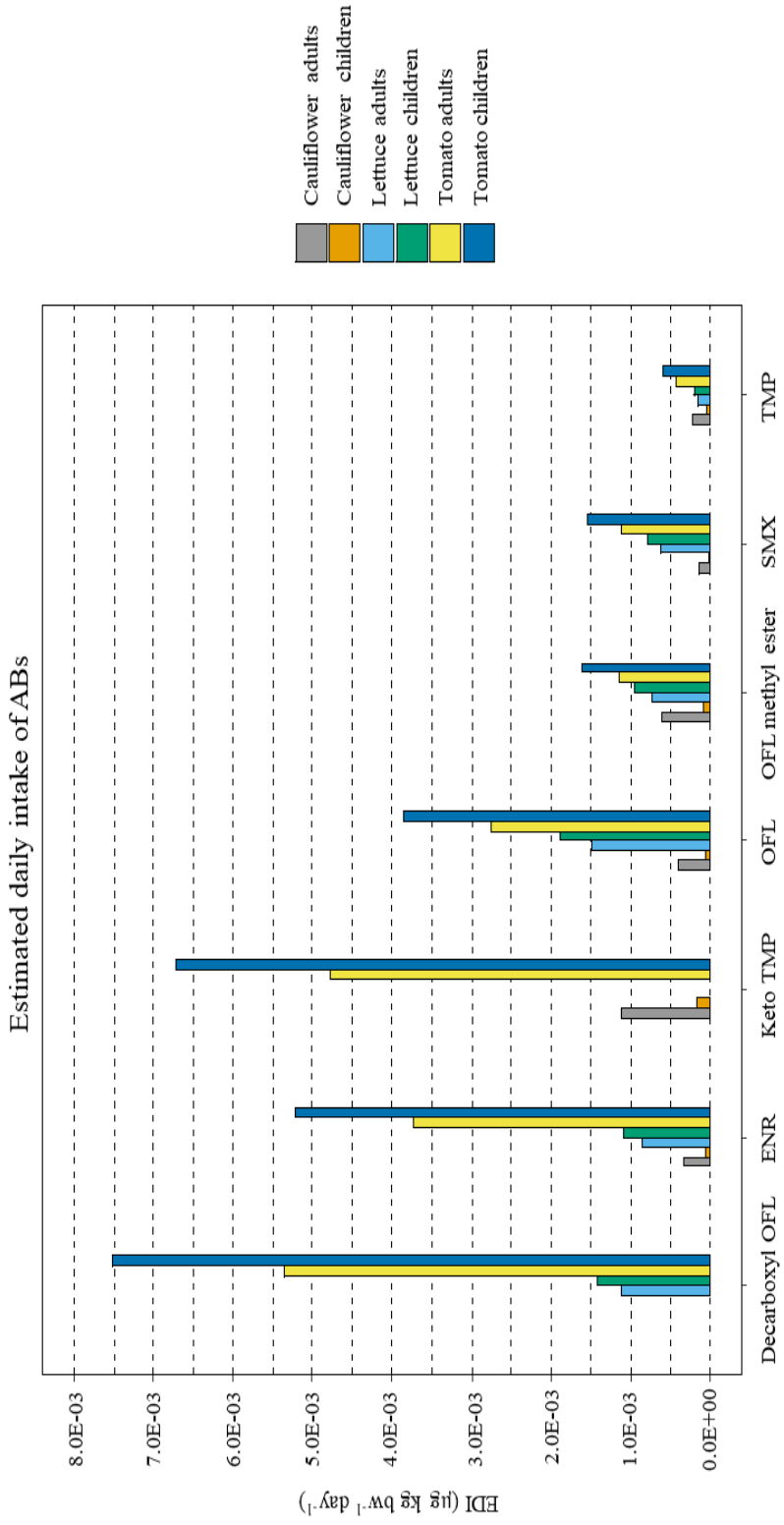
Supplementary Figure S3.1. Linear prediction of 75th percentile multivariate regression model for all vegetables in zones 3 and 4.



Supplementary Figure S3.2. Two dimensional plots of the first three principal components



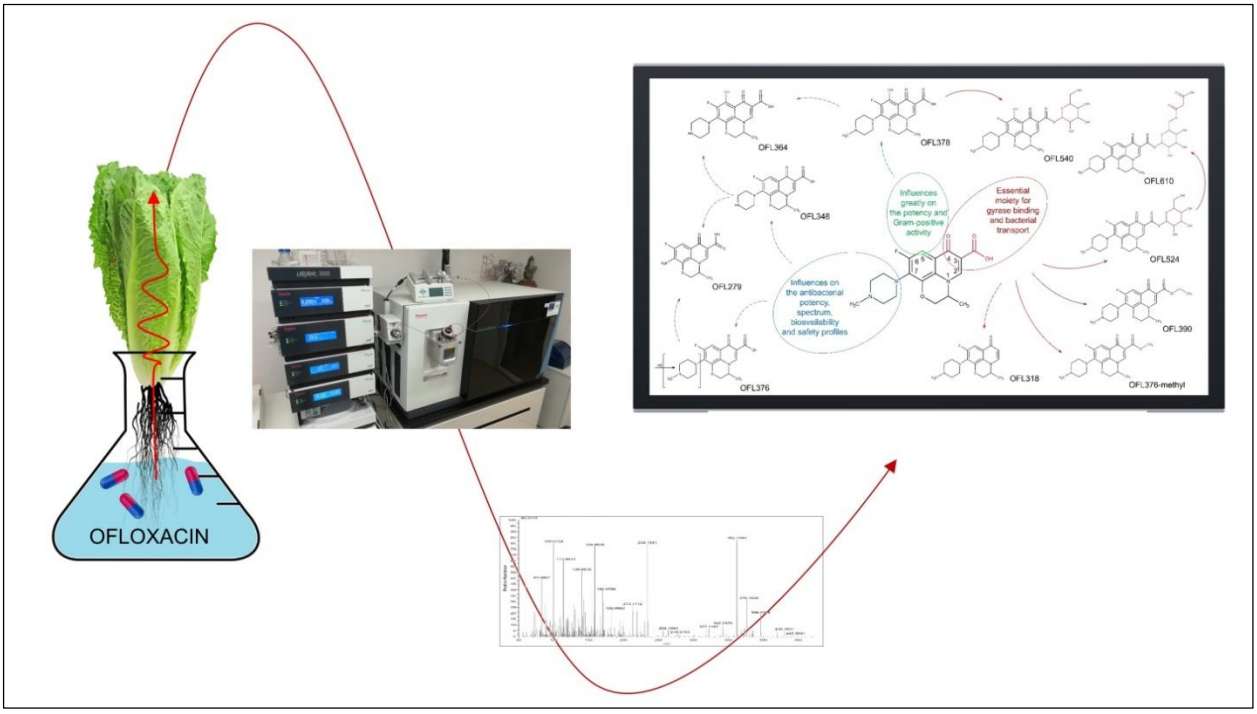
Supplementary Figure S3.3. EDIs of AB compounds through vegetable consumption for children and adults



# Chapter IV: Biotransformation pathways of ofloxacin in lettuce

This Chapter is based on the article:

Đorđe Tadić, Michal Gramblicka, Robert Mistrik, Cintia Flores, Benjamin Piña, Josep Maria Bayona. Elucidating biotransformation pathways of ofloxacin in lettuce (*Lactuca sativa* L). Environmental pollution, 260 (2020) 114002



***In brief:***

ABs can be uptaken by plants from soil desorption or directly from irrigation water, but their metabolization pathways in plants are largely unknown. In this paper, an analytical workflow based on high-resolution mass spectrometry was applied for the systematic identification of TPs of OFL in lettuce. The targeted metabolites were selected by comparing the mass chromatograms of exposed with control samples using an advanced spectra-processing method (Fragment Ion Search). The innovative methodology presented allowed us to identify a total of 11 metabolites, including 5 OFL metabolites that are being reported for the first time in plants. Accordingly, major transformation pathways were proposed revealing insight into how OFL and related chemicals are metabolized in lettuce. Furthermore, the influence of biotransformation on potential residual antimicrobial activity of identified compounds was discussed. Human exposure to ABs at doses below the MICs is crucial in human risk assessment, including food ingestion; however, in the case of OFL presented results reveal that plant metabolites should also be considered so as not to underestimate their risk.

## 4.1. Introduction

Organic xenobiotics (e.g., pharmaceuticals, personal care products, pesticides, polycyclic aromatic hydrocarbons) can be uptaken and metabolized in plant tissues, which can affect the plants' metabolism, as well as the organisms that feed on them, including humans [169,170]. Xenobiotic detoxification by plants can be classified into three phases: I) transformation, usually hydrolysis, reduction, or oxidation; II) conjugation with a polar molecule (e.g., sugars, amino acids, malonic acid, glutathione) and esterification; and III) internal compartmentation and storage in vacuoles or the cell wall [47]. If the xenobiotic already have a functional group suitable for phase II metabolism, then detoxification can precede without the need for phase I metabolism. Unlike phase I, which can produce phytotoxic metabolites, phase II products are either nontoxic or less toxic than the parent compound [171]. The occurrence of xenobiotic metabolites has already been investigated. For instance, the concentration of 10,11-dihydro-10,11-dihydroxy-carbamazepine and the biological activities of 2-hydroxy-carbamazepine and 3-hydroxy-carbamazepine are similar or even higher than those of the parental compound (carbamazepine) [172]. In their investigation of glycosylated conjugates of 11 contaminants of emerging concern in lettuce, Hurtado et al. [173] demonstrated the need for metabolite identification and quantification since the conjugated fraction of some of the investigated compounds accounted for a significant fraction of the free parent compound (between 27 and 83%).

ABs are a class of xenobiotics of special concern. AB residues in the environment exert a selection pressure on the environmental microbiome and, thus, generate environmental reservoirs of ARB and ARGs. AB resistance is recognized as a serious and growing global threat in modern human and veterinary medicine [13].

Numerous studies have demonstrated that ABs can be taken up and bioaccumulated in plants [98,102,130,134,144,146,174]. In contrast, little attention has been paid to the identification of AB phytometabolites [49,50,52,175,176]. Moreover, the concern over the occurrence of AB metabolites is not limited to their toxicity; they can also retain antimicrobial activity and promote AB resistance. For instance, some SMX TPs (e.g., hydroxyl-SMX, acetyl-SMX, nitro-SMX) have been shown to exhibit the targeted mechanism of action of the parent compound [105]. Strikingly, Majewsky et al. [105] even found that two SMX TPs (4-$NO_2$- and 4-OH-SMX) inhibit *Vibrio Fischeri* growth to a clearly greater extent than the parent compound. A similar phenomenon has been observed for fluoroquinolones. Čvančarová et al. [106] investigated the residual antimicrobial activities of fluoroquinolones (OFL, norfloxacin, and ciprofloxacin) TPs produced by ligninolytic fungi. The biotransformation took place on the

piperazinyl moiety of the fluoroquinolone scaffold, resulting in rather high residual activity remaining after the biodegradation [106]. Additionally, some metabolites, such as the glucose conjugates, may incorporate the complete AB structure. These metabolites may remain inactive until they are transformed in the human digestive tract by the cleavage of the conjugates, releasing the “trapped” AB in the free form [177].

Concerns have been raised regarding the occurrence of AB metabolites (i.e., decarboxyl OFL and keto TMP) in vegetables, and an analytical method for their quantification has been reported (Chapter II). However due to the nature of the target screening approach used, the methodology can only be used to analyze commercially available metabolites. Consequently, other metabolites and TPs not included in the targeted list would remain overlooked. To overcome this shortcoming, this study applies non-target screening to provide further insight into AB TPs and metabolites in plants. More specifically, bench scale experiments were performed and a novel high-resolution mass spectrometry methodology was applied to identify OFL TPs and metabolites in lettuce grown in hydroponic conditions.

OFL was selected for this study as it is frequently detected in WW and RWW, has high persistence in soil, and has a high uptake potential [136,178]. Moreover due to their usage in livestock farming, fluoroquinolones have been classified as “critically important” by the WHO, requiring the immediate development and implementation of risk-management strategies [179].

Along with plants, microorganisms are involved in ecosystem self-purification processes, and microbial biodegradation is considered as important process for eliminating (transforming) most xenobiotics [180]. Microorganisms can degrade contaminants through metabolic and/or co-metabolic pathways [180]. However, Singh et al. [181] discovered that plant (duckweed) metabolism is the primary mechanism of OFL removal from media. But our knowledge of its biotransformation pathways in plants remains essentially incomplete. A better understanding of the OFL biotransformation pathways could improve metabolism predictions. Specifically, we can apply this knowledge about plant transformation to other fluoroquinolones and chemicals that have the same functional groups and/or structure as OFL.

Accordingly, the main objectives of this study, intended to shed light on these chemical and biological problems, are, respectively: 1) to identify plant and/or microbial OFL metabolites (phase I and phase II) and OFL TPs in lettuce using high-resolution LC-HRMS$^n$ spectra elucidation; and 2) to assess OFL biotransformation pathways in lettuce.

## 4.2. Materials and methods

Information about used chemicals, sample extraction and methodologies for metabolite quantification is listed in Section 4.5.

### 4.2.1. Experimental setup

Lettuce (*Lactuca sativa* L) seeds were germinated in plastic trays in the dark. After the seventh day, the seedlings were transferred to a hydroponic system. Control plants were grown in Hoagland's nutrient solution, while the exposed seedlings were irrigated with Hoagland's nutrient solution spiked with OFL (5 µg $mL^{-1}$). Because it can sometimes be impossible to acquire characteristic fragments due to the low intensities in the mass spectra of the corresponding precursor ions, high enough concentration of ABs were added to the nutrient solution to enable comprehensive $MS^n$ screening [182,183], without overlooking the "low-intensity metabolites" that may have antimicrobial properties or toxicity. All treatments were performed in triplicate. Fresh growing solution fortified with OFL was added every three days. Hydroponic containers were wrapped in aluminum foil to prevent OFL photodegradation. Thirty days after AB exposure, the plants were harvested and separated into leaves and roots. All samples were comminuted with liquid nitrogen and stored at −20°C until analysis. Experiment was done in nonsterile conditions in order to simulate as much as possible real field scenario.

### 4.2.2. LC separation

The UltiMate 3000 UPLC station (Dionex, Thermo Fisher Scientific, Waltham, MA, U.S.A.) was equipped with a Kinetex C18 column (particle size 2.6 µm, ID 2.1 mm, length 50 mm; Phenomenex, Torrance, CA, U.S.A.), including a pre-column. The column was thermostated at 25°C, and the samples were kept at 15°C in the autoinjector. The flow rate of the mobile phase was set to 0.25 mL $min^{-1}$. Mobile phase A was water with 0.1% v/v formic acid. Mobile phase B was acetonitrile with 0.1% v/v formic acid. A linear gradient elution was used: 0 min – 97% A, 3 min – 97% A, 15 min – 80% A, 17 min – 5% A, 19 min – 5% A, 20 min – 3% A, 30 min – 97% A. An injection volume of 5 µL was used for all samples.

### 4.2.3. MS analysis

The Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was equipped with heated ESI. The measurement was performed in the positive ionization mode at a spray voltage of 3500 V. Nitrogen was used as the sheath and auxiliary gas with flow rates of 4.1 L $min^{-1}$ and 7.97 L $min^{-1}$, respectively. The sweep gas was turned off. The ion transfer tube and vaporizer temperature were set at 325°C and 275°C, respectively. The full scan ($MS^1$) was performed using an Orbitrap detector at a resolution of 60,000, in the mass range from 50 to 1000 Da, with a maximum injection time of 100 ms, automatic gain control target of 500,000, and 3 averaged microscans. To obtain the $MS^2$ and $MS^3$ scans, two fragmentation techniques were performed, namely: collision-induced dissociation (CID) at normalized collision energies of 20, 25, 40, and 60 (maximum = 100); and higher-energy collisional dissociation (HCD) at normalized collision energies of 20, 30, and 60 (maximum = 200). The collision pressure was $8 \cdot 10^{-3}$ Torr. The isolation width was 1 Da in both $MS^2$ and $MS^3$; the automatic gain control target was lowered to 40,000 ion capacity, and the maximum injection time was 10 ms.

### 4.2.4. Selection of candidate m/z values for $MS^n$

The mass chromatograms ($MS^1$ full scans only) of the samples grown on OFL-rich medium were compared with those of the metabolite-free blank samples grown in the absence of the OFL. The ions whose peak intensity exeed 70% between the exposed and control samples were targeted. The second approach for identifying possible metabolites in plants consisted of using advanced spectra-processing methods, namely Fragment Ion Search (FISh) feature of the Mass_Frontier™ 8.0 software (HighChem, Bratislava, Slovakia). More specifically, comprehensive fragmentation pathways based on a set of general ionization, fragmentation, and rearrangement rules are applied to generate possible fragments of selected parent compound. Those fragments are further on utilized to filter out the majority of matrix related ions. With FISh, metabolite peaks can be selectively resolved and identified from background matrix ions by searching for *in-silico* predicted fragments of known ABs. It utilizes the parent compound structure to filter out the majority of matrix related background ions. Using the FISh algorithm, all spectral and subsequent chromatographic peaks related to a metabolite are “fished out” and possible sub-structures for detected metabolites are displayed. It also provides extensive list of biotransformation pathways.

### 4.2.5. Data-processing and interpretation

High-resolution extracted ion chromatograms of the parent OFL and its potential phase I and II metabolites were obtained by processing the full scan data using Xcalibur™ 4.1 software (Thermo Fisher Scientific, Waltham, MA, U.S.A.) with a 5 ppm mass tolerance. FISh and MS/MS fragmentation prediction, both features of the Mass_Frontier™ 8.0 software (HighChem, Bratislava, Slovakia) were used to extract structurally related spectral peaks (peaks related to the parent AB in metabolite) and to simulate compounds' fragment ions, which can be compared with the fragmentation information from samples, respectively.

### 4.2.6. Identification, confirmation, and annotation

Elemental composition of the detected ions was proposed based on the accurately measured mass, Ring Double Bond (RDB) equivalent value, and comparison with the theoretical isotope pattern of the proposed sum formula [184]. The structure elucidation method was based on spectral trees according to MS/MS and $MS^n$ data, since $MS^2$ and $MS^3$ acquisitions generally produce mass spectra with minimal interferences as only ions within a selected m/z window are fragmented.

The level of confidence for the identification of the detected compounds was classified according to Schymanski et al. [185], namely: Level 1 - confirmed structure through matching with a reference standard; Level 2 - probable structure (this category was divided into two sublevels: 2a - probable structures through matching with literature or library spectrum; and 2b - probable structures based on diagnostic MS/MS fragments and/or ionization behavior, parent compound information, and the experiment's diagnostic evidence); Level 3 - tentative candidate(s), for which there is evidence for a possible structure or structures, but insufficient information for one exact structure only; Level 4 - unequivocal molecular formulas, which can be unambiguously assigned using the spectral information; and Level 5 – an exact mass of interest that can be measured in the sample and be of specific interest for the investigation. Accordingly, the molecular structures were primarily searched in ChemSpider (www.chemspider.com), whereas structure elucidation was based on the use of characteristic fragmentation during data-dependent MS/MS fragmentation. Finally, if there was a commercially available authentic standard of a given compound, the RT of sample ions and fragmentation spectra were matched to the measured reference substances.

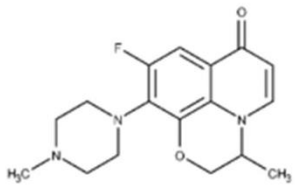
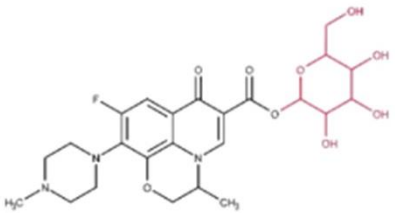
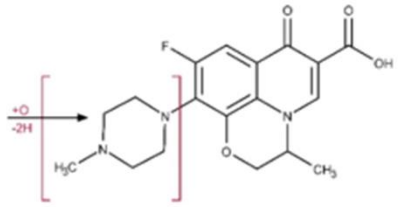
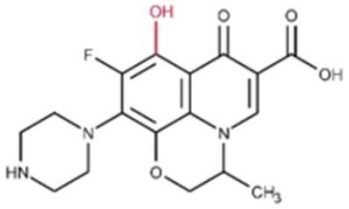
## 4.3. Results and discussion

### 4.3.1. Identification of metabolites using HRMS$^3$

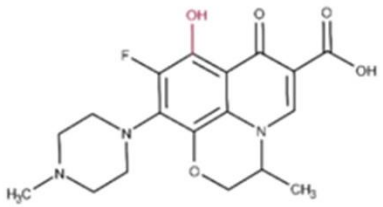
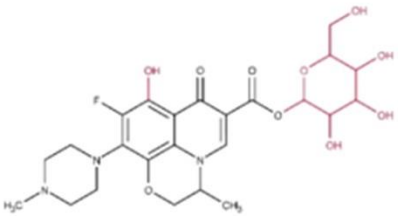
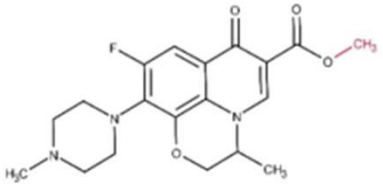
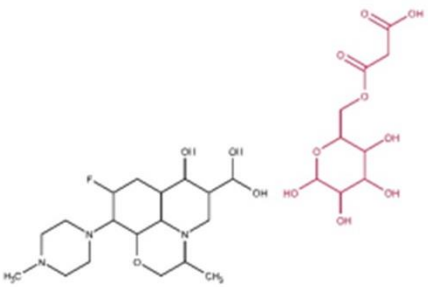
FISh algorithm revealed itself as handy tool when it comes to structural elucidation of unknown compounds. This novel integrated approach provides the functionality required for the elucidation of metabolites, even if their spectra are not present in the libraries (e.g., MassBank and mzCloud™) and their analytical standards are not available, as was the case with the OFL phase II metabolites. This *in-silico* feature reduced the number of unnecessary acquisitions, which would be performed if only the chromatogram spectra comparison (exposed vs. control) were applied. Finally, a list of 78 candidate metabolites with their m/z values was compiled. Following the exclusion of the peaks identified with the aforementioned two methods showing S/N ratios less than 3 and intensities lower than 1 x $10^5$ counts, as the intensity would not be sufficient to acquire reasonable quality MS/MS data [108]. These m/z values were the most likely to be associated with the compounds related to OFL and were subsequently targeted for $MS^2$ and $MS^3$ fragmentation analyses, intended for molecular confirmation/identification. Ultimately molecular structures were assessed for 11 compounds.

The mass spectral information and structures of the OFL and its proposed metabolites are shown in Table 4.1. In all, 11 metabolites are described, with Level 1 (n=4), Level 2b (n=3), Level 3 (n=3), and Level 4 (n=1) confirmation confidence. The mass measurement error was lower than 3 ppm for all compounds except OFL348 (-3.42 ppm). The CID spectra and descriptions of the identified metabolites and their prominent fragments, containing proposed structures and molecular formulas with relative errors and RDB equivalents, are shown in the supplementary information of this Chapter.

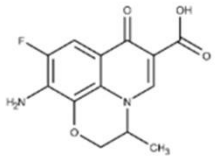
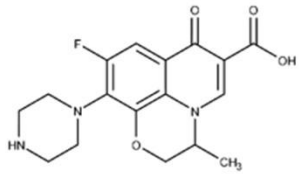
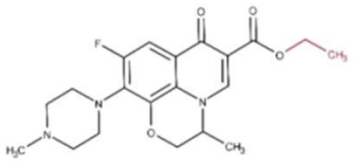
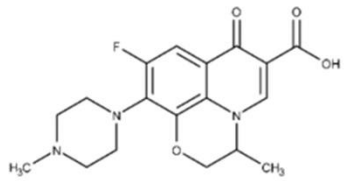
Table 4.1. Mass-spectral information of ofloxacin and proposed metabolites

| Compound ID | Predicted formula (assignment) | Structure proposed | RT (min) | Observed $[M+H]^+$ (m/z) | Error (ppm) | Characteristic fragments[a] (m/z) | Level[b] |
|---|---|---|---|---|---|---|---|
| OFL318 | $C_{17}H_{20}O_2N_3F$ (M $-CO_2$) |  | 1.0 | 318.1609 | -1.04 | 298.1548<br>261.1031<br>205.0970<br>188.0705 | 1 |
| OFL524 | $C_{24}H_{30}O_9N_3F$ (M $+C_6H_{10}O_5$) |  | 2.1 | 524.2029 | -1.89 | 480.2133<br>404.1606<br>362.1502<br>318.1605<br>261.1028 | 3 |
| OFL376 | $C_{18}H_{18}O_5N_3F$ (M +O $-2H$) |  | 4.7 | 376.1293 | -2.81 | 358.1185<br>333.0748<br>315.0641<br>287.0693<br>272.0459<br>259.0744 | 3 |
| OFL364 | $C_{17}H_{18}O_5N_3F$ (M $-CH_2$ +O) |  | 5.0 | 364.1293 | -2.82 | 346.1184<br>316.1284<br>298.1178<br>279.0767<br>261.0662 | 2b |

(continuation) Table 4.1

| Compound ID | Predicted formula (assignment) | Structure proposed | RT (min) | Observed $[M+H]^+$ (m/z) | Error (ppm) | Characteristic fragments[a] (m/z) | Level[b] |
|---|---|---|---|---|---|---|---|
| OFL378 | $C_{18}H_{20}O_5N_3F$ (M +O) |  | 5.6 | 378.1450 | -2.68 | 361.1422<br>334.1553<br>317.1525<br>247.0873 | 2b |
| OFL540 | $C_{24}H_{30}O_{10}N_3F$ (M +$C_6H_{10}O_5$ +O) |  | 6.7 | 540.1973 | -2.75 | 523.1946<br>378.1448 | 3 |
| OFL376-methyl | $C_{19}H_{22}O_4N_3F$ (M +$CH_2$) |  | 8.3 | 376.1660 | -1.91 | 362.1500<br>319.1079<br>305.0923<br>265.0612 | 1 |
| OFL610 | $C_{27}H_{32}O_{12}N_3F$ (M +$C_9H_{12}O_8$) |  | 8.3 | 610.2034 | -1.39 | 362.1498 | 4 |

(continuation) Table 4.1

| Compound ID | Predicted formula (assignment) | Structure proposed | RT (min) | Observed $[M+H]^+$ (m/z) | Error (ppm) | Characteristic fragments[a] (m/z) | Level[b] |
|---|---|---|---|---|---|---|---|
| OFL279 | $C_{13}H_{11}O_4N_2F$ (M $-C_5H_9N$) |  | 10.0 | 279.0768 | -2.73 | 261.0663<br>238.0378<br>191.0248<br>164.0378 | 2b |
| OFL348 | $C_{17}H_{18}O_4N_3F$ (M $-CH_2$) |  | 10.4 | 348.1353 | -3.42 | 330.1247<br>304.1454<br>284.1393<br>261.1034 | 1 |
| OFL390 | $C_{20}H_{24}O_4N_3F$ (M $+C_2H_4$) |  | 10.7 | 390.1825 | 0.35 | 362.1509<br>344.1403<br>333.1244<br>305.0931<br>287.0826 | 1 |
| OFL | $C_{18}H_{20}O_4N_3F$ (M) |  | 11.4 | 362.1506 | -1.27 | 318.1609<br>298.1546<br>261.1030<br>219.0562 | 1 |

M-parental drug; RT-retention time; [a] CID spectra are shown in supplementary material; [b] According to Schymanski et al.[186]: Level 1 - confirmed structure; Level 2a - probable structures through matching with literature spectrum; Level 2b - probable structures based on diagnostic MS/MS; Level 3 - tentative candidate(s); Level 4 - unequivocal molecular formulas; and Level 5 – an exact mass of interest

## Major biotransformation pathways

To date, this is the first study to report OFL metabolites in lettuce. The pathways responsible for the metabolization of OFL, schematically shown in Figure 4.1, will be further discussed.

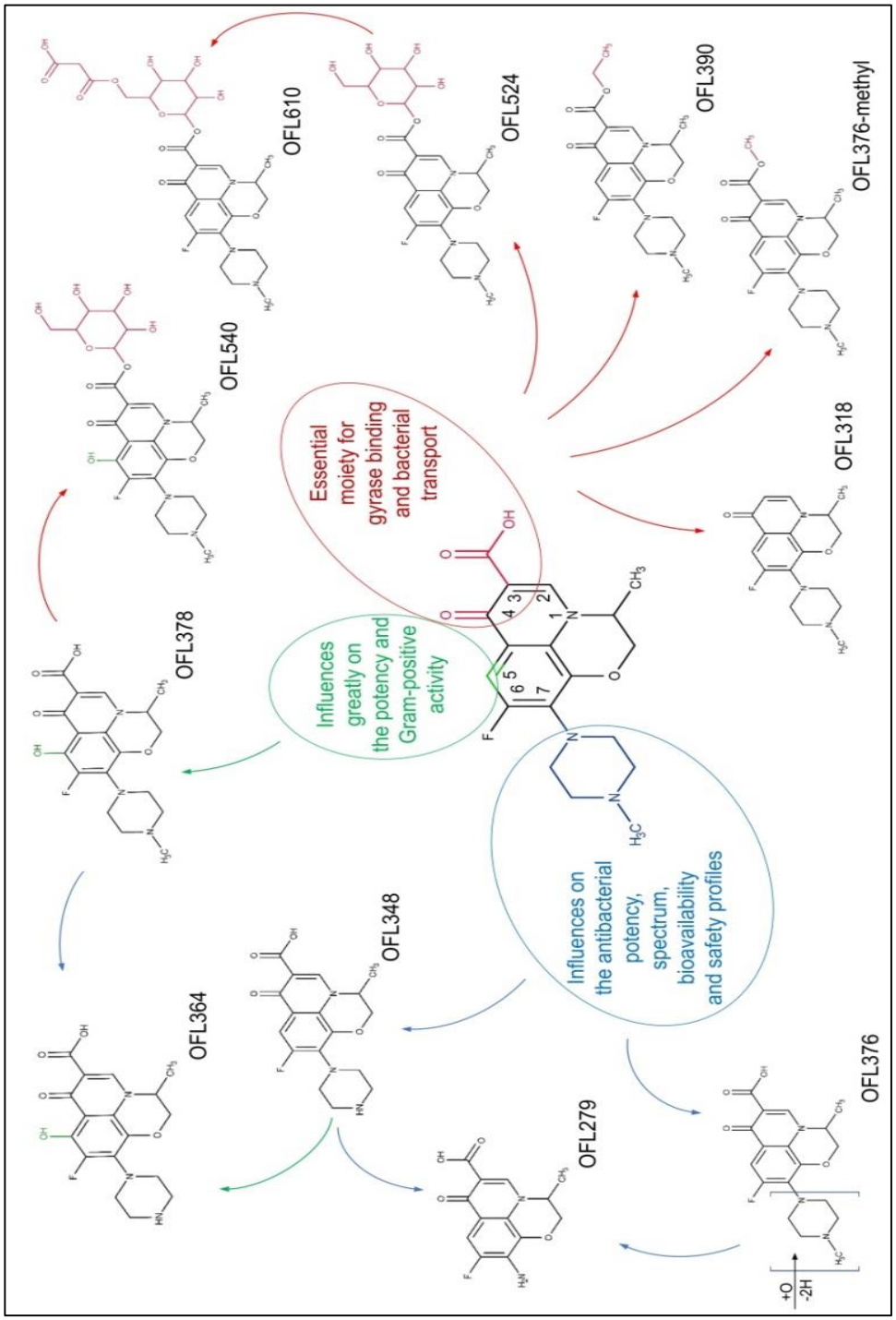



Figure 4.1. Proposed biotransformation pathways of ofloxacin and impact on antimicrobial activity. Metabolization was observed at three positions: on the piperazine ring at C7 and C5 and in the carboxylic group at C3, marked in blue, green, and red, respectively. The structure-activity relationship with the modifications of different positions of the ofloxacin nucleus has been modified from Anderson et al. [199] and Gao et al. [212].

The fluorine at position C6 and the fused ring connecting the N1 to the C8 position remained intact. Hydroxylation, at the C5 position on the aromatic ring (OFL364 and OFL378) and on the methyl-piperazine ring (OFL376), led to the formation of OFL metabolites. The hydroxylation of OFL in lettuce was expected to be enzyme-catalyzed, as reported for diclofenac in barley and horseradish [123]. In fact, the hydroxylation reactions to the diclofenac proved to be catalyzed by cytochrome P450 as in mammals [123]. Additionally, the results presented by He et al. [186] show the correlation of the occurrence of two hydroxy intermediates of ibuprofen with the trend of higher P450 activity. Hydroxylation of ENR (Figure 4.2) has been found in cow's milk, suggesting that it is the major phase I metabolization pathway in cows [187]. Similarly, Zhao et al. [188] observed rapid and efficient hydroxylation of ciprofloxacin in Chinese flowering cabbage as presented in Fig. 4.2. Additionally, both studies reported metabolites of ENR and ciprofloxacin that underwent transformation on the piperazine ring, resulting in the remaining amino group substituent at the C7 position of the quinolone structure (Fig. 4.2). Similarly, OFL279 may be formed after several consecutive reactions, e.g., the cleavage of the methyl-piperazine ring, after either prior N-demethylation (OFL348) or oxidation (OFL376) of the OFL, which is consistent with the metabolites reported by Čvančarová et al. [106]. The OFL279, OFL348, and OFL376 metabolites have also been described as products of OFL degradation by *Trametes versicolor* [189].

N-demethylation, which was noted for OFL348 and OFL364, was also one of the OFL biodegradation processes by mixed bacterial culture [77] and ligninolytic fungi [106]. Moreover, cytochrome CYP450-dependent N-demethylation has been detected as a means of detoxification of herbicides in plants [48]. As can be seen in Fig. 4.1, OFL364 can be formed from two forerunners (OFL378 and OFL348). Specifically, OFL364 underwent multistep reactions at two different sites of the OFL molecule, namely, hydroxylation at C5 and N-demethylation at the C7 substituent. All the aforementioned metabolites were classified as phase I metabolites, since dealkylation and oxidation reactions are mainly catalyzed by cytochrome P450 enzymes [190]. As a potential biotransformation enzyme in plant and fungi [191,192], aryl acid decarboxylase may catalyze decarboxylation of OFL and the formation of another phase I metabolite: OFL318.

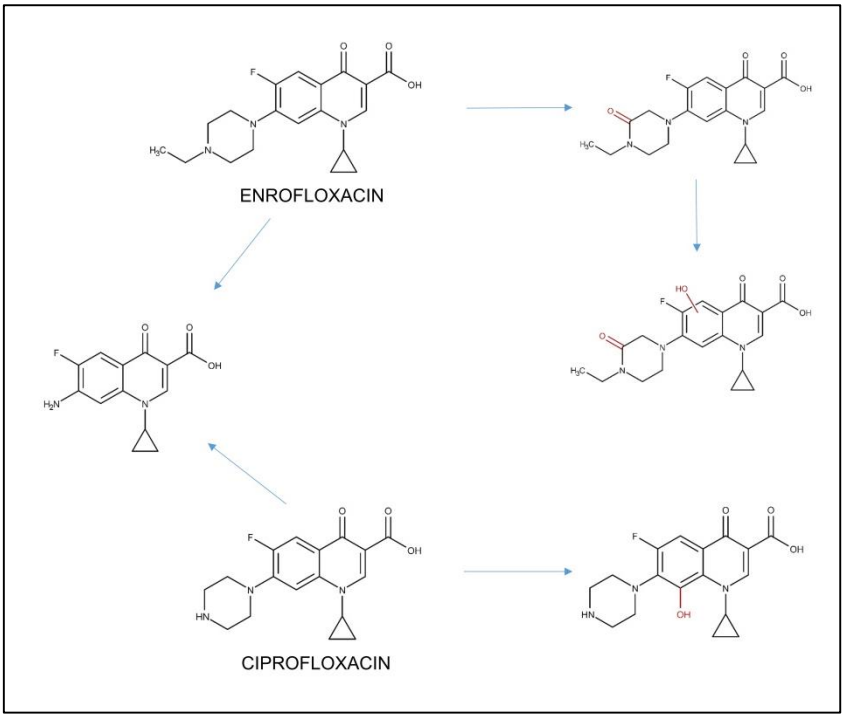



Figure 4.2. Extracted metabolization pathways for enrofloxacin [187] and ciprofloxacin [188] in cow's milk and in Chinese flowering cabbage, respectively

Three out of four of the observed reactions are well established in phase II metabolism. Specifically, methyltransferase was considered to be related to the methylation of clarithromycin, which forms a phase II metabolite in lettuce [49]. Thus, O-methyl-transferase can be considered to transfer a methyl group from the lettuce to the OFL in the case of OFL376-methyl. Glycosylation, catalyzed by O-glucosyl-transferase, is a common plant detoxification strategy against xenobiotics that leads to the formation of phase II glycosylated metabolites [50,123,186,193–195]. The conjugation of glucose with OFL or hydroxyl-OFL forming OFL524 or OFL540, respectively, was triggered by the carboxylic acid at the C3 position, which appeared to be the most active site on the OFL scaffold. Additionally, O-malonyl-β-D-glucoside conjugates are often formed in plants and have been proposed to be equivalent to animal β-D-glucuronides [46]. Early literature on xenobiotic metabolism in plants indicates that xenobiotic-ß-D-glucosides are frequently further metabolized. No direct malonyl conjugation of herbicides has been reported, but several examples are available for a malonyl transfer after an activation or primary conjugation [194]. Thus, OFL524 may undergo further conjugation reactions with malonic acid, catalyzed by malonyl-CoA-transferases, forming OFL610 as a stable end product in the vacuole. Lastly, OFL390 was identified as a conjugate with ethanol. The enzyme involved in this reaction, which has not been well characterized yet, could be acyl ethyl ester synthase.

To the best of authors' knowledge, all OFL phase II metabolites (n=5) presented in this study are being reported for the first time in plants. Although the presence of one (decarboxyl OFL) out of 3 targeted OFL metabolites was confirmed in lettuce irrigated with reclaim water (Chapter II), the reported results here suggest that there are other metabolites and TPs which are overlooked and should be considered in a comprehensive assessment.

Among the OFL metabolites identified in this study, three of them (OFL318, OFL376-methyl and OFL390) were able to be quantified according to authentic standard availability showing that OFL318 (decarboxyl OFL) with a concentration of 1.2 µg $g^{-1}$ fw was the most prominent among the quantified metabolites, followed by OFL376-methyl (0.7 µg $g^{-1}$ fw) and OFL390 (0.2 µg $g^{-1}$ fw). Detected OFL concentration was the highest with concentration of 5.6 µg $g^{-1}$ fw. Although growing medium was not analyzed, bioconcentration factor (BCF) was roughly estimated as the ratio of the OFL concentration in lettuce (µg $g^{-1}$ fw) to the spiked (nominal) concentration in the growth medium (µg $mL^{-1}$). BCF estimated in such way serves as a theoretical minimum and with a value of 1.12 mL $g^{-1}$ raises concern related to the uptake of OFL by lettuce in field scale conditions. The estimated BCF value is almost 15 times higher than the one reported by Marsoni et al. [100] OFL in *Eruca sativa* leaves. Thus, a follow up study to investigate uptake and translocation factors of OFL and its metabolites would be highly beneficial.

One important difference between animal and plant metabolism is that pharmaceuticals, in the final phase of plant metabolism (phase III), are not excreted but stored in cell walls and vacuoles. Consequently, identified TPs and metabolites need to be investigated and incorporated in the monitoring schemes to better assess environmental and human health risk.

### 4.3.2. Potential residual antimicrobial activity

The relationship between the chemical structure and the biological activity of fluoroquinolones is well established, and significant moieties for antimicrobial potency have been identified. Based on the OFL mechanism of action and the fact that structurally related compounds often display similar biological activity; it was possible to conclude that the transformation of OFL influences antimicrobial activity. In this regard, the followed discussion provides reasons to conduct experiments to investigate the biological activity of identified metabolites *in vivo*.

Specifically, the carboxylic acid side chain at the C3 position and the exo-cyclic oxygen at position C4 have been found to be essential for antimicrobial activity [196,197]. Five metabolites (OFL279, OFL348, OFL364, OFL376, and OFL378) retain this region of the OFL molecule, which serves as the chelation site for a divalent magnesium +2 ion (Mg) that binds to the DNA gyrase of the bacterial cell and also plays a role in bacterial membrane transport. However, some phase II metabolites that undergo bulky substitution in this sensitive area, such as OFL524, OFL540, and OFL610, may retain the complete, if dormant, parental antimicrobial potency. For instance, it could be judiciously concluded that the *O*-glycosylation of the carboxylic acid greatly interferes with the stereochemistry around that crucial area, leading to substantial loss of the parental activity during drug metabolization. Conversely, this may lead to its being overlooked, since the dormant antimicrobial activity can simply be reclaimed by deconjugation in the presence of a suitable enzyme, which may, in fact, take place in the human gut [173,190].

According to previous studies, changes at C5 position had a moderate influence on potency; an amino, hydroxyl, or methyl group can markedly increase in vitro activity against gram-positive bacteria [196,198]. For some fluoroquinolones, additions at C5 have already been proven to be beneficial for antimicrobial activity, as with sparfloxacin ($NH_2$) and grepafloxacin ($CH_3$) [199]. Therefore, since no bulky substituents were observed at this position, except for the aforementioned hydroxyl group, it can be concluded that the antimicrobial potency of OFL364 and OFL378 was not critically decreased, but there is a solid theoretical basis to experimentally check for the exact opposite scenario.

It is known that certain combinations of ABs may have synergistic effect, means that each new compound added lowers the MSC of the others [200]. Additionally, the knowledge of environmental concentrations that might exert selection pressure for ARB is limited [58]. Due to aforementioned results, attention should be called to the possible contribution of a multi-drug mixture of AB metabolites that retain antimicrobial activity to the selection of ARGs, especially in complex environmental conditions.

## 4.4. Conclusions

There is a growing concern about AB contamination of vegetables as a factor of AB resistance promotion. Thus, simple bench scale experiments were performed followed by comprehensive screening using Fusion Lumos, the advanced performance mass spectrometry system, and the foremost data analysis software in order to identify and to assess the possible structures of TPs of OFL in lettuce. In total 11 TPs, of which 5 metabolites were reported for the first time

in plants, are identified and their molecular structures are proposed with different confidence levels along with their CID spectra. Additionally, to date this is the first study that investigates the major biotransformation pathways involved in lettuce metabolization strategy that consequently led to biotransformation of OFL. As reported, glycosylation among others, catalyzed by O-glucosyl-transferase, was a common detected plant detoxification strategy detected in case of 3 plant metabolites. The conjugation of glucose with OFL or hydroxyl-OFL was triggered by the carboxylic acid at the C3 position, which appeared to be the most active site on the OFL scaffold. The proposed biotransformation pathways of OFL can be useful in the prediction of metabolization of other fluoroquinolones and chemicals with similar structure and functional groups. Finally, as the main risk of AB occurrence in the environment is promotion of antimicrobial resistance, plausible effect of biotransformation on residual antimicrobial activity was considered. Although it has to be experimentally confirmed, there is a theoretical indication of antimicrobial activity persistence.

The contribution of AB biotransformation to the dissemination of environmental sources of AB resistance has to be investigated especially since a certain number of the new medicines are, in fact, only structural modifications of existing drugs. Thus, future research should be focused on quantification of identified biotransformation compounds in vegetables, as well as on investigating their antimicrobial activity on relevant bacteria in order to prioritize which TPs should be included in AB contamination monitoring schemes.

## 4.5. Supplementary information

### 4.5.1. Materials and methods

#### 4.5.1.1. Chemicals

OFL Methyl Ester (CAS 108224-82-4), OFL Ethyl Ester (CAS 177472-30-9), deacrboxyl OFL (CAS 123155-82-8), and OFL Impurity E (demethylated OFL) (CAS 82419-52-1) were purchased from LGC standards S.L.U. (Barcelona, Spain). Selection of metabolite standards for purchasing was based on their availability and price. All standards were high-purity (95% or higher). More information regarding chemicals is provided in the Section 2.2.1.

#### 4.5.1.2. Sample extraction

Sample extraction procedure is described in Section 2.2.3.

#### 4.5.1.3. Analysis of OFL methyl ester and metabolite quantification

In order to prove that OFL methyl ester was not produced during sample preparation due to the usage of methanol in the analytical method additional experiments were done. Namely, the same procedure as used for procedural blank was applied with additional spiking step at the very beginning of the analytical procedure. Three extraction vials were spiked (1000 ng) with: (1) OFL; (2) OFL and OFL methyl ester; and (3) OFL methyl ester. Same sample preparation procedure was conducted as for the lettuce samples. Target screening revealed that OFL methyl ester in the first sample (spiked only with OFL) was below detection limit, proving that there was no detectable generation of methyl ester metabolite. Moreover, there was no observed difference in concentration of OFL methyl ester between the second and the third experiment, confirming the previous results. Aforementioned instrumentation and methodology were used to quantify identified metabolites in lettuce for which analytical standards were available.

Supplementary Figure S4.1. Most common fragmentation reactions

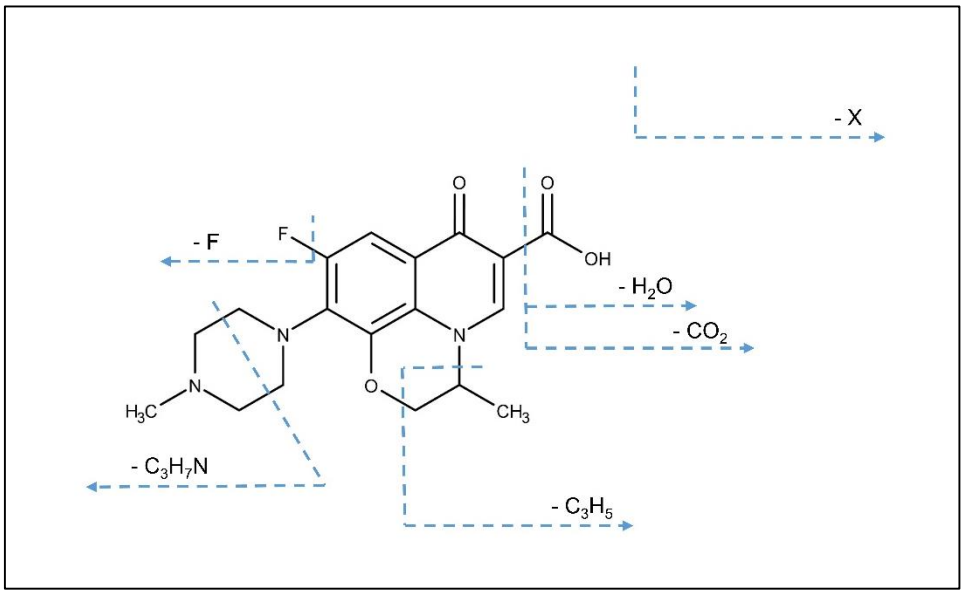
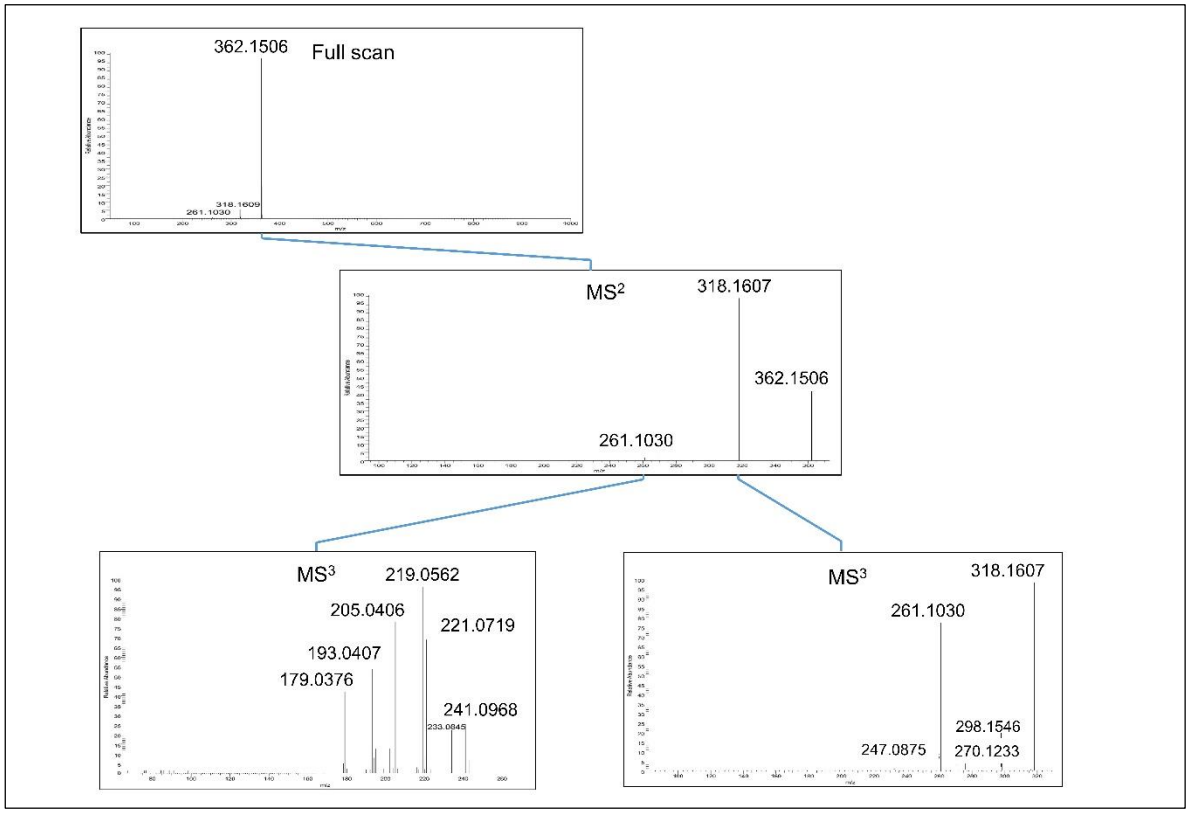


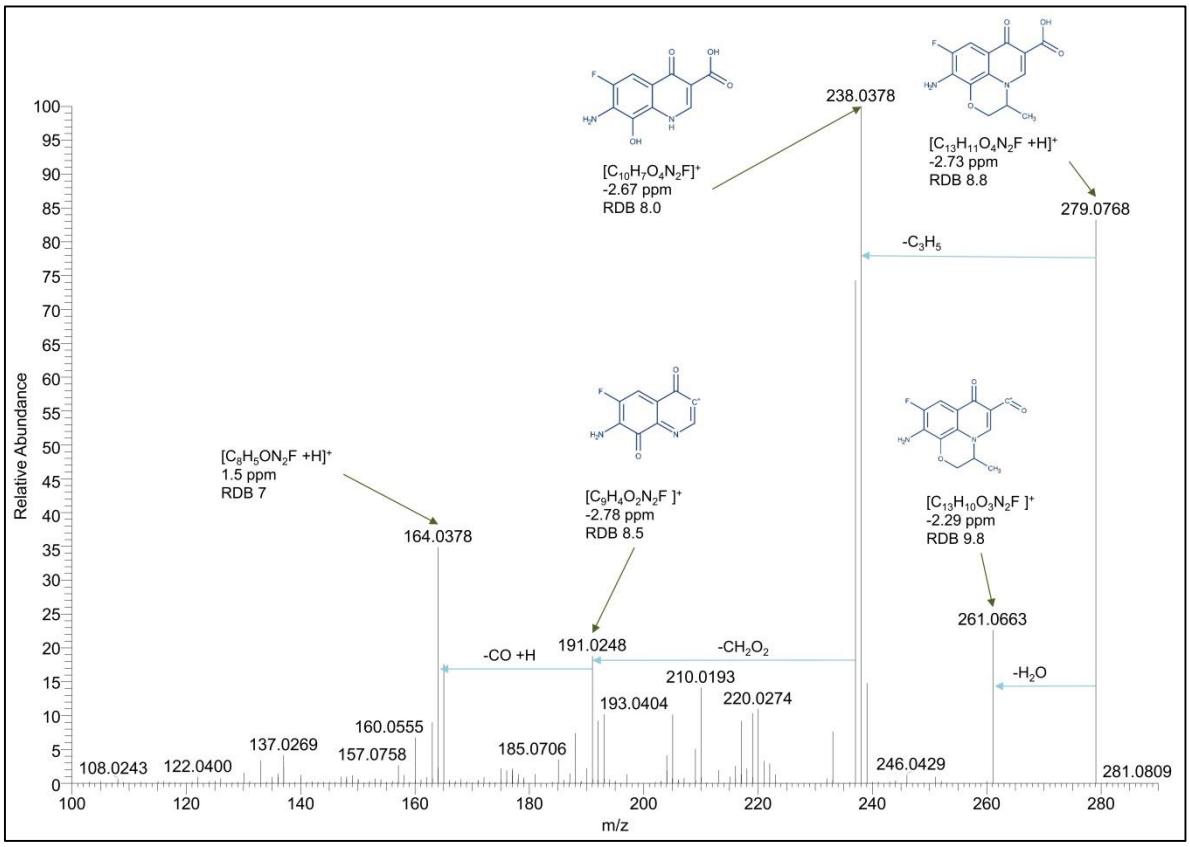

Figure S4.1 summarize the most common fragmentation reactions observed for detected compounds. One of the most common fragmentation processes for conjugated metabolite is the elimination of the added moiety (presented with “X” on the Fig. S4.1) revealing the parent OFL molecule. This was at the same time the most precious reactions in term of unambiguously confirmation of metabolite parent structure. Cleavages were observed on oxazine and methyl-piperazine rings, served as a back bone in analysis of fragmentation patterns. Although, deflourinted fragments were detected in spectra, fluorine played more important role in the structure assignment process. Namely, fluorine decreased the number of possible molecular formulas for a given m/z value. Finally, water loss and decarboxylation, typical for carboxylic group, were detected.

Supplementary Figure S4.2. Mass spectral tree of ofloxacin



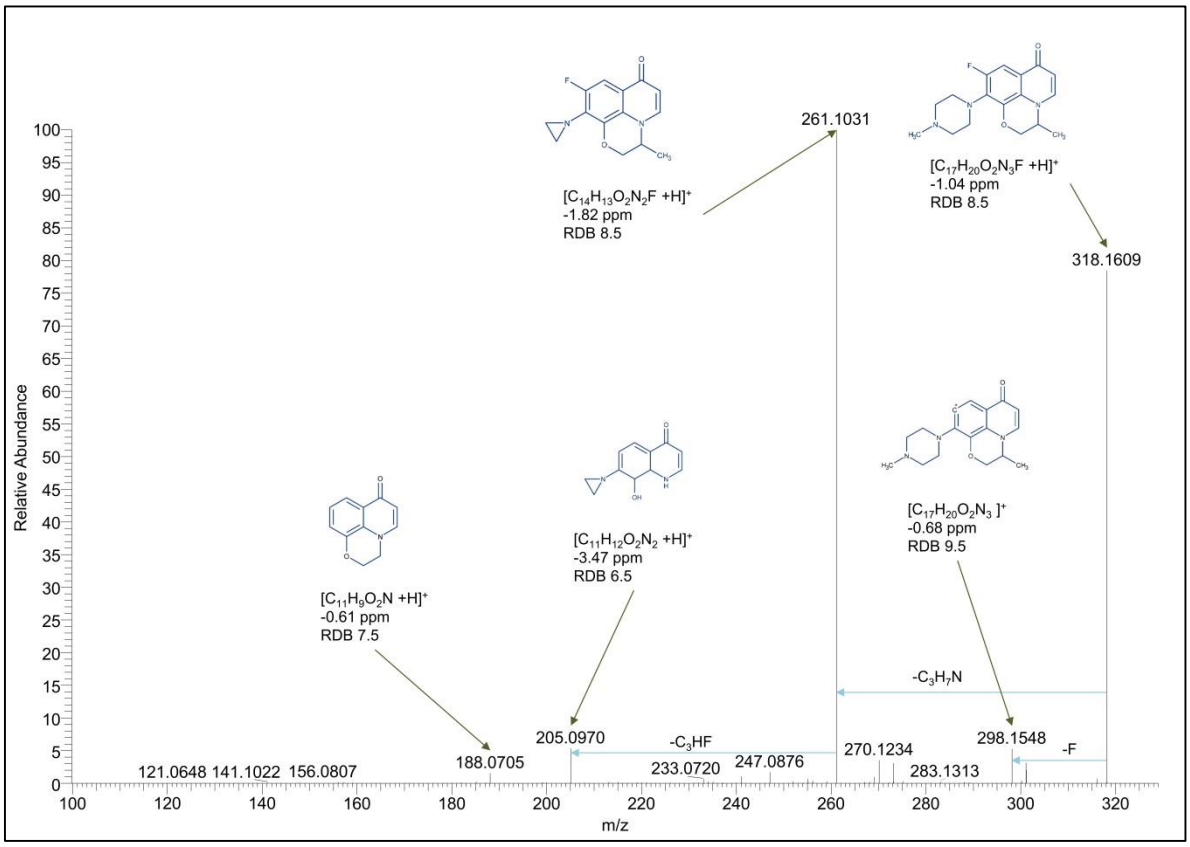

The OFL was eluted at 11.4 min giving the pseudo molecular ion $[M+H]^+$ at m/z 362.1506 (error: -1.27 ppm). The $MS^n$ spectra of OFL (Figure S4.2) with the $MS^3$ of its two main characteristic product ions at m/z 318.1607 and 261.1030 corresponded to the loss of the carboxyl group and methylaziridine, respectively. Further fragmentation of those two product ions yielded fragments at m/z 298.1546, 270.1233, 247.0875, 241.0960, 233.0845, 221.0719, 219.0562, 205.0406, 193.0407, and 179.0376.

Supplementary Figure S4.3. CID spectra of OFL279



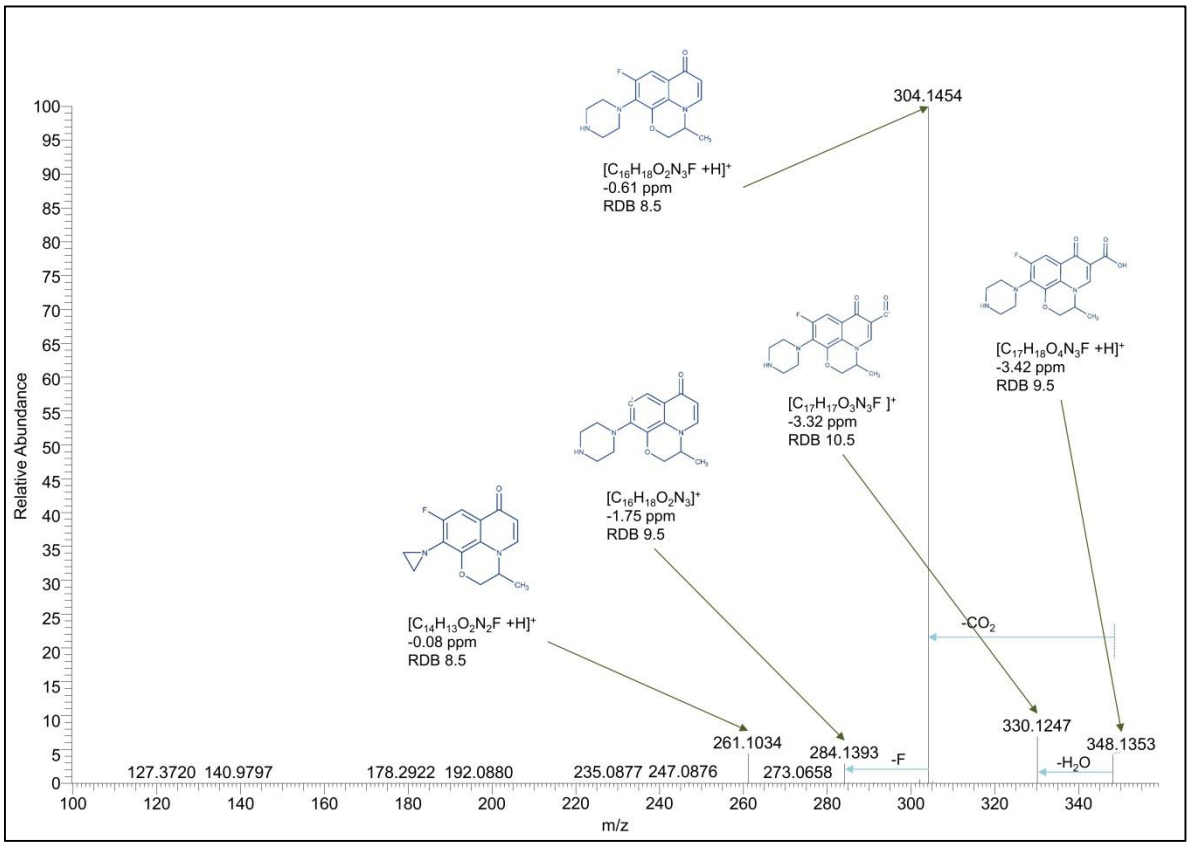

The proposed elemental composition, based on the fragmentation pattern (Level 2b identification) shown in Figure S4.3, for OFL279 (m/z 279.0768, RT=17.1 min) was $C_{13}H_{11}O_4N_2F$ (error: -2.73 ppm). Two prominent fragments at m/z 261.0663 ($C_{13}H_{10}O_3N_2F$) and 238.0378 ($C_{10}H_7O_4N_2F$) are the result of the neutral loss of water (-18.0105 Da) due to dehydration of the carboxyl group and the neutral loss of the propyl group (-41.0390 Da) due to oxazine ring cleavage. The latter ion underwent additional fragmentation, resulting in m/z 191.0248 ($C_9H_4O_2N_2F$) and 164.0378 ($C_8H_5ON_2F$), which were formed from m/z 237.0300 (dehydrogenated m/z 238.0378) through the consecutive neutral losses of 46.0053 Da ($-CH_2O_2$) and 26.9870 Da (-CO; +H).

Supplementary Figure S4.4. CID spectra of OFL318



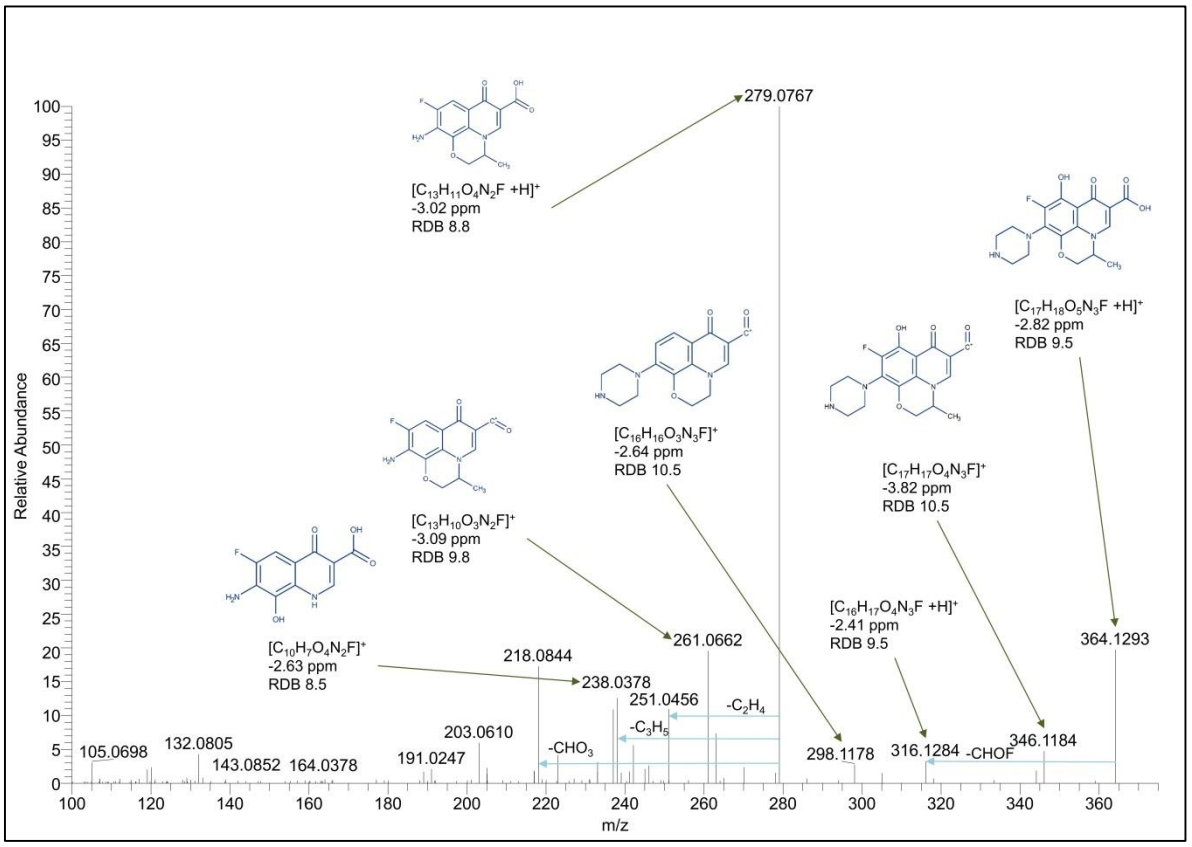

The mass spectrum of OFL318 (m/z 318.1609, RT at 1.0 min), shown in Figure S4.4, corresponds to the formula for decarboxyl OFL ($C_{17}H_{20}O_2N_3F$, error: -1.04 ppm). Three fragments (m/z 298.1548, 261.1031, and 205.0970) matched fragments obtained from the parental compound. Moreover, the base peak at m/z 261.1034 showed the same fragmentation pattern when $MS^3$ acquisition was conducted as the one obtained from the OFL with the same m/z value. Finally, OFL318 was confirmed by comparing the RTs and fragmentation patterns with those of the purchased authentic standard, resulting in a Level 1 identification.

Supplementary Figure S4.5. CID spectra of OFL348



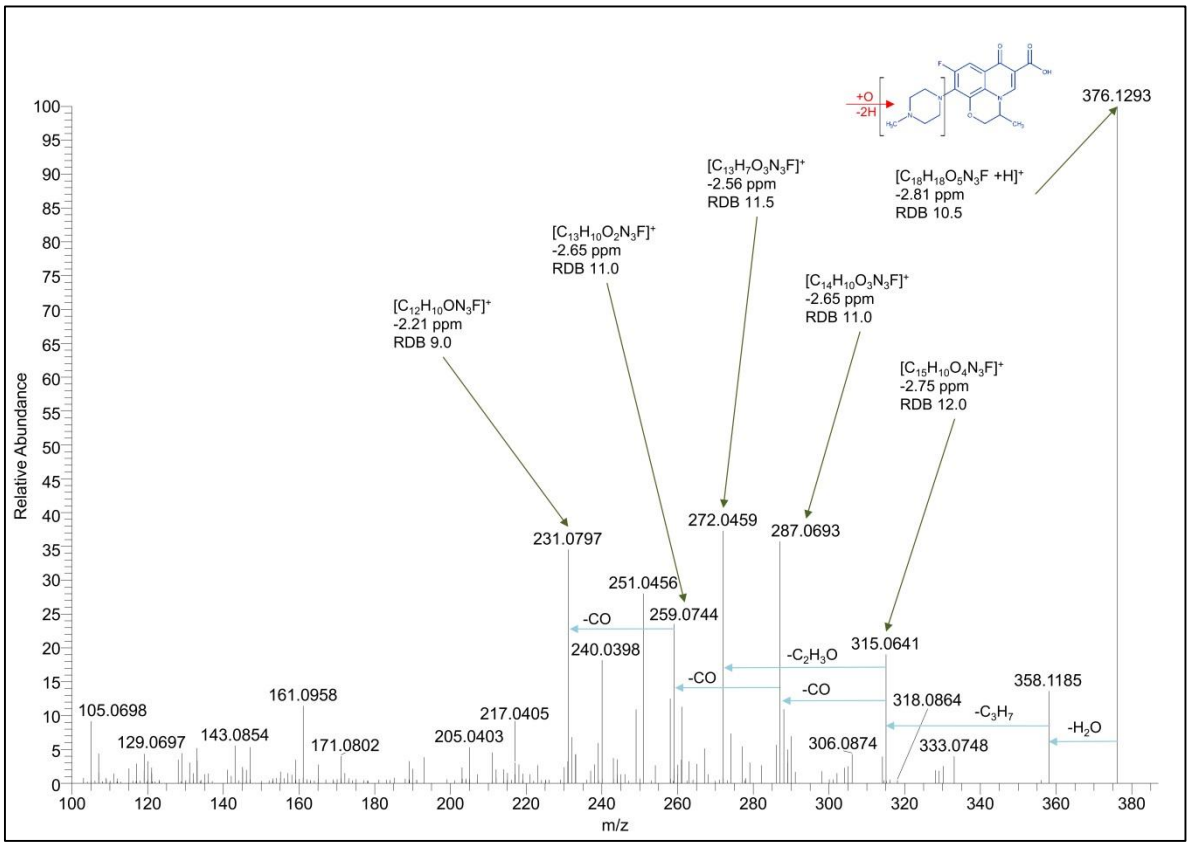

The proposed structure for OFL348 (m/z 348.1353, RT=10.4 min) was N-desmethyl OFL ($C_{17}H_{18}O_4N_3F$, error: -3.42 ppm) with its 4 characteristic fragments: m/z 330.1247, 304.1454, 284.1393, and 261.1034 (Figure S4.5). The fragments at m/z 330.1247 ($C_{17}H_{18}O_2N_3$) and 304.1454 ($C_{16}H_{18}O_2N_3F$) can be explained by the loss of the water and carboxylic group from the parent peak, whereas m/z 284.1393 ($C_{16}H_{18}O_2N_3$) corresponds to the defluorinated fragment of the base peak (m/z 304.1454). A characteristic fragment of OFL was detected (m/z 261.1034) as well. The proposed structure was conclusively confirmed with the reference standards, with Level 1 confidence.

Supplementary Figure S4.6. CID spectra of OFL364



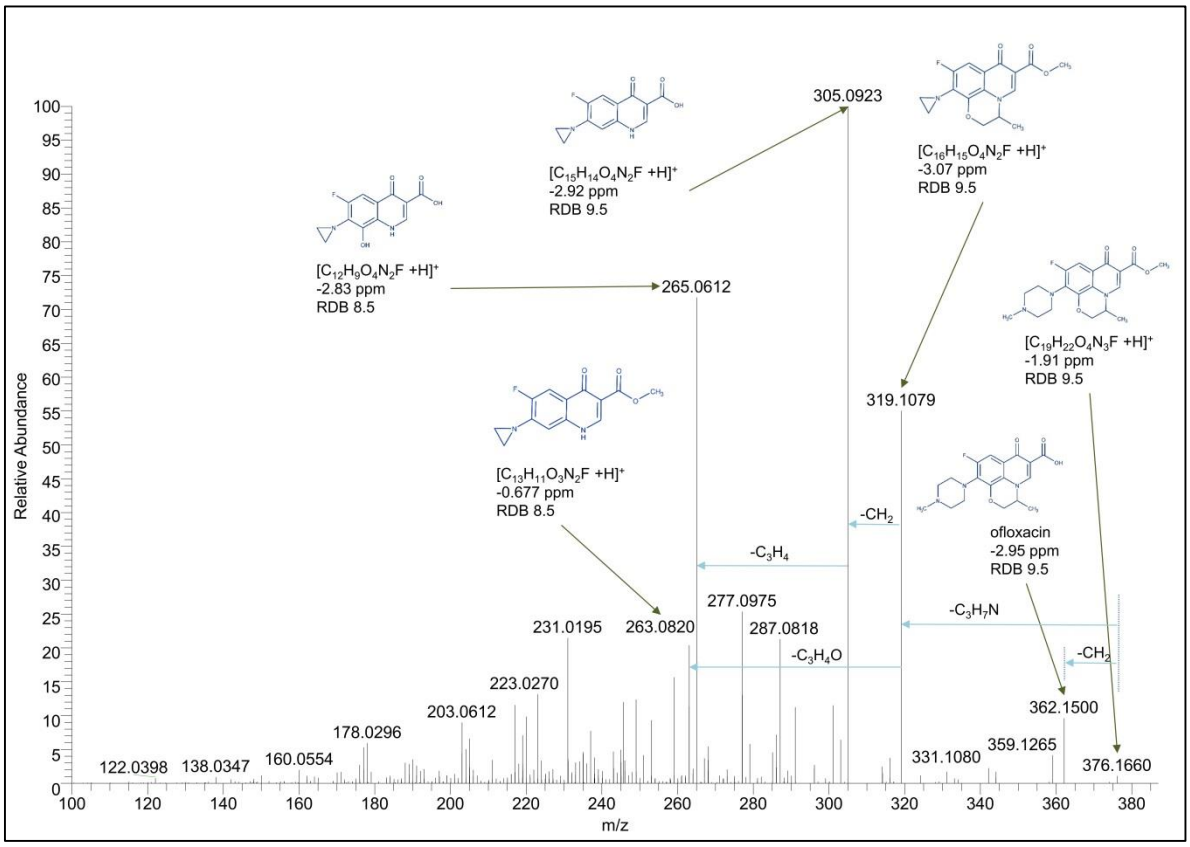

The exact observed m/z value of the $[M+H]^+$ of OFL364 (m/z 364.1293, RT= 5.0 min) corresponds to demethylation plus hydroxylation. The structure of N-desmethyl hydroxy-OFL ($C_{17}H_{18}O_5N_3F$, error: -2.82 ppm) was thus assigned to this metabolite, with a confidence Level of 2b. Neutral loss of water (-18.0109 Da) produced the m/z 346.1184 ($C_{17}H_{17}O_4N_3F$) fragment. Further on, tentative structures were assigned to the fragments at m/z 298.1178, 279.0767, and 261.0662, as shown in Figure S4.6. The neutral loss of 48.0006 Da, corresponding to the sum of the demethylation (14.0156 Da; $-CH_2$), fluorine exchange (17.9906 Da, -F), and reduction (15.9949 Da; -O), indicates the formation of m/z 298.1178 ($C_{16}H_{16}O_3N_3F$). The ion detected at m/z 279.0767 corresponds to the elemental composition of OFL279 ($C_{13}H_{11}O_4N_2F$), whereas the ions at m/z 261.0662 and 238.0378 represent its fragments, as previously described.

Supplementary Figure S4.7. CID spectra of OFL376



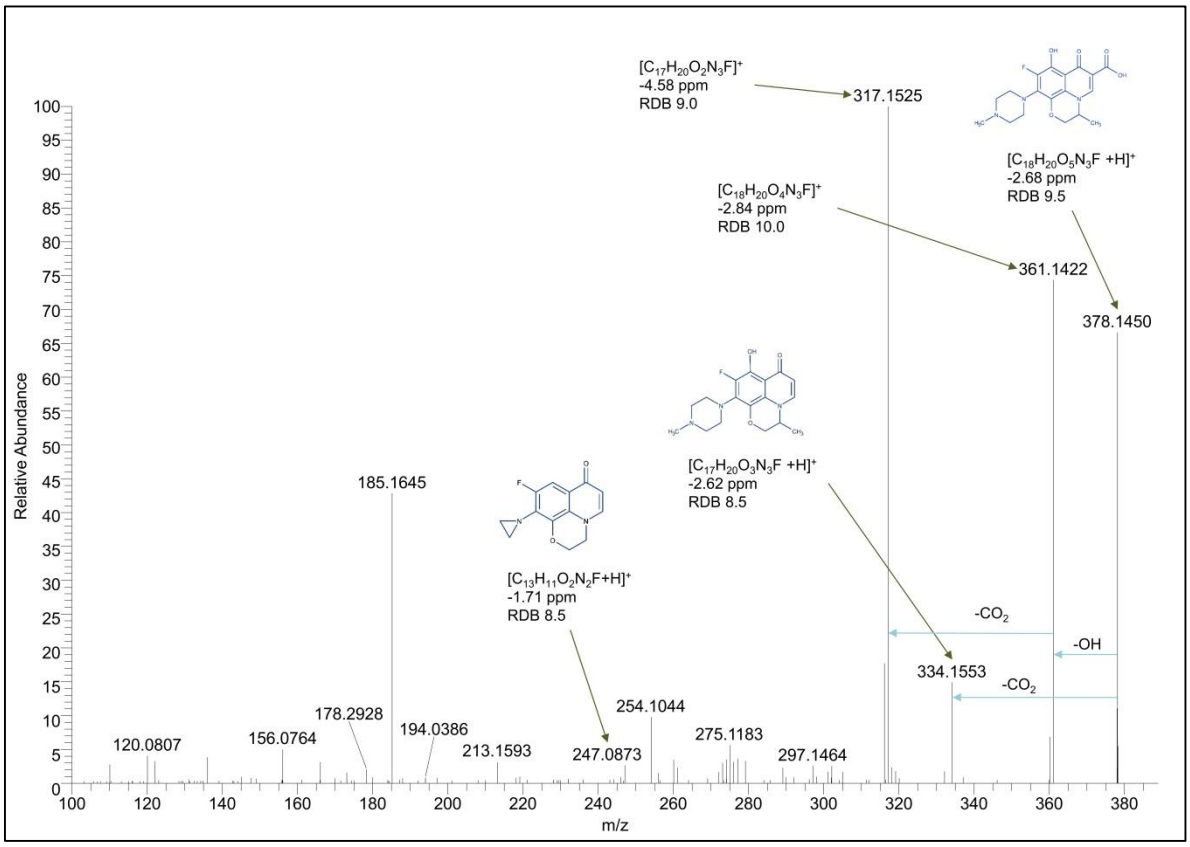

For OFL376 (m/z 376.1292, RT= 4.7 min), the formula of oxidized OFL ($C_{18}H_{19}O_5N_3F$, error: -2.81 ppm) was assigned based on the exact observed m/z value of $[M+H]^+$. Specifically, the exact observed m/z value was 13.9783 Da higher than that of the OFL, which indicates additional oxygen and the loss of two hydrogens. Several prevalent product ions were observed at m/z 315.0641 ($C_{15}H_{10}O_4N_3F$), 287.0693 ($C_{14}H_7O_3N_3F$), 272.0459 ($C_{13}H_7O_3N_3F$), 259.0744 ($C_{13}H_{10}O_2N_3F$), and 23.0797 ($C_{12}H_{10}ON_3F$); nevertheless, the exact position of the oxygen could not be assigned, resulting in a Level 3 identification. Given the structure of OFL, we suggest the pyrimidine ring as a reasonable site for oxidation (Figure S4.7).

Supplementary Figure S4.8. CID spectra of OFL376-methyl



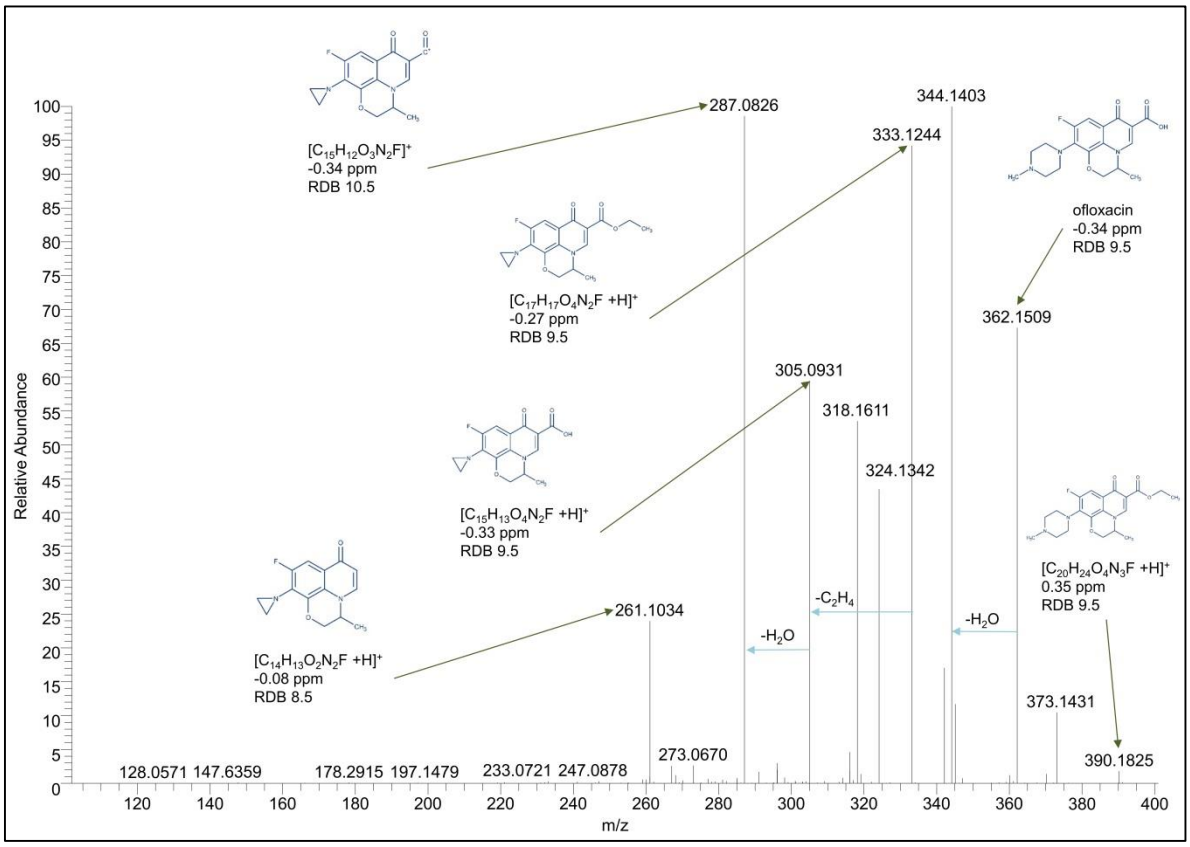

Fragmentation of OFL376-methyl (m/z 376.1660, RT= 8.3 min) revealed the presence of OFL (m/z 362.1500) as a product ion, confirming that this metabolite contains the structure of the parent molecule. Furthermore, the mass-to-charge difference of 14.0160 Da between OFL376-methyl and OFL indicates conjugation with the methyl group; hence, the elemental formula of OFL methyl ester was proposed ($C_{19}H_{22}O_4N_3F$, error: -1.91 ppm). As can be seen in Figure S4.8, two fragment ions arise from the neutral loss of the methyl group: m/z 362.1500 (OFL) as a product ion of m/z 376.1660 (OFL376-methyl), and a base peak at m/z 305.0923 ($C_{15}H_{14}O_4N_2F$) as a product ion of m/z 319.1079 ($C_{16}H_{15}O_4N_2F$). Structures were assigned to two more fragments at m/z 265.0612 ($C_{12}H_9O_4N_2F$) and 263.0820 ($C_{13}H_{11}O_3N_2F$), proving the proposed structure, which was eventually confirmed with Level 1 confidence, since the RT and fragmentation profile matched the injected reference standard. Additional experiments were performed in order to confirm that there is no generation of OFL methyl ester due to the usage of methanol in the analytical procedure (as describe above).

Supplementary Figure S4.9. CID spectra of OFL378



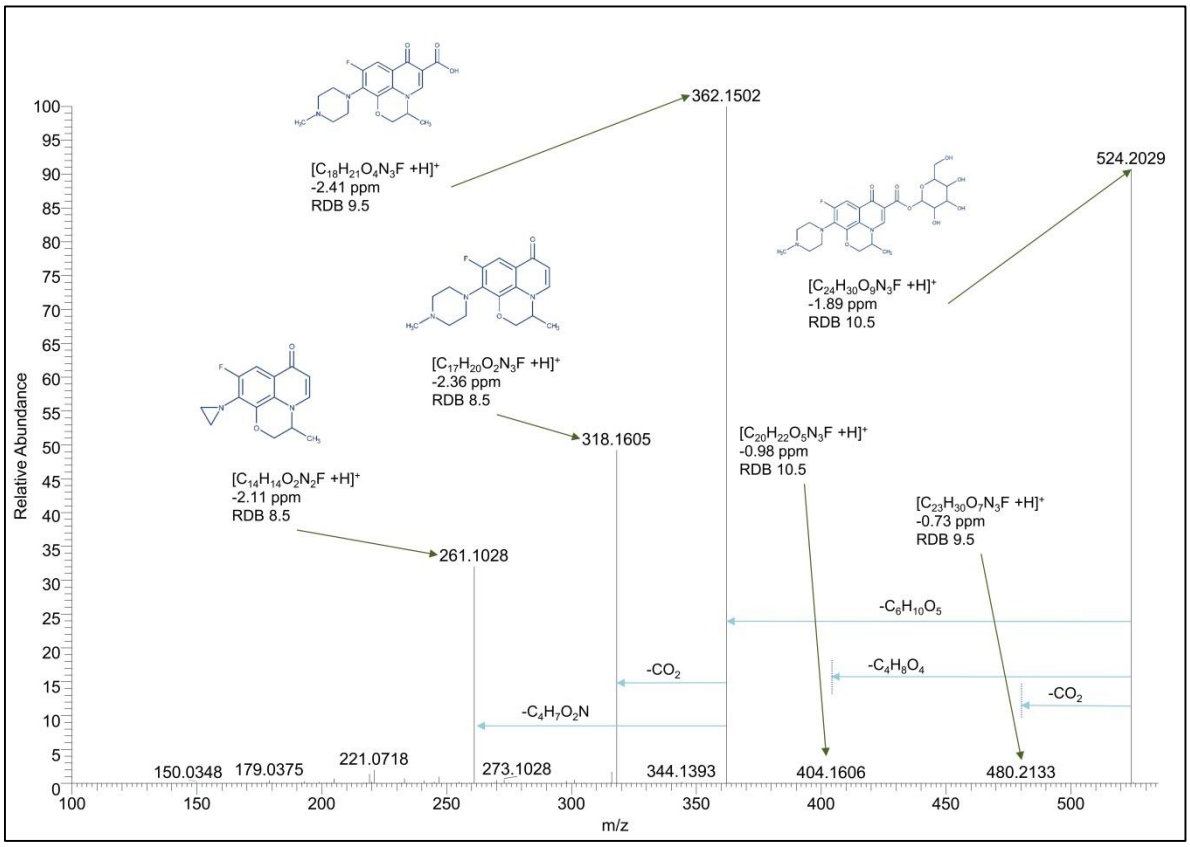

The mass difference (15.9944 Da) detected for OFL378 (m/z 378.1450, RT=5.6 min) when it was compared with the mass of the parent compound indicates additional oxygen; thus, the formula of hydroxyl-OFL ($C_{18}H_{20}O_5N_3F$, error: 2.68 ppm) was assigned to this metabolite (Figure S4.9). Decarboxylation (–$CO_2$) of OFL378 led to the formation of a characteristic ion product at m/z 334.1553 ($C_{17}H_{20}O_3N_3F$), while the neutral loss of the hydroxyl group formed an m/z 361.1422 ($C_{18}H_{20}O_4N_3F$) ion, which was later decarboxylated to m/z 317.1525 ($C_{17}H_{20}O_2N_3F$). Finally, a structure was suggested for m/z 247.0873 ($C_{13}H_{11}O_2N_2F$). According to the observed fragments, Level 2b identification was achieved.

Supplementary Figure S4.10. CID spectra of OFL390



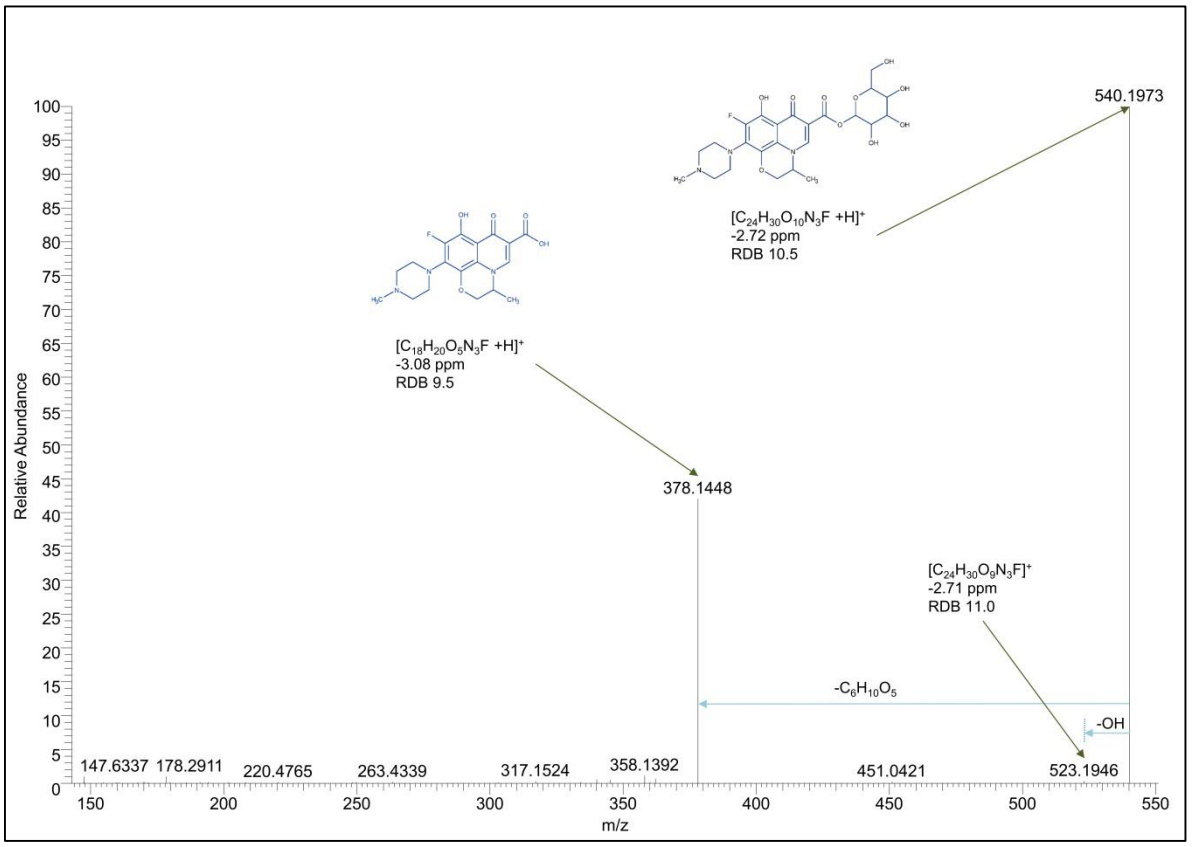

Based on the mass-to-charge difference (28.0319 Da) between OFL390 (m/z 390.1825, RT=10.7 min) and OFL, the proposed structure and formula ($C_{20}H_{24}O_4N_3F$, error: 0.35 ppm) correspond to OFL ethyl ester. Fragmentation (Figure S4.10) revealed an OFL ion (m/z 362.1509) and its characteristic fragments (m/z 344.1403, 318.1611, and 261.1034), along with m/z 333.1244 ($C_{17}H_{17}O_4N_2F$), 305.0931 ($C_{15}H_{13}O_4N_2F$), and 287.0825 ($C_{15}H_{12}O_3N_2F$). Product ions and the RT of the purchased OFL ethyl ester analytical standard confirmed the assigned structure. OFL390 was thus identified with a confidence Level of 1.

Supplementary Figure S4.11. CID spectra of OFL524



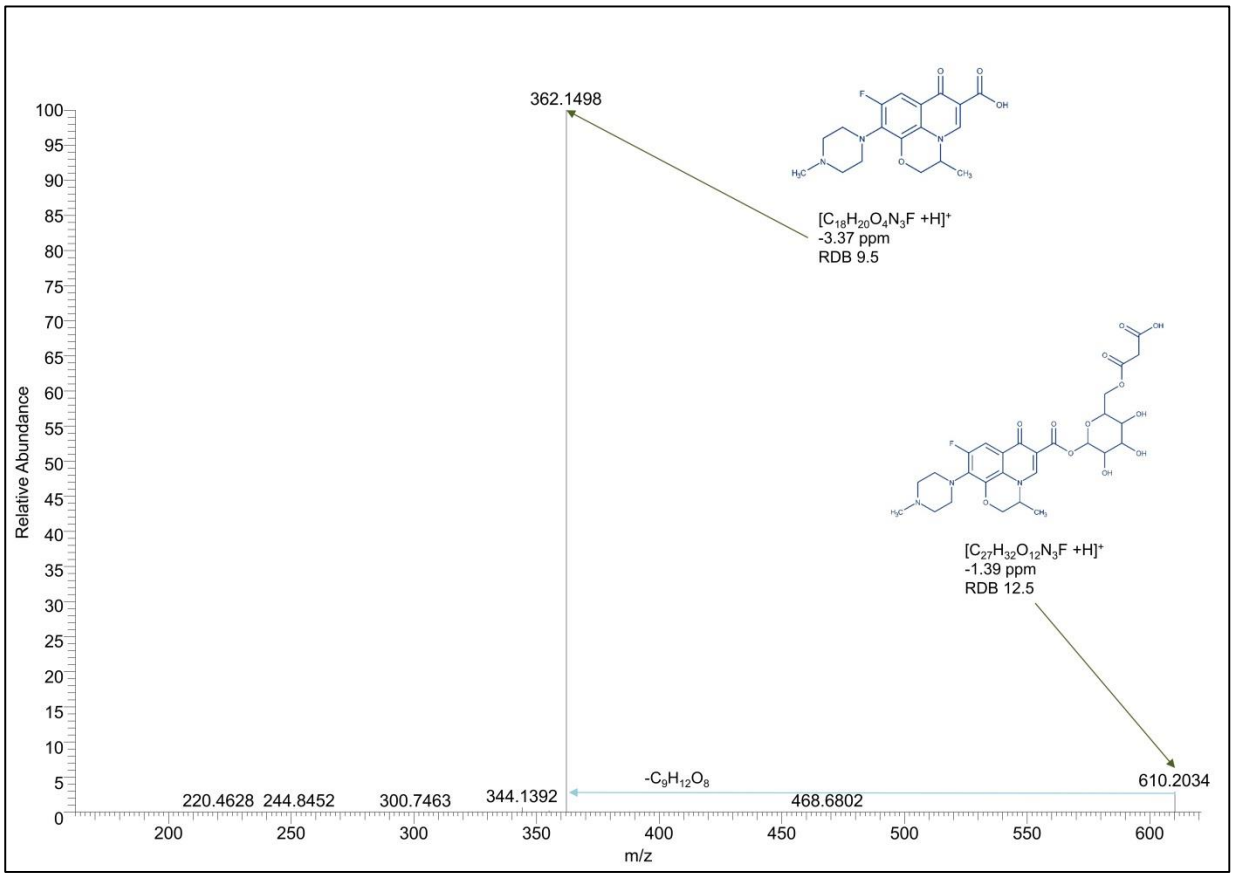

Fragmentation of OFL524 (m/z 524.2029, RT=2.1 min), shown in Figure S4.11, revealed an OFL fragment (m/z 362.1502) formed by the neutral loss of anhydroglucose moiety (162.0527 Da), indicating that the compound corresponds to O-glycosyl OFL ($C_{24}H_{30}O_9N_3F$, error: 1.89 ppm). Two OFL product ions (m/z 318.1605 and 261.1028) indicated its structure in the OFL524 molecule, which was eventually confirmed with $MS^3$. Two more fragments were observed with poor intensity at m/z 480.2133 ($C_{20}H_{22}O_5N_3F$) and 404.1606 ($C_{23}H_{30}O_7N_3F$), formed by the neutral loss of 43.9896 Da ($-CO_2$) and 120.0423 Da ($-C_6H_{10}O_5$). A tentative structure was suggested with Level 3 confidence.

Supplementary Figure S4.12. CID spectra of OFL540




Another glycosylated metabolite was detected. Specifically, fragmentation of OFL540 (m/z 540.1973, RT=6.7 min) gave one dominant fragment (m/z 378.1448), which can be attributed to the neutral loss of anhydroglucose moiety (162.0525 Da), as shown in Figure S4.12. $MS^3$ of the m/z 378.1448 product ion gave the same fragmentation pattern as OFL378. Thus, the molecular formula of O-glycosyl-hydroxy-OFL ($C_{24}H_{30}O_{10}N_3F$, error: -2.75 ppm) was assigned to OFL540. The neutral loss of the hydroxyl group led to the formation of m/z 523.1946 ($C_{24}H_{30}O_9N_3F$). Due to the relatively poor mass spectra, a tentative structure was assigned with a confidence Level of 3.

Supplementary Figure S4.13. CID spectra of OFL610




In Figure S4.13, the spectra for OFL610 (m/z 610.2034, RT=8.3 min) reveal one dominant product ion (m/z 362.1498) of OFL. The OFL structure that is incorporated in OFL610 was unequivocally confirmed with $MS^3$ acquisition. The neutral loss of 248.0536 Da matched malonyl-glucoside adduct. Thus, the formula and structure of O-malonyl-β-D-glycosyl-OFL ($C_{27}H_{32}O_{12}N_3F$, error: -1.39 ppm) was suggested for this metabolite, with a confidence Level of 4.

# Chapter V: General Discussion

At the time of wiring this Thesis, the SARS-CoV-2 pandemic is currently dominating every aspect of healthcare across the globe. The pandemic is putting various longer-term public health issues, including the steady rise of antimicrobial resistance in the shadow [201]. Scientific community warns not to lose sight of problems that will persist in time after SARS-CoV-2 pandemic. In fact, the situation with pandemic can get even more difficult, in lower and middle-income countries, which currently have a higher burden of AB resistance than developed countries [202].

Escalating evolution of resistance coupled with a diminished antibiotic pipeline has led some to claim that a post-antibiotic era is eminent [203]. According to WHO antibiotic resistance is one of the biggest threats to global health, and food security. Several countries are adopting strategies to fight the antimicrobial resistance. However, a global view of the problem of antibiotic resistance should be adopted in order to come to terms with the problem [204].

Europe's water resources are increasingly becoming under stress. Pressure from climate change, droughts, and urban development have put a significant strain on freshwater supplies. In this context, Europe’s ability to respond to the increasing risks to water resources could be enhanced by a wider reuse of RWW. RWW is commonly used to irrigate agricultural land in arid and semi-arid regions because of water shortages. There is major public concern regarding agricultural applications of RWW due to the introduction of ABs from irrigation waters into crops via plant uptake. In addition, manure application in agricultural practices as organic fertilizer, is another source of ABs in soils and vegetables. Efforts are on-going across the EU to develop manure and WW processing technologies that allow turning them into safe and valuable agronomical resources [205].

There is abundant evidence of the complex, yet clear relationship between the occurrence of antimicrobial resistances and the use of ABs. Thus, multidisciplinary studies focusing on occurrence and fate ABs as well as ARB&ARGs are necessary. Within this context, it has

become increasingly important to gather data from realistic field-scale conditions in order to uncover knowledge that can help up us mitigating this issue.

The main difficulties in the analysis of ABs in vegetables are associated to the relatively low AB's concentrations in crops, and the complexity of the vegetal material. These matrices, which contain large amounts of endogenous components, like organic matter, pigments and fatty or waxy materials, which can interfere in the determination [15]. Actually, matrix effects are major concerns in quantitative LC-MS, since co-eluting compounds affect ESI, therefore affecting method sensitivity, precision, and accuracy. Addition of surrogate standard, an analyte chemically similar to target compound (e.g., isotope label compound) may compensate the matrix effect. However, surrogate standards are costly and not always commercially available, especially in case of TPs and metabolites. In this Thesis, deuterated analytical standard of parent AB was intended to be used as surrogate standards for AB metabolites, too. But this was not completely feasible due to the sample-specific matrix interference. More specifically, TMP-d3 was suitable as surrogate standard for two investigated TMP metabolites in all matrices except in lettuce. SMX-d4 served as surrogate standard for all analyzed sulfa drugs only in cauliflower, but not for: SDI in lettuce; SDI and SMZ in tomato; SDI, SMZ, STZ, and N-acetyl SMX in broad beans. While OFL-d3 was used as surrogate standard for OFL metabolites only in lettuce. Applied approach indeed compensated matrix effect, but this approach failed to overcome the loss in sensitivity due to the matrix effects. To address this issue, extract clean-up was tailored in such way that interfering, co-eluting substances were removed as much as possible (Section 2.3.2). Due to the prudent optimization, it was possible to detect ABs and their metabolites in all sampled vegetable matrices. Finally, achieved selectivity and sensitivity of the developed analytical methodology, described in the Chapter II, allowed MDLs in the low ng $g^{-1}$ level. More specifically, MDLs and MQLs ranged from 0.02 ng $g^{-1}$ fw to 0.73 ng $g^{-1}$ fw and from 0.04 ng $g^{-1}$ fw to 1.22 ng $g^{-1}$ fw, respectively.

Hydroponic experiments are more intuitive for understanding uptake, translocation and metabolization mechanism, such as the one described in Chapter IV. But these experimental set-ups do not represent the complexity of a genuine agricultural environment. Data obtained from greenhouse or simulated field scale experiments can better explain the actual uptake, and accumulation. Although, all these studies do not include all conditions that are present in the real fields, they can help us understand the behavior of ABs in the agricultural environments. Thus, in this Thesis data obtained from experiments conducted under controlled conditions served as a valuable source for the explanation of phenomenon observed in the performed real field monitoring study.

Over the past decade, there has been important progress on the knowledge about ABs&ARB&ARGs fate in the environment. Occurrence of ABs&ARB&ARGs in crops, and related risks, are determined by various factors such as type of soil, climate, type of crops, daily intake, preparation of food (raw vs. cooked), agricultural practices, time frame of cultivation, geographical location, and irrigation water quality among others. Due to the comprehensive sampling campaign, and the fact that investigated vegetables were grown alongside commercially available ones, a realistic insight in the level of contamination presented in Chapter III was ensured. HCA and PCA clearly showed that manure application, irrigation with water from the River Llobregat and the crop type were main factors affecting ABs pollution loads. Data published by Margenat et al. [168] showed that occurrence of chemical contaminants in vegetables depended on the type of vegetable rather than the location. Similarly, the type of vegetable was the main determinant for both ARG distribution and microbiome composition, with minor contributions of geographic location and irrigation water source. Thus, the plant genotype plays a significant role to their load. As an example, pharmaceuticals in lettuce are transported to shoots with transpiration flow, which means that for accumulation and transport of ABs, their lipophilic characteristics are not crucial, as water movement is [44]. In this regard, estimated net irrigation requirements for tomato (6136 $m^3$ $ha^{-1}$ $year^{-1}$), green beans (5696 $m^3$ $ha^{-1}$ $year^{-1}$), haricot beans (4300 $m^3$ $ha^{-1}$ $year^{-1}$), and lettuce (2268 $m^3$ $ha^{-1}$ $year^{-1}$) may illustrate variability of one important factor for different types of vegetables [104]. Not just uptake, but metabolization varied among different vegetables as presented in Section 3.3.5. Thus, we may attribute concentrations of AB metabolites to the plant physiology. In fact, the main biotic factors that may influence the uptake of chemical pollutants are the plant itself, and the soil microorganisms. While, climatic conditions, the physicochemical properties of contaminants and soil composition constitute the main abiotic factors [37].

Seasonality may affect ABs pollution loads through many different ways. Microclimates determined by season affect spatial and temporal variations in meteorological parameters and evapotranspiration [104]. The fluctuation of concentration level of ABs in manure and WW over the year was reported. For instance, Hu et al. [134] investigated the effect of manure application and observed that the occurrence of ABs were higher in winter time than in summer. Similarly, seasons affect concentration of ABs in WW, which were higher during winter in this particular area [206]. On the other hand, data on the seasonal fluctuation of pollutants in the Llobregat River showed the opposite trend [166]. Another factor affected by season is the growing time. As mentioned in Section 3.3.4, growing time for lettuce in summer was more than three times shorter than during spring. This is an important observation, since a relation between plant grow rate and uptake of ABs was confirmed [143]. Keeping all this

data in mind, results presented in Chapter III suggest that each agricultural system should be observed separately in order to predict the ABs occurrence.

The causal relationship between the emergence of antibiotic resistance and the widespread use of ABs cannot be disputed. Exposure to ABs is the principal risk factor in the emergence and selection of ARB. Further elucidation of this factor requires consideration of the situations where ABs are frequently used. Knowledge of the ABs environmental concentrations that might exert selection for ARB is still limited. CCA analysis showed that ABs were an important factor driving ARG abundances in the different vegetable species. Moreover, ARB and ABs residues, together under favorable conditions may promote antibiotic resistance dissemination [207]. Presence of AB metabolites and other pollutants which can contribute to the resistance promotion was confirmed e.g., heavy elements [168], yet their synergetic behavior in the environment is largely unknown. We are exposed to cocktail of chemicals from our food, as shown in Section 2.7. Dose-response assessment of the relationship between the evolution and emergence of antibiotic resistance and the AB concentration requires a metric to indicate the potential of AB concentrations to promote the development of ARB in their complex communities [16]. Thus, it is of utmost importance to investigate minimal selective concentrations of ABs in the environment, since at concentration lower than MSC susceptible bacteria will outcompete the resistant strain, as shown in the Figure 5.1.

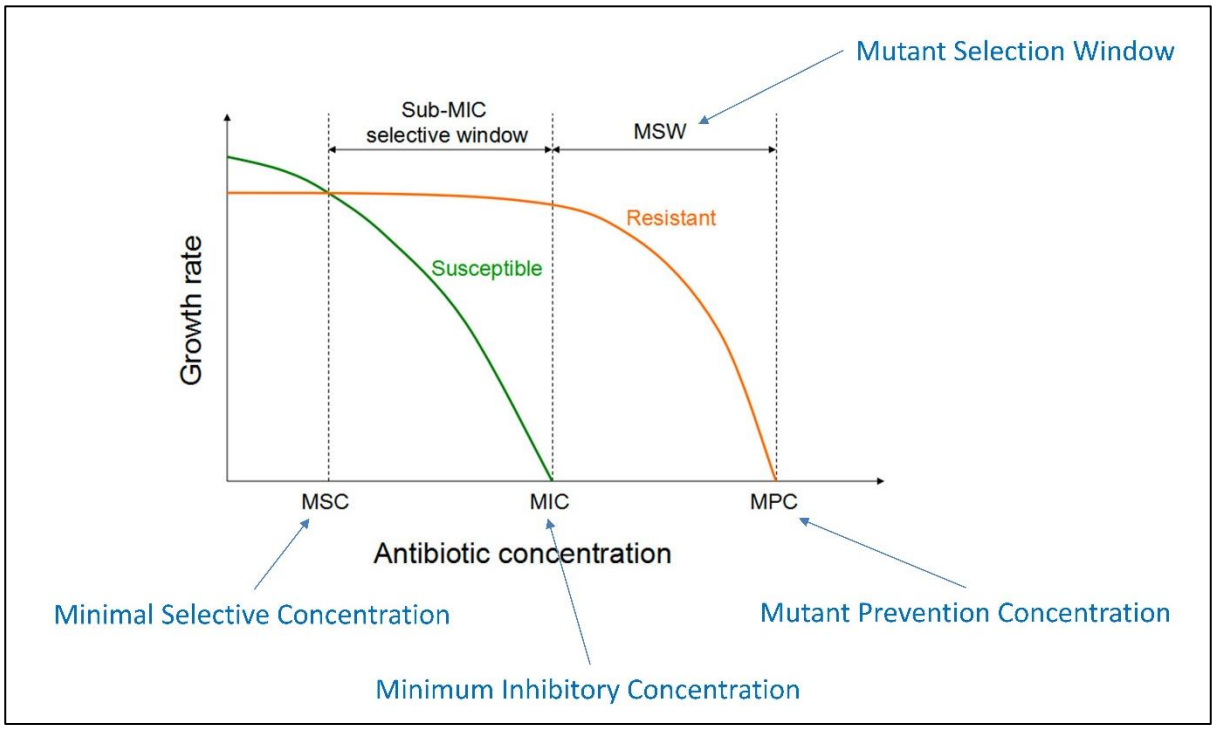



Figure 5.1. Grow rate of resistant and susceptible bacteria in the sub-MIC and traditional MSW. Figure modified from [57]

Moreover, the predicted no effect concentration values represent risks of AB resistance development, but still it is not clear what would be the risks from the developed resistance [208]. In this regard, this Thesis supports the idea of health risk assessment readjustment as a way to estimate the risk of resistance promotion caused by ABs occurrence in the environment and the risks related to the co-occurrence of ARB and ARGs in vegetables.

AB metabolites draw special attention since they were quantified in higher concentration than their parent compound. Identification of antibiotic metabolites is a crucial step in shifting metabolites from non-target to target screening methodologies. Chapter IV presents novel multi-step approach for the detection and identification of metabolites in plants based on high resolution LC/HRMS$^n$ spectra.

Although MS/MS spectra-based identification of plant metabolites has already been developed, structural elucidation of unknown metabolites from MS and MS/MS data is still a challenging task. Product ion spectra can be reproducible across similar instruments, although the reproducibility depends on many factors, including the configuration of the mass analyzer. Publicly available databases (e.g., mzCloud, Metlin) can also be good sources of product ion spectra [81]. To generate data that can provide more structural information, MS$^n$ analysis was performed. Namely, multistage mass spectrometry (MS$^3$), which involved subsequent fragmentations of previously produced fragments, generated a fragmentation trees. In this way, more specific information about the structure of each fragment can be obtained. However, we must have in mind that structure elucidating using non-targeted screening is a game of confidence where all results reported can be assigned a Level of confidence [75,185,209]. For instance, fragmentation trees allowed us to univocally confirm presence of OFL in the molecular structure of following metabolites: OFL610, OFL524, OFL390 and OFL376-methyl. Namely, MS$^2$ of these metabolites revealed product ion with m/z 362.1498, which indicated presence of incorporated OFL. This product ion was selected for further fragmentation and its spectra perfectly matched spectra of OFL. Nevertheless, MS$^3$ spectra provided valuable information for structure elucidation.

An advantage of mass spectrometry is that can detect an almost infinitely wide range of molecules. This broad applicability, in combination with its intrinsically high sensitivity and high throughput, results in very rich and complex data sets from which the analyte(s) of interest must be extracted [76]. This huge influx of information provided by HRMS is at the same time opportunity and challenge. For instance, prioritization of masses selected for further investigation and finally data processing workflow are still not harmonized. Chapter IV presents a multi-step approach for the detection and identification of metabolites in plants

utilizing advanced spectra processing methods (FISh), and comparison between exposed and reference sample. Comparison between exposed and control sample is an executive technique that generate large number of potential metabolites. With FISh, metabolite peaks can be selectively resolved and identified from background matrix ions by searching ions related with *in-silico* predicted fragments of known pharmaceuticals. This fresh integrated approach, that combines different strategies, provides the functionality required for the annotation and elucidation of metabolites, even if their spectra are not present in the library. Moreover, this way processing software will not significantly inflate the number of signals detected. Software for *in-silico* prediction of metabolization appeared to be more suitable for transformation and breakdown products, than for predictions of complex metabolites such as phase II conjugates.

Despite the uptake of contaminants of emerging concern such ABs by crops has been already documented, their metabolization in plants is largely unknown. Chapter IV describes metabolization pathways of OFL in lettuce and supposedly of structurally related quinolones. Hydroxylation, cleavage of the methyl-piperazine ring, N-demethylation, oxidation, and decarboxylation were identified as phase I reactions involved in metabolization of OFL for the first time. Whereas the following reactions were observed in phase II metabolization: methylation, glycosylation, conjugation with malonic acid and conjugation with ethanol. Tian et al. [49] reported similar reactions in the metabolization of CLI in plants, i.e., demethylation, methylation, and oxidation. Main phase II reaction for metabolization of OFL was glycosylation, which was previously reported as main biotransformation pathway in plant for SMZ and SMX as well [50,51]. However, recent studies [50–52] discovered other conjugation reactions as part of phase II metabolism (e.g., pterin, glutathione, and methylsalicylate conjugates) that were not identified in case of OFL. Conjugation of OFL with malonic acid, although well studied metabolization pathways, was reported for the first time for AB in plant. Conjugation with ethanol was a rather unique finding, since the current available information about this pathway in plants is still scarce.

The main question that needs to be answered is whether or not metabolization of ABs affects their antimicrobial activity. Analysis based on the structure-activity relationship provides valid reasons for further investigations as described in Section 4.3.3. More specifically, the cellular targets for quinolones are bacterial type II topoisomerases, gyrase, and topoisomerase IV. However, OFL itself displays an improved activity against gyrase, a greater penetration into gram-positive organisms, and an enhanced pharmacokinetics and pharmacodynamics [210]. Based on the OFL mechanism of action and the fact that structurally related compounds often display similar biological activity, influence of transformation on antimicrobial activity was

discussed. The electron-withdrawing fluorine at C6 was present in for all detected metabolites, influencing the antibacterial potency of the metabolites, since it facilitates cell penetration and target binding of the quinolone [198,199]. The oxygen atom in the oxazine ring (which also remains intact) has been found to be an important element in the structure, endowing such compounds with an antibacterial effect [211]. As mentioned in Section 4.3.3, OFL279, OFL348, OFL364, OFL376, and OFL378 may retain antimicrobial activity since no modifications were observed at the C2, C3, or C4 positions. Specifically, C2, C3, or C4 positions have been found to be essential for antimicrobial activity [196,197]. Because the carboxylic acid is a major factor in drug binding, its substitution is generally detrimental to the antibacterial activity of 4-quinolones [197,198,210]. Hence, OFL318, OFL376-methyl, OFL390, OFL540, OFL524, and OFL610 are not expected to have the same biological activity as the other group of metabolites ("C3/C4 free"). However, these metabolites may still retain the complete, parental antimicrobial potency. For instance, glycosyl conjugates may be cleaved by glucosidases and, in the case of acyl glycosides (-COOH substitution), by esterases [194]. Substituents at the C5 position appear to have the capacity to alter the molecule's overall steric configuration, although this phenomenon was only observed in the case of OFL364 and OFL378. Four metabolites (OFL279, OFL348, OFL376, and OFL364) underwent changes at the C7 position. Substituents at C7 of the quinolone molecular nucleus, especially the ones that increase bulkiness, appear only to support rather than to be pivotal to, the overall antimicrobial activity of the fluoroquinolone molecule. They influence potency by conferring protection from the efflux exporter proteins of bacteria, facilitating penetration into the bacterial cell, diminishing the likelihood of bacterial resistance in wild-type bacterial strains, and increasing anti-anaerobic activity [196,197,210]. Thus, for OFL348 and OFL364, a slight variation in antimicrobial activity can be expected due to demethylation on the methyl-piperazine ring. It is particularly relevant for OFL348, since the demethylation was the only structural alteration, leaving intact the piperazine ring, which is known to increase the ability of quinolones to penetrate the bacterial cell wall [197]. In this regard, the analysis provides additional reasons to conduct experiments to investigate the biological activity of metabolites identified *in vivo*, especially since a certain number of the new medicines are, in fact, only structural modifications of existing drugs. The formation of AB biotransformation products with a high potential for antimicrobial activity suggests that they cannot be overlooked since they can contribute exerting selective pressure to microbial communities.

**Hypothesis validation**

**Hypothesis I:** Different agricultural practices determine pollution loads of ABs in vegetables.

Different agricultural practices influenced AB pollution in vegetables thus hypothesis was validated. Namely, among observed manure application, type of irrigation water, type of crops and growing season (which impacted growing period, hence affecting ABs loads) are the main factors that could affect occurrence and fate of ABs in agricultural systems.

**Hypothesis II:** Through vegetable consumption humans are exposed to ABs TPs alongside with the parent AB.

The hypothesis is validated. Results showed in the Thesis investigate ABs metabolites in field grown vegetables. Applying target screening techniques, their occurrence was confirmed in the edible parts of field grown vegetables, even in concentrations higher than the parent compound. Moreover, non-target screening revealed presence of other AB biotransformation products.

**Recommendation for future research and further actions**

- Investigate ecotoxicological effects of ABs, their metabolites and TPs, their synergistic and antagonistic effects as well as interactions with other contaminants frequently found in vegetables (e.g., heavy elements, pesticides). More specifically, to investigate their persistence, bioaccumulation and toxicity, hence provide fundamental knowledge for their prioritization. In case of AB metabolites and TPs, special attention should be given to their potential residual antimicrobial activity.

- Determination of dose responses for promotion of antimicrobial resistance in the agricultural systems. Multidisciplinary and comprehensive experimental set-ups will be required in order to shred some light on this issue.

- A thorough investigation of the factors affecting fate of ABs in real agricultural environments to predict ABs occurrence in field-scale conditions. Strategies and guidelines (e.g., fertilizer-crop pairing, irrigation schemes) can be developed, consequently helping farmers to mitigate AB pollution. This notwithstanding, the problem of ABs occurrence in the environment should be addressed from its source, e.g., applying advanced treatment processes in the treatment of wastewater.

- AB pollution is not a regional, but a global problem, and it should be addressed accordingly. There is still space for improvement of the legislation worldwide regulating ABs. Moreover, decision makers should find a way to stimulate and support pharmaceutical industry that works on the discovering of new ABs. Finally, it is a duty of scientific community to raise awareness about the consequences of the inappropriate use of ABs. For instance, governments, health facilities, schools and communities across the globe participate in the yearly World Antibiotic Awareness Week, which was initiated by WHO. Since the internet and other forms of media connect and update people worldwide, short films, documentaries, animations, advertisement, lectures, discussion forums, campaigns, etc. are useful and available tools that scientific community can use.

# Chapter VI: Conclusions

- Multi-residue analytical method was developed for the analysis of ABs and their metabolites in vegetables. The relative recovery values for all compounds were in acceptable range (from 70 to 118%). MDLs and MQLs were sufficiently low and ranged from 0.02 ng $g^{-1}$ fw to 0.73 ng $g^{-1}$ fw and from 0.04 ng $g^{-1}$ fw to 1.22 ng $g^{-1}$ fw, respectively.

- SMX, TMP, OFL, ENR, decarboxyl OFL, OFL methyl ester and keto TMP were detected above their MQLs in field grown vegetables and ranged from 0.09 ng $g^{-1}$ fw to 3.61 ng $g^{-1}$ fw. AB metabolites in 73% of all cases were detected in the concentrations higher than the parent compounds. OFL and ENR were the most frequently detected ABs, above their MQLs in 94 and 81% of total samples, respectively. AB metabolites followed the distributions of their parent compounds and the varied according to vegetable type.

- Estimated HQs were two orders of magnitude lower than one indicating that the risk associated with vegetables consumption deemed to be negligible. However, the present results support the need to readjust the risk assessments approaches.

- The potential relationship between the distribution of the ARGs and AB compounds was analyzed with CCA. The ARG distribution and abundance indicate that the ABs had a significant impact (selective pressure), as they explained 54% of the total variation (inertia proportion) in ARG abundance.

- Among the observed farm plots, zone 1 and zone 4 showed the highest AB pollution loads, which can be attributed to the manure application and irrigation with the water from the River Llobregat, respectively. In addition, type of crops determined occurrence of ABs and especially their metabolites. That was confirmed by both HCA and PCA.

- State-of-the-art instrumentation (Orbitrap Fusion Lumos) and data processing tools (Fragment Ion Search and in-house developed algorithms) were applied in identification and annotation of OFL metabolites in lettuce. Out if 11 metabolites, five were reported for the first time in plants. Glycosylation, as a metabolization pathway, was detected in case of three plant metabolites. Pointing out that phase II metabolization reaction catalyzed by O-glucosyl-transferase was a common detected plant detoxification strategy.

- Metabolization of OFL was observed at three positions: in the carboxylic group at C3 position, and at C5 and C7 positions of the piperazine ring; of which carboxylic acid appeared to be the most active site on the OFL scaffold. The proposed biotransformation pathways can be used in the prediction of biotransfomation of other ABs that belong to the fluoroquinolone group as well as to the any other chemical that shares structure and functional groups with OFL.

- A theoretical indication of residual antimicrobial activity after biotransformation was discussed based on the OFL mechanism of action and the fact that structurally related compounds often display similar biological activity. Potential antimicrobial activity persistence is clear in case of conjugation, since the dormant antimicrobial activity can simply be reclaimed by deconjugation. In addition, the contribution of other biotransformation products to the promotion of antibiotic resistance has to be investigated especially since a certain number of the new medicines are, in fact, only structural modifications of existing drugs.

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