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Assessment of the presence of transformation products of pharmaceuticals in agricultural environments irrigated with reclaimed water by wide-scope LC-QTOF-MS suspect screening --Manuscript Draft--

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Abstract:	<p>The transformation that pharmaceuticals can undergo during the water reclamation cycle, or by biotic/abiotic reactions when reclaimed water (RW) is used for irrigation, can lead to the presence of transformation products (TPs) in agricultural environments. However, data on TPs in real crops are scarce. Herein, a suspect screening approach was applied for the comprehensive investigation of 262 potential TPs, associated with 20 prioritised pharmaceuticals found in real tomato crops exposed to long-term RW irrigation. The occurrence and fate of the TPs was evaluated by the retrospective analysis of RW, soil, leave and tomato samples from 4 intensive production greenhouses. Sample analysis was accomplished by liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS). Up to 18 TPs were tentatively identified, of which 2 were not previously reported. 7 TPs were finally confirmed with analytical standards. 5 TPs were determined in RW, 15 TPs in soil and 2 TPs in leaves. Remarkably, the investigated TPs were not found in tomato fruits. These results shed light on the variety of TPs that can be found in the water reuse cycle and contribute to the assessment of the global risks of wastewater reuse and the safety of the vegetable and fruit production system.</p>

9th November, 2020

Dear Editor,

Please, find enclosed the manuscript entitled “**Assessment of the presence of transformation products of contaminants of emerging concern in agricultural environments irrigated with reclaimed water by wide-scope LC-QTOF-MS suspect screening**” by A. B. Martínez-Piernas, P. Plaza-Bolaños and A. Agüera, for consideration of publication in *Journal of Hazardous Materials*. A list of suggested reviewers is also provided.

The research presented in the submitted manuscript relates to the Aims and Scope of the journal as it deals with the occurrence and fate of transformation products (TPs) of contaminants of emerging concern (CECs) in real field agricultural environments irrigated with reclaimed water.

The overall objective of this work was to identify and evaluate the occurrence of CEC TPs derived from reuse practices in real field agricultural systems. To this aim, a retrospective LC-QTOF-MS suspect screening analysis of 262 TPs from 20 prioritised CECs was applied to samples from 4 greenhouses (crop: tomato), considering the complete water reuse cycle: reclaimed water, perlite/agricultural soil, plant material and tomato fruits. Combining the support of spectral libraries, *in silico* prediction tools and own MS² structural elucidation, a total of 18 TPs from 9 parent CECs were allocated in different identification confidence levels. 7 TPs were confirmed with their analytical standards. The occurrence study revealed the presence of 15 TPs in perlite, 8 TPs in agricultural soils, 4 TPs in reclaimed water and 2 TPs in plant leaves. Remarkably, none of the investigated TPs was found in tomato samples. Interestingly, some of the detected TPs derived from the antibiotics azithromycin and clarithromycin. Some of these TPs maintained intact the antibacterial moiety in their molecule and, thus, some potential antimicrobial activity, stressing the likely spread of antibiotic resistance in the water reuse cycle. Furthermore, 2 TPs were tentatively identified which were not reported before. To our knowledge, this study represents the first wide-scope identification of CEC TPs in real-field agroecosystems irrigated with reclaimed water.

I hope that the reviewing process finds the manuscript acceptable for publication in the journal.

Yours Sincerely,

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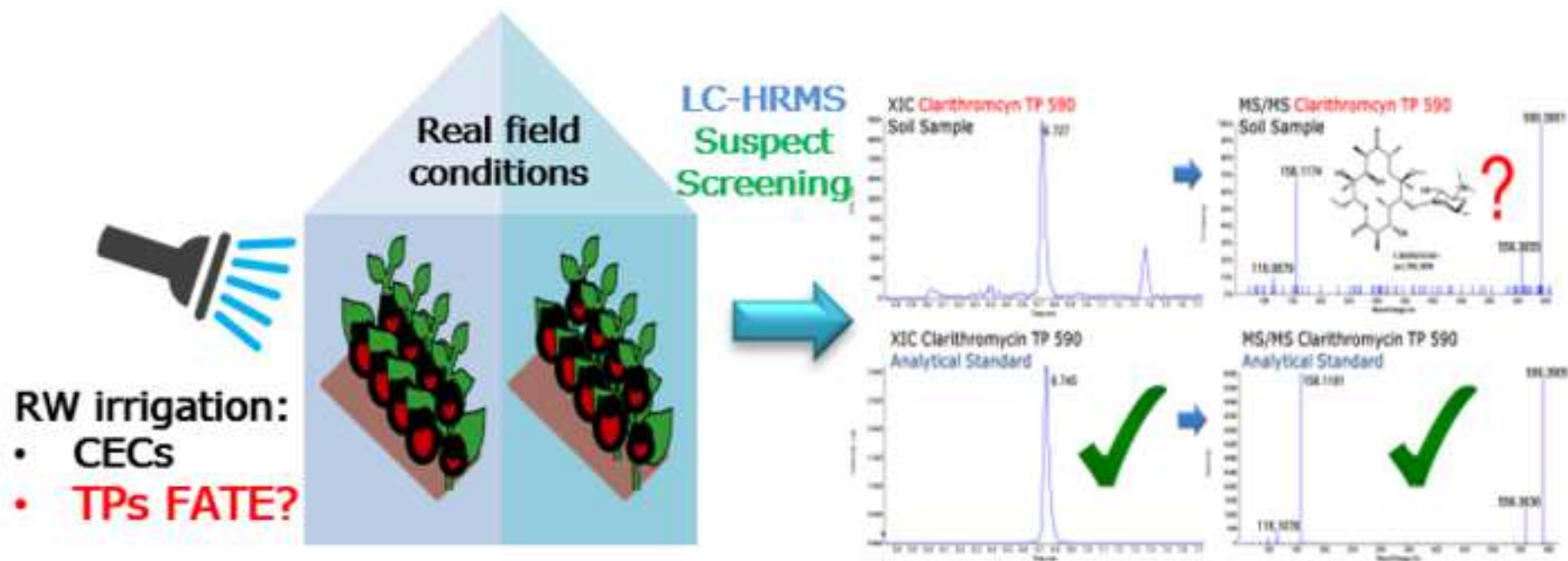
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Novelty statement

This study represents the first wide-scope analysis of transformation products (TPs) of prioritised contaminants of emerging concern (CECs) in real agricultural environments exposed to long-term water reuse practices. A retrospective analysis based on a suspect screening strategy was applied for the identification of TPs in samples from the whole water reuse cycle: reclaimed water, soil, plant leaves and tomato fruits.

Among the detected TPs (7 confirmed with standards and 2 TPs not reported), the occurrence study revealed presence of TPs in soil but not in tomato. Antibiotic TPs with potential activity were detected, indicating also a potential antibiotic resistance spread.



Highlights

- LC-QTOF-MS suspect screening to evaluate 262 TPs in agroecosystems
- 18 TPs tentatively identified and 7 confirmed in reclaimed water, soil and leaves
- None of the investigated TPs were detected in the analysed tomato fruits
- Potential spread of antibiotic resistance due to detected active antibiotic TPs
- Additional research is needed in other crops and reclaimed water agro-ecosystems

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Dear Editor,

We would like to thank honestly to the reviewers for the time that they devoted to reading our manuscript, as well as for their valuable and constructive comments which greatly helped us to improve its quality. Please, find below a detailed answer to each of the comments raised by the reviewers.

REVIEWER # 1

In their manuscript entitled 'Assessment of the presence of transformation products of contaminants of emerging concern in agricultural environments irrigated with reclaimed water by wide-scope LC-QTOF-MS suspect screening' the authors provide the results of an intensive retrospective analysis of transformation products of CECs that were identified from complex agricultural matrices. This paper provides a significant contribution in efforts to evaluate the extent of CEC TP exposure in the environment. The authors provide important and relevant detail into their workflow for unknown identification and confirm tentative assignments with standards in a number of instances. With that said, there are significant structural/organisational issues that should be addressed prior to acceptance and a thorough grammatical review is also necessary. Below I have provided comments with the aim of improving the clarity of this manuscript.

Comment 1: There are significant grammatical errors throughout. I have recommended specific suggestions for the abstract, but the paper in its entirety would benefit from careful revision.

RESPONSE: The manuscript was sent to language check.

Line 19: I would suggest using a more technical term in place of 'suffer'

RESPONSE: The wording has been changed accordingly.

Line 23: change 'to 20 prioritised...' to 'with 20 prioritised'

RESPONSE: The wording has been changed accordingly.

Line 28: change 'being' to 'with' or similar

RESPONSE: The wording has been changed accordingly.

Line 30: change 'evidences' to 'results' or similar

RESPONSE: The wording has been changed accordingly.

Line 31: change 'assess' to 'the assessment of'

RESPONSE: The wording has been changed accordingly.

Line 31: add 'wastewater' before 'reuse'

RESPONSE: The wording has been changed accordingly.

Line 32: change to '... the safety of the vegetable and fruit production system' or similar

RESPONSE: The wording has been changed accordingly.

Comment 2: In the introduction (lines 66-69), the description of transformation product toxicity should be expanded and less general as it is the major focus of this study. What TPs have been found to be more toxic than their parents. There is literature on this topic that also describes why many/most TPs will likely be less toxic/bioactive. This should be included.

RESPONSE: According to the reviewer's comment, more literature has been included in the Introduction section to expand the description of TPs toxicity (Lines 79-96 revised Manuscript with tracked changes).

Comment 3: *This reviewer is unfamiliar with the term 'wide-scope'. I would suggest removing it from the title and/or consider using an alternate word that is more commonly used.*

RESPONSE: Using the term "wide-scope" we want to emphasize the large number of TPs derived from a wide variety of pharmaceuticals that have been studied in this work. We believe that it is a term widely used in suspect and non-target screening analysis.

Comment 4: *Please provide the latin name with each vegetable/fruit crop at its first mention.*

RESPONSE: The information has been added accordingly (Lines 152 and 154, revised Manuscript with tracked changes).

Comment 5: *Section 2.2. What does 'W' stand for? Please ensure that this abbreviation is necessary. Line 154: change 'form' to 'from'*

RESPONSE: We agree with the reviewer. The term 'W distribution' is not properly explained in the text. The authors wanted to describe that subsamples were taken following a W sampling route in the cultivation area. In order to clarify it, the term 'W distribution' has been replaced by 'zig-zag sampling' (Line 159, revised Manuscript with tracked changes).

Also, please provide additional details of the QuEChERS methods in the main body of the text. Why were 2 different methods needed and for which of the samples?

RESPONSE: Text S1 has been moved to the main body of the Manuscript as Paragraph '2.3. Sample preparation'.

In previous works, we optimised and validated two different QuEChERS-based methods for soil and for plant material (tomato leaves and fruit) (Martínez-Piernas et al. Anal. Chim. Acta. 1030 (2018) 115–124; and Martínez-Piernas et al. J. Agric. Food Chem. 67 (2019) 6930–6939, respectively). In both methodologies, acetate buffer is used to adjust the extraction pH, although slight protocol modifications had to be made due to the differences between both matrices. The main differences are: i) the plant material is extracted in wet-weight (10 g) while the soil is processed freeze-dried (1 g), ii) an additional rehydration step before soil extraction is necessary, iii) the addition of different amounts of MgSO₄ during salting-out due to the different water content of the matrices, and iv) various adsorbent mixtures for the d-SPE step.

Line 154: change 'form' to 'from'

RESPONSE: The mistake has been corrected.

Comment 6: *Section 2.5. please describe what was used as a procedural blank*

RESPONSE: Procedural blanks were prepared according to the Eurachem Guide on 'Blanks in method validation'. For the preparation of the procedural blank, the matrix was replaced by Milli Q water and subjected to the same analytical process as the real samples. A reference of the Guide has been added in line 248 (revised Manuscript with tracked changes).

Line 191: *Please clarify if the entire MassBank database was accessed or if the Norman Network curated list was used.*

RESPONSE: The entire MassBank Europe collection of mass spectra was used. The text and the reference have been modified accordingly.

Line 206-207: please provide more information on what is meant by spatial occurrence? Does this just mean between the 4 different growth facilities? If so, I do not think that constitutes a spatial distribution but rather a location occurrence.

RESPONSE: The reviewer is right. This sentence has been deleted.

Comment 7: The presentation of the TPs could be significantly improved. There is significant redundancy in the description of the approach for every TP. This is already presented in the method. I would suggest moving much of this to the SI and presenting a more general presentation of the results that is organised by parent compound. You may also consider moving Table S2 to the main text or merging Table 1 and Table S2 and presenting this in the manuscript.

RESPONSE: According to reviewer's comment, section '3.1. Tentative identification of TPs' has been significantly reduced and organised by parent compound for a more general discussion of these results. Table S2 has been merged with Table 1 in the body of the text.

Comment 8: Why is semi-quantification (line 444) rather than quantification used? Is this because no internal standard was used? Whatever the reason, the procedure used for this should be incorporated into the methods.

RESPONSE: The authors used the term "semi-quantification" considering that the results obtained represent only an approximation of the concentration of the analytes, considering the following aspects:

- This work has been carried out by retrospective analysis and, consequently, samples and standards have been analysed in different days. Unfortunately, samples cannot be reinjected and IS were not used when they were analysed.
- The extraction efficiency of the method has not been tested for the confirmed TPs.

A new description has been added to 2.6 section to clarify this point (lines 267-271, revised Manuscript with tracked changes).

Comment 9: Lines 452-455, should extraction efficiency from the SP compared to soils also be considered? Additionally, it would be worth some discussion on the relationship between soil pore water and uptake that has been shown by many studies to be more strongly correlated than bulk soil and plant uptake.

RESPONSE: In a previous work (Martínez-Piernas et al. Anal. Chim. Acta. 1030 (2018) 115–124), the extraction method was fully validated for parent compounds in agricultural soil matrix and the recoveries for all CECs were in the range 70-120%. Therefore, the difference in the number of detected analytes in both matrices is not expected to be related to a bad performance of the extraction method in soil. The reasons why this commodity showed a higher number of analytes and concentrations could be related to the large surface area of perlite which allows it to retain a large amount of water. In addition, it should also be noted that perlite was contained in pots, which would limit migration or vertical transport of TPs. However, since no specific sorption/desorption studies of CECs were carried out in perlite, these hypotheses could not be evaluated.

Comment 10: Figure 3 and Figure 1 could be combined into a single figure.

RESPONSE: We consider that Figure 1 should remain in the Materials and Methods section for clarification on the specific periods in which the sampling events took place, while Figure 3 should be kept in the Results and discussion section.

REVIEWER # 2

This paper examines transformation products of several pharmaceutical parent chemicals that have been found in soils and some plants (e.g., tomatoes). While the chemical analyses appear to be very thorough, it is unclear how this research will be used by ecological or human health risk assessors or agricultural research. The pharmaceuticals would be assumed to be associated with sludge from wastewater treatment plants and at very low concentrations in treated wastewater or recycled wastewater - this is the case in the U.S. and in many other countries. From my perspective there is a significant gap in our understanding of concentrations and distribution of the parent pharmaceutical chemicals in soils, recycled water, and edible crops. The status of transformation products may be of chemical interest but those data may not be readily usable because we know so little about their toxicology and therefore risk to biota or people.

RESPONSE: Critical information of CECs and their mixtures is needed by many disciplines to gain a better understanding of the ecological impacts of water reuse on organisms of different trophic levels. However, and although the information in this regard is increasing, a full picture of the problem is not possible if the presence of TPs is ignored, especially when it is known that toxicity of some TPs may exceed that of the parent compounds [Escher and Fenner, Environ. Sci. Technol. 45(9) (2011) 3835-3847]. Furthermore, they can interact with each other resulting in additive or potentially even synergistic mixture effects. This work aims to contribute to identifying the presence of relevant TPs as a first step in the study of their risk to biota and/or consumers. Thus, this work represents a step forward and complements previous studies about the presence and fate of parent compounds in agricultural environments exposed to reuse practices.

I was unclear about the results from the perlite samples - isn't that a "soil" that should be free of contaminants? Are the pharmaceuticals coming from recycled water that is used to water the tomatoes grown in perlite? If so, then that would explain why the frequency and types of TPs in perlite and greenhouse soil were similar.

RESPONSE: Perlite is an inert, porous, and lightweight material widely used in soilless cultures since provides adequate aeration and proper water retention and drainage capabilities. As it should be initially a “blank” matrix, TPs found in perlite are derived from reclaimed water irrigation, which contains parent CECs and TPs. Furthermore, it cannot be discarded that the formation of TPs may take place by reactions produced by biological organisms present in the perlite ecosystem.

Still, the concentrations of TPs measured appear to be extremely low (< 20 ng/L and most below 1 ng/L).

RESPONSE: Please, note that the concentrations of the TPs in soil/plant material are expressed as ng/g instead of ng/L. The relevance of TP's concentration depends on their ecological impacts, which are still unknown. The initial steps to study TPs' agricultural implications are linked to the knowledge of their structures and environmental concentration levels reached after long-term exposure in real-field conditions. Therefore, this work provides useful information to begin to fill the ecotoxicological knowledge gaps related to TPs in reuse practices.

Table 3 should list detection limits for each TP.

RESPONSE: According to reviewer's comment, LOQs haven been included in Table 3.

Much of the Discussion in the paper is speculative regarding potential effects such as anti-microbial resistance and potential effects on people or biota. For the most part, as the authors indicate, the TPs that could be identified have very little information in this regard.

RESPONSE: Indeed, as indicated by the reviewer, there is a lack of ecotoxicological information for the identified TPs. It is expected that the proven evidence of their presence in agricultural environments will contribute to expanding the available information on the extent of exposure and fate of TPs in an agricultural ecosystem subjected to long-term irrigation with reclaimed water.

It is also noteworthy that, unlike most of the scientific literature usually focused on experiments in artificially contaminated soils, the present study has been carried out on a real tomato crops long term-irrigated with reclaimed water.

I also suggest specifying pharmaceuticals in the title of the paper - this paper did not examine all types of CECs, only certain pharmaceuticals.

RESPONSE: According to reviewer's comment, the title has been modified.

REVIEWER #3

The paper is providing valuable information on the occurrence of transformation products (TPs) of contaminants of emerging concern (CECs) in soil, leave and tomato samples irrigated by the reclaimed water. The study is a continuation of previous work by the authors where 57 pharmaceuticals and their TPs were detected in the crops exposed to the long-time irrigation. The available high-resolution mass spectrometry data were retrospectively re-analysed for presence of 262 potential TPs of the prioritised 20 parent CECs. As a result, none of the TPs was detected in the tomato samples, however, a few were present in the soil and leaves.

Despite the paper provides so much needed information on the fate of CECs in the agricultural ecosystems, it is written in a way more suited for dedicated mass spectrometry journal, with lengthy discussion on the process of identification of TPs, which were finally of no relevance from the human health point of view. In the discussion are often addressed ecotoxicology effects of the identified TPs and even hints are made that there is a potential threat from the transfer of antimicrobial resistance. The reasoning behind such assessments and statements is inadequate.

Therefore, the paper is recommended for publication only after significant shortening and major revision.

General

An in-house retention time (RT) prediction model has been used to support the process of identification of TPs. As claimed by the authors (lines 202 - 204) 'due to the rough RT estimation, the prediction approach was not considered as exclusion criteria for structure allocation, due to the reliability requirements and the inherent limitations of the method'. Nevertheless, it has been used rather opportunistically in the discussion, discarded when wrong value came out and praised when it matched the RT of the identified TP.

The discussion should not include references to this model.

RESPONSE: An error window of ± 2 min was assumed considering the possibility of making large errors in the predicted log K_{ow} , according to our in-house experience. RTs were predicted for all TPs for which a unique structure could be suggested. This information was available in Table S2 (now merged with Table 1, revised Manuscript) and it was not discussed for almost all TPs to avoid redundancy in the identification description. For TPs for which insufficient information could not be compiled to propose a unique structure (most of the TPs included in identification level 3 due to the possibility of several positional isomers), the log K_{ow} could not be calculated and, therefore, their RTs were not estimated. According to reviewer's comment, the discussion of RTs has not been included in the revised Manuscript. However, expected RT information has been kept in Table 1.

The English should be improved.

RESPONSE: The manuscript was sent to language check.

Line 28 - ... being 2 TPs not previously reported... Please, rephrase.

RESPONSE: The wording has been changed accordingly.

Line 117 - ... Physical-chemical characterization... Should read ...physico-chemical...

RESPONSE: The wording has been changed accordingly.

Line 191 - ... MassBank database (NORMAN network) [25]...Please, use proper description and reference to both MassBank Europe and NORMAN network. MassBank Europe is wrongly addressed several times throughout the text.

RESPONSE: The text and the reference have been modified accordingly.

Line 202 - ... the EPI Suit software... Should read EPI Suite...

RESPONSE: The wording has been changed accordingly.

Line 444 - ...Table 3 depicts the average semi-quantified concentrations...There is no mention in the text before how the semi-quantification works. Should be explained.

RESPONSE: The authors used the term "semi-quantification" considering that the results obtained represent only an approximation of the amount of the analytes. Considering that:

- This work has been carried out by retrospective analysis and, consequently, samples and standards have been analysed in different days. Unfortunately, samples cannot be reinjected and IS were not used when they were analysed.
- The extraction efficiency of the method has not been tested for the confirmed TPs.

A new text has been added to 2.6 section (lines 267-271, revised Manuscript with tracked changes) to clarify this point.

Line 488 - 492 - ...An estimated environmental risk assessment in aquatic environments carried out by Beretsou et al. (2016) reported that no individual risk is expected for CIT 343 at a semi-quantified concentration in wastewater of 0.01 $\mu\text{g L}^{-1}$. However, other study developed by Osawa et al. (2019) found that CIT 343 showed positive results in two of the three in silico carcinogenicity prediction models applied... This is confusing. Not clear, if the authors are addressing environmental ecotoxicity or human toxicity when assessing the results of the study. Please, clarify.

RESPONSE: Due to the scarce information available on the general toxicological implications of the TPs identified in this work, in these lines we set out to collect, identify and highlight both environmental and human repercussions found in the literature. In our opinion, it contributes to provide a preliminary vision of the impact associated with the presence of these compounds in agricultural environments, but according to the reviewer's comment, we have deleted the sentence referring to the results published by Osawa et al. to avoid misunderstandings.

Line 494 - ...commonly used as brain scanner... Please, rephrase.

RESPONSE: According to the reviewer's comment, the term has been rephrased (lines 727-728, revised Manuscript with tracked changes).

Line 496 - 497 - ...no ecotoxicological details or hazard information were found for DIP 267 in real environmental samples... Not clear, what was the purpose of this assessment. Pollutants in tomatoes, as crops intended for human consumption, should not be assessed based on ecotoxicological criteria (adverse effects to ecosystems). Please, clarify.

RESPONSE: We agree with the reviewer. The toxicological evaluation of crops intended for consumption should be based on health risk assessment. However, we refer to ecotoxicological aspects in the case of DIP 267 as it was not found in tomato fruits but in perlite samples. Since none of the TPs detected were found in tomato, a discussion of their health risk assessment has been avoided. The term "ecotoxicological details" has been replaced by "environmental risk assessment" to clarify this point. (line 735, revised Manuscript with tracked changes).

Line 503 - 504 - ...the high frequency of detection observed in this work would require further study of the presence and potential ecotoxicological effects in agricultural environments...Please, clarify why 'ecotoxicological effects' are relevant for this study.

RESPONSE: From our point of view, critical information is required for many disciplines to obtain a better understanding of the ecological impacts of water reuse on (micro)organisms that take part of agricultural ecosystems (from reclaimed water to edible crops). CECs, TPs, and their mixtures may affect biological biodiversity, biogeochemical cycles of nutrients, ecosystem functions and services, and their resilience to environmental stressors. For instance, a study carried out by Heye et al. found that a TP of the antiepileptic drug carbamazepine exhibited a higher chronic toxicity on the midge *Chironomus riparius* in comparison to the parent compound (Heye et al. Water Res. 98 (2016) 19-17). The opinions of experts on reclaimed water reuse point out that there is a knowledge gap on the agricultural impact of TPs on (micro)organisms (Carter et al. Environ. Sci. Process. Impacts. 21 (2019) 605–622 & Deviller et al. Chemosphere. 240 (2020) 124911). The identification of CEC TPs will contribute to studying their fate in agricultural environments and quantifying them when feasible, which aids to evaluate TPs assessment as exposure- or effect-driven on agricultural compartments. To clarify this point and to provide a more generic discussion, the sentence has been rephrased (lines 738-739, revised Manuscript with tracked changes).

Line 522 - 523 - ...Since the majority of the tentatively identified TPs in this study have been only investigated in aquatic environments, all potential ecotoxicological evidences are referred to this media...Please, clarify why this discussion is relevant for the study.

RESPONSE: Due to the lack of available information on the impact of TPs on agricultural ecosystems, a review on environmental implications on aquatic microorganisms is included, which may shed light on the potential impact of TPs on agricultural microorganisms. According to reviewer's comment, the sentence has been deleted.

Line 527 - 530 - ...effects on microorganisms, terrestrial wildlife and plant stress inducers, spread of antibiotic resistance, toxicological synergistic effects related to mixtures,

transformation of parent compounds in plant metabolism, introduction into the food chain and human low-level exposure...None of these points is properly addressed in the 'ecotoxicology' assessment in the text.

RESPONSE: None of these points have been addressed in the discussion since they are unknown for the scientific community. This is what has been highlighted in the manuscript. This work represents the first study that reveals the wide variety of pharmaceutical TPs that can be present in agricultural environments irrigated with reclaimed water, so that no specific (eco)toxicological tests at agricultural level have been performed so far.

Line 555 - 557 - ...the need for specific knowledge to evaluate TP ecotoxicological effects, including the spread of antibiotic resistance in agricultural environments submitted to RW irrigation...This statement in the conclusions is not sufficiently backed-up in the discussion and should be either supported by more evidence or deleted.

RESPONSE: New evidence about the risk associated to the presence of antibiotics and their TPs in agricultural systems has been included in the text (lines 719-724, revised Manuscript with tracked changes). Tadic et al. [J. Hazard. Mater. 401 (2021) 123424] reported that “*detected ABs explained 54 % of the total variation in AB resistance genes abundance in vegetable samples. Thus, further studies are needed to assess the risks of antibiotic resistance promotion in vegetables and the significance of the occurrence of their metabolites*”.

The transformation that pharmaceuticals can undergo during the water reclamation cycle, or by biotic/abiotic reactions when reclaimed water (RW) is used for irrigation, can lead to the presence of transformation products (TPs) in agricultural environments. However, data on TPs in real crops are scarce. Herein, a suspect screening approach was applied for the comprehensive investigation of 262 potential TPs, associated with 20 prioritised pharmaceuticals found in real tomato crops exposed to long-term RW irrigation. The occurrence and fate of the TPs was evaluated by the retrospective analysis of RW, soil, leave and tomato samples from 4 intensive production greenhouses. Sample analysis was accomplished by liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS). Up to 18 TPs were tentatively identified, of which 2 were not previously reported. 7 TPs were finally confirmed with analytical standards. 5 TPs were determined in RW, 15 TPs in soil and 2 TPs in leaves. Remarkably, the investigated TPs were not found in tomato fruits. These results shed light on the variety of TPs that can be found in the water reuse cycle and contribute to the assessment of the global risks of wastewater reuse and the safety of the vegetable and fruit production system.

1 **Assessment of the presence of transformation products of**
2 **pharmaceuticals in agricultural environments irrigated with reclaimed**
3 **water by wide-scope LC-QTOF-MS suspect screening**

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16

17 **Abstract**

18 The transformation that pharmaceuticals can undergo during the water reclamation cycle, or by
19 biotic/abiotic reactions when reclaimed water (RW) is used for irrigation, can lead to the presence
20 of transformation products (TPs) in agricultural environments. However, data on TPs in real crops
21 are scarce. Herein, a suspect screening approach was applied for the comprehensive investigation
22 of 262 potential TPs, associated with 20 prioritised pharmaceuticals found in real tomato crops
23 exposed to long-term RW irrigation. The occurrence and fate of the TPs was evaluated by the
24 retrospective analysis of RW, soil, leave and tomato samples from 4 intensive production
25 greenhouses. Sample analysis was accomplished by liquid chromatography coupled to
26 quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS). Up to 18 TPs were tentatively
27 identified, of which 2 were not previously reported. 7 TPs were finally confirmed with analytical
28 standards. 5 TPs were determined in RW, 15 TPs in soil and 2 TPs in leaves. Remarkably, the
29 investigated TPs were not found in tomato fruits. These results shed light on the variety of TPs
30 that can be found in the water reuse cycle and contribute to the assessment of the global risks of
31 wastewater reuse and the safety of the vegetable and fruit production system.

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34 **Keywords:** Water reuse, transformation products, contaminants of emerging concern, LC-
35 QTOF-MS, suspect screening

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40 **1. Introduction**

41 Reuse of reclaimed water (RW) for agricultural purposes is one of the solutions to reduce water
42 stress in arid and semi-arid regions. In these areas, the lack of water is a widespread problem, and
43 it is particularly important when agriculture represents the main economic activity. Consequently,
44 water reuse in agriculture contributes to an efficient water usage and the preservation of the
45 environment [1]. In this sense, the European Union (EU) is promoting a circular economy strategy
46 through urban wastewater reuse as a reliable alternative water source for agricultural irrigation
47 [2]. Thus, the EU Regulation (EU) 2020/741 [3] on minimum requirements for water reuse
48 (including agriculture irrigation), establishes a common framework based on physico-chemical and
49 microbiological parameters. However, reference levels for contaminants of emerging concern
50 (CECs), term which also includes pharmaceuticals, are not defined in this document.

51 One of the possible risks derived from water reuse practices is due to the presence of CECs in
52 RW and their release into agricultural systems. Water monitoring legislation at EU level
53 (Directive 2013/39/EU [4]) only focuses on a set of 45 priority substances (PS) and priority
54 hazardous substances (PHS). Additionally, the so-called EU 2020 Watch List proposes the
55 monitoring of 19 compounds for their consideration as possible PS [5]. Despite this, current
56 European legislation is still insufficient to manage the risks derived from wastewater reuse in
57 agriculture due to the occurrence of CECs and their TPs in RW and their possible plant/fruit
58 uptake [6].

59 One of the main knowledge gaps in addressing general risk assessment of water reuse is the
60 determination of the levels of pharmaceutical TPs in the water-soil-plant nexus. These TPs can
61 be generated by different biotic and abiotic processes during wastewater treatments [7,8] and by
62 parent compound biotransformation [9]. In addition, some TPs can be present in RW at
63 concentration levels similar to those of the parent pharmaceuticals [10]. Available data indicate
64 that, in most cases, TPs are as toxic as or less toxic than their parents [11]. Nevertheless, some
65 TPs may pose increased environmental risks than parent compounds: i) if they are formed at
66 >10% the concentration of the parent compounds, ii) if they show higher persistence and mobility
67 than their parents, and iii) if they exhibit toxicity due to the preservation of the bioactive moiety
68 or result in a different and more bioactive action than parents [11,12]. A recent study has reported
69 that TPs of sulfamethoxazole, trimethoprim, diclofenac, tetracycline, and ibuprofen, which were
70 generated during wastewater treatments, exhibited higher toxicity to aquatic microorganisms than
71 parent compounds [13]. Besides, recent works pointed out that the potential synergistic effects of
72 parent antibiotics and their TPs cannot be obviated, including their role in antibiotic resistance
73 spread in agricultural ecosystems [14,15]. The negative effects on the composition of soil
74 microbial community due to the presence of CECs have also been reported [16], but effects
75 associated with their TPs at environmental concentrations still requires further investigation. In
76 plants, the highest risk is related to the possible uptake of CECs and TPs and the subsequent health

77 risks when consuming the fruit or final product, which are still under discussion [15,17].
78 Considering these facts, additional investigation of TPs and their fate in the water reuse cycle is
79 needed.

80 Research efforts have mainly been focused on the identification of pharmaceutical TPs in the
81 water-soil-plant nexus including the study of the TPs generated in secondary and tertiary
82 wastewater treatments [18,19], after CEC degradation in soils, and as a consequence of in-plant
83 biotransformation [20–22]. The mechanisms explaining the formation of TPs have not been
84 thoroughly described yet. Studies dealing with the identification of TPs in agro-ecosystems are
85 usually performed under controlled conditions. Thus, single or mixtures of a few parent CECs are
86 used to spike the investigated medium at considerably higher concentrations than those expected
87 in a real agroecosystem. These strategies are essential to identify new TP structures and predict
88 their potential ecotoxicological effects under the evaluated conditions. However, the number and
89 nature of TPs reported in real agricultural environments is still scarce [15,23–25]. To our best
90 knowledge, studies dealing wide-scope search of TPs in real agricultural ecosystems have not
91 been reported. Due to the high number of unknown TPs that can be present in agricultural
92 compartments, their identification is a considerably complex and difficult task. In this sense,
93 liquid chromatography (LC) coupled to high-resolution mass spectrometry (HRMS) has
94 demonstrated its potential and capabilities as a powerful analytical tool for the detection of
95 unknown compounds at trace levels by the application of suspect screening methodologies and
96 retrospective analysis [26].

97 The present study aims to investigate the presence of pharmaceutical TPs due to the use of RW
98 in agricultural irrigation in real field crops. The selected agricultural systems consisted of plastic-
99 based greenhouses devoted to the intensive production of tomato and showing a long-term
100 exposition to RW irrigation. Samples from these greenhouses, including RW, soil, plant leaves
101 and tomato fruits, were studied in detail. A retrospective analysis to search and identify TPs from
102 prioritized pharmaceuticals was performed by using LC-HRMS data and a suspect screening
103 approach.

104

105 **2. Materials and methods**

106 *2.1 Chemicals and reagents*

107 Ultrapure water, acetonitrile (MeCN), glacial acetic acid and formic acid (LC-MS grade) were
108 purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was produced using a
109 Milli-Q water purification system from Millipore (Darmstadt, Germany). PTFE syringe filters (25
110 mm diameter, 0.22 µm pore size) were from SinerLab Group (Madrid, Spain). A total of 7
111 analytical reference standards were acquired for confirmatory purposes, namely atenolol acid
112 (CAS 56392-14-4, Toronto Research Chemicals, North York, Canada), 4-bromoantipyrine (CAS
113 5426-65-3, Sigma-Aldrich), chlorothiazide (CAS 58-94-6, TRC), citalopram amide (CAS 64372-

114 56-1, TRC), de(cladinosyl)clarithromycin (CAS 118058-74-5, TRC), N-desmethyltramadol
115 (CAS 1018989-94-0, LGC Standards (Middlesex, United Kingdom) and SR-49498 (CAS
116 748812-53-5, TRC) with purity > 98%). Individual stock solutions of each compound were
117 prepared in concentrations ranging from 1000 to 2000 mg L⁻¹ in MeOH. All standard solutions
118 were stored in amber glass vials at -20°C. Working solutions were prepared at appropriate
119 concentrations in MeCN:H₂O (10:90, v/v).

120

121 2.2 Sample collection

122 RW was supplied by a private regeneration plant facility, which treats municipal wastewater
123 secondary effluents by filtration (sand and anthracite filters) and chlorination (NaClO). This plant
124 provides RW to greenhouses (GH) of Almería province (Spain). Among them, 4 GHs devoted to
125 intensive production of tomato (13000–25000 m²) were selected and sampled due to RW has been
126 used for irrigation for more than 10 years. One of the GHs was dedicated to an experimental
127 soilless culture of cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) grown in pots filled
128 with perlite substrate (SP), while the other 3 produced tomato ramyle and retinto varieties
129 (*Solanum lycopersicum* L.) ground in real agricultural soils (GH1-3). Physico-chemical
130 characterization of the sampled soil can be found elsewhere [23]. Figure 1 shows the sampling
131 timeline followed for each matrix in the four sampling sites (GH1, GH2, GH3 and SP). For soil,
132 two sampling events were scheduled in consecutive years coinciding with the end of the tomato
133 cultivation (May 2016 and May 2017). In every sampling event, 500 g of soil were taken
134 (composite sample, five soil cores, zig-zag sampling, depth 10-15 cm close to the plant root). The
135 subsamples were mixed to conform the homogeneous composite sample which was sieved, freeze
136 dried until constant weight, grinded and kept in the dark at -20°C until analysis. For plant material
137 (plant leaves and tomato fruit), up to 4 sampling events were fixed in different periods throughout
138 a commercial tomato campaign (from January 2016 to May 2016). In each sampling event,
139 tomatoes at mature stage of growth and leaves of similar size (500 g each) were taken from
140 different plants of the greenhouse following a zig-zag sampling route. The subsamples were
141 chopped and mixed to form a homogeneous composite sample and were kept in the dark at -20°C
142 until analysis. Regarding RW, only a single RW sample was taken in November 2015.

143

144 2.3. Sample preparation

145 Sample extraction (soil, leaves and tomato) was carried out using two different versions of
146 QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) acetate methodology,
147 which were previously published by our group [23,24]. For leaves and tomatoes, a portion of 10
148 g of plant material were placed into a 50-mL polypropylene centrifuge tube. Then, 10 mL of 1%
149 acetic acid in MeCN and 20 µL of the extraction quality control solution (100 µg/L) were added
150 and the tube was shaken (5 min). After that, 6 g of anhydrous MgSO₄ and 1.5 g of NaOAc were

151 added and the tube was shaken again (5 min) and centrifuged at 3500 rpm (2054xg, 5 min).
152 Following this, a dispersive solid-phase extraction (d-SPE), used as clean-up step, was carried
153 out. To this purpose, 5 mL of the upper organic layer were placed into a 15-mL centrifuge tube
154 containing 750 mg of anhydrous MgSO₄, 125 mg of primary-secondary amine (PSA) and 125 mg
155 of C18. Then, the tube was vortexed (30 s) and centrifuged at 3500 rpm (5 min). Finally, 4 mL of
156 extract were transferred to screw-cap vials where 40 µL of MeCN at 1% of formic acid were
157 added. Prior to injection into the HPLC-QTOF-MS system, 100 µL of the extract were evaporated
158 until dryness and reconstituted in 100 µL of MeCN:H₂O (10:90, v/v).

159 In case of soil samples, 1 g of freeze-dried sample was weighed in a 50-ml polypropylene tube.
160 After that, 4 mL of Milli-Q H₂O were added, then shaken in a vortex (30 s) and left for 15 min
161 for rehydration. Afterwards, 10 mL of 1% acetic acid in MeCN and 20 µL of the extraction quality
162 control solution were added and the tube was shaken (5 min). Following this, 5 g of anhydrous
163 MgSO₄ and 1.5 g of NaOAc were added and the tube was shaken again (5 min) and centrifuged
164 (3500 rpm, 2054g, 5 min). In this case, the d-SPE consisted in a mixture of 750 mg of MgSO₄
165 and 125 mg of C18. Then, the protocol followed the same steps as for plant material described
166 before. RW sample was filtered and 100 µL of MeCN were added to an aliquot of 900 µL
167 previously to direct injection in the HPLC-QTOF system, as it is described in [23].

168

169 *2.4. LC-QTOF-MS analysis*

170 Analysis of samples was carried out using a LC Agilent 1260 Infinity system (Agilent
171 Technologies, Foster City, CA, USA) equipped with a Poroshell 120 EC-C18 analytical column
172 (50 x 4.6 mm, 2.7 µm particle size, Agilent Technologies) operated at a flow rate of 0.5 mL min⁻¹
173 and using an injection volume of 20 µL. Ultrapure water (0.1% formic acid, eluent A) and MeCN
174 (eluent B) were used as mobile phases. The gradient used ranged from 10% to 100% of solvent
175 B: initially it was kept constant at 10% for 2 min, then increased linearly from 10% to 100% for
176 9 min and finally it remained constant for 4 min before returning to the initial conditions. The
177 total analysis run time was 22 min. The LC system was connected to a QTOF mass analyzer
178 Triple TOF 5600+ from Sciex Instruments (Foster City, CA, USA) with a DuoSprayTM ion source
179 consisting of an electrospray (ESI) interface for sample injection and an atmospheric-pressure
180 chemical ionization interface (APCI) for calibrant solution delivery. Samples were analyzed in
181 both ESI⁺ and ESI⁻ modes. The ESI source parameters were: ionspray voltage, 4500 V; curtain
182 gas, 25 (arbitrary units); GS1, 60 psi; GS2, 60 psi; and temperature, 575°C. The acquisition was
183 made via TOF MS survey scan (resolving power of 30000) with an accumulation time of 250 ms
184 followed by four IDA (Information Dependent Acquisition) TOF MS/MS scans with an
185 accumulation time of 100 ms. The *m/z* scan range for both TOF MS and IDA was 50-1000.
186 Dynamic background subtraction was considered for IDA experiments. For MS² fragmentation,

187 a collision energy of 30 eV with a ± 15 eV spread was applied. Analyst TF 1.5 software was used
188 for data acquisition, and Sciex OS 1.5 for data processing (both from Sciex).

189

190 2.5. Prioritization of CECs and TPs suspect screening list

191 The previous analysis of the agricultural samples (RW, soil, leaves, tomato), retrospectively
192 evaluated in this work, revealed the presence of 57 CECs (pharmaceuticals and some of their
193 major and more frequently reported TPs) [23,24]. To investigate the presence TPs, a previous
194 selection of the parent analytes was carried out based on the following criteria: (i) compounds
195 found at high concentration; (ii) analytes most frequently detected in the different commodities;
196 (iii) drugs suspected to have an environmental/human toxic impact, according to literature
197 evidences; and (iv) those included in current regulations [4,5]. Based on these criteria, 20 parent
198 CECs were selected: amitriptyline (AMI), atenolol (ATE), azithromycin (AZI), caffeine (CAF),
199 cetirizine (CET), citalopram (CIT), clarithromycin (CLA), dipyrone (DIP), flecainide (FLE),
200 fluoxetine (FLU), hydrochlorothiazide (HCT), irbesartan (IRB), lamotrigine (LAM), lidocaine
201 (LID), mepivacaine (MEP), propranolol (PRP), telmisartan (TEL), tramadol (TRA), trimethoprim
202 (TRI) and venlafaxine (VEN).

203 Once the selection was performed, a literature search focused on their relevant and previously
204 reported TPs was carried out, minded diverse formation sources such as wastewater treatments
205 and biological processes. For certain CECs, reported TPs were not found in literature, and thus,
206 the *in silico* prediction tool EAWAG-BBD Pathway Prediction System [27] was used to obtain
207 possible TP structures. The suspect list finally developed contained 262 TPs and is shown in Table
208 S1. 8 TPs from the list had already been analysed in our laboratory [23,24]. Accordingly, their
209 retention time (RT) were included in the TP processing list to exclude them from possible isomer
210 candidates and were not considered as suspect TPs onwards. This was the case of paraxanthine
211 (CAF TP); N-desmethylcitalopram (CIT TP); 4-acetylaminoantipyrine, 4-formylaminoantipyrine
212 and antipyrine (DIP TPs); o-desmethyltramadol and tramadol-N-oxide (TRA TPs); and o-
213 desmethylvenlafaxine (VEN TP) (Table S1).

214

215 2.6. Suspect screening workflow

216 The analysis of HRMS data was entirely performed by Sciex OS software. The suspect list was
217 processed using the $[M+H]^+$ and $[M-H]^-$ adducts in samples analyzed by ESI+ and ESI-
218 respectively. The criteria considered to obtain tentative candidates included an absolute intensity
219 threshold of 1000 cps, a S/N ratio >10 , a tolerance of 5 ppm mass accuracy and an isotope ratio
220 difference (IRD) $<10\%$. Only chromatographic peaks with an intensity response ten times higher
221 than that of the procedural blanks [28] were further studied. The algebraic calculation of the best
222 formula to fit accurate masses of the product ions was performed by the Sciex OS software tool,
223 Formula Finder, and fragment alignment was also verified. Acquired MS² spectra were primarily

224 compared with two spectral libraries, the internal library All-in one HRMS (Sciex) and the open-
225 access database of mass spectra MassBank Europe [29]. In case of match with libraries, a score
226 >80% was set for the tentative assignation of candidates. Additionally, the *in silico* fragmentation
227 tool ChemSpider database [30] (integrated into the Sciex OS software) was checked to enhance
228 spectra interpretation. A minimum score of 70% was considered for candidates. TPs for which no
229 match was found in libraries or using the *in silico* fragmentation tool, the criteria adopted for their
230 further investigation as candidates was the presence of at least two product ions with a mass
231 accuracy error <5 ppm. Furthermore, their acquired MS² spectra were checked with literature
232 evidence, when available. To use the chromatographic retention behavior of the TPs to help with
233 their structural elucidation, an in-house RT prediction model was applied using a linear
234 correlation of the measured RTs and the estimated log *K_{OW}* values ($RT=0.8363 \times \log K_{OW} + 4.2853$,
235 $R^2=0.4705$) of 120 analytical standards analyzed in the same conditions. A window of ± 2 min
236 was considered. TP log *K_{OW}* were estimated by the EPI Suit[31]. Due to the rough RT estimation,
237 the prediction approach was not considered as exclusion criteria for structure allocation, due to
238 the reliability requirements and the inherent limitations of the method. TPs tentatively identified
239 were grouped according to the identification confidence levels proposed by Schymanski et al.
240 [32]. The concentration of the TPs confirmed with the analytical standard (identification level 1),
241 was estimated by external standard calibration curves. Experimental limits of quantification
242 (LOQs) were set as the lowest acceptable concentration in the calibration curve which yielded a
243 signal-to-noise (S/N) ratio close to 10 with a mass error < 5 ppm and an IRD <10%.

244

245 **3. Results and discussion**

246 *3.1. Tentative identification of TPs*

247 The identification of TPs in agro-ecosystems presents some analytical difficulties, which
248 include: (i) the complexity of the matrices; (ii) the frequent lack of high-quality MS² spectra,
249 mainly due to the low TP concentrations in samples; (iii) the lack of MS² spectra for many of
250 these TPs in libraries; and (iv) the lack of commercially available analytical standards for final
251 confirmation of the structure. For these reasons, it is often necessary to carry out structural
252 elucidation of TPs based on a laborious and detailed observation of the spectra, which in many
253 cases leads to a tentative identification.

254

255 The suspect screening strategy applied in this study yielded a first list of 44 TP candidates in
256 RW, soil, leaves and tomato samples. After comparing the acquired spectra with spectral
257 databases and literature, and scrutinizing the fragmentation pattern of each compound, a total of
258 18 TPs could be tentatively identified in the samples. 6 TPs were grouped in the high confidence
259 level 2 and, eventually, 7 TPs could be confirmed with the RT of their analytical standards. Table
260 1 shows the proposed structures, identification levels (according to Schymanski et al

261 [32]), chromatographic, spectral information and a summary of the criteria considered for
262 structure allocation in each case. A thorough discussion devoted to structure assignment of the
263 identified TPs has been performed and is shown below.

264

265 3.1.1 ATE TP

266 The formation of the only detected ATE TP, ATE 268 ($[M+H]^+$ C₁₄H₂₁NO₄, m/z 268.1543),
267 involves the hydrolysis of the amide group of the parent compound (Figure S1A), which results
268 in the formation of an acid. The acquired MS² spectrum of ATE 268 (Figure S1B) showed similar
269 fragmentation pattern as ATE and matched with the reported spectrum in literature [21]. A score
270 of 92% with ATE acid in MassBank Europe database supported the purchase of the analytical
271 standard and its RT was finally confirmed (Figure S1C).

272

273 3.1.2 AZI TPs

274 Up to three AZI TPs (Figure S2) were identified in the samples. AZI 592 (C₃₀H₅₇NO₁₀, $[M+H]^+$,
275 m/z 592.4055) and AZI 434 (C₂₂H₄₃NO₇, $[M+H]^+$, m/z 434.3112) kept the macrolactone ring intact
276 and were formed by the consecutive enzymatic removal of the desosamine (Figure S3) and
277 cladinose (Figure S4) moieties, reported in biotransformation reactions [2]. Both compounds were
278 included at identification level 2b, due to a 92 % of score with the structure proposed for AZI 434
279 in ChemSpider, and the match of three diagnostic fragments of AZI 592 with the most intense
280 ions found in the literature [18]. AZI 374 (C₁₉H₃₅NO₆, $[M+H]^+$, m/z 374.2534) represents a further
281 step in the AZI degradation process, which mainly results in the opening of the macrolide ring
282 and subsequent losses and oxidative reactions in the resulting structure. MS² information (Figure
283 S5) coincided well with two different isomers [18], AZI 374 B and 374 C in Table S1, but not
284 sufficient spectral evidence was found for an unambiguous assignment. For this reason, AZI 374
285 was included in identification level 3.

286

287 3.1.3. CIT TPs

288 The TPs CIT 339 (C₂₀H₁₉FN₂O₂, $[M+H]^+$ m/z 339.1503) and CIT 325 (C₁₉H₁₇FN₂O₂, $[M+H]^+$
289 m/z 325.1346) identified were formed by an oxidation reaction of CIT (Figure S6) in the furan
290 ring and further N-demethylation (Figures S7 and S8, respectively). Detection of the neutral loss
291 of CO from the product ion at m/z 276.0819 was indicative of the presence of a carbonyl group
292 in the furan ring, which produced 3-oxo-citalopram, a CIT human metabolite, as confirmed by
293 Beretsou et al [33]. The subsequent N-demethylation was supported by the loss of the NH₂CH₃
294 group observed in CIT 325 [34]. The full agreement of the MS² spectra of both compounds with
295 those reported in the literature placed them in the identification level 2b.

296 CIT 343 (C₂₀H₂₃FN₂O₂, $[M+H]^+$ m/z 343.1816) was confirmed as CIT amide. This TP is formed
297 by hydrolysis reaction of the nitrile group of CIT to yield a primary amide. This proposal was

298 supported by the presence of the product ion corresponding to the loss of the amide moiety at m/z
299 237.1074 (Figure S9A) and similarities of the MS² fragmentation pattern found in literature
300 [33,34]. Lastly, structure confirmation was carried out by reference standard analysis (Figure
301 S9B).

302

303 3.1.4. CLA TPs

304 CLA 590 (C₃₀H₅₅NO₁₀, [M+H]⁺ m/z 590.3898) would be formed by cleavage of the cladinose
305 group of CLA (Figure S10), typical of macrolide antibiotics, which led to the formation of a
306 hydroxy derivative. The CLA 590 MS² spectrum (Figure S11A) matched with the fragmentation
307 pattern reported in the literature [20,35] and by the ChemSpider database (85% of score). Its RT
308 was finally confirmed with the analytical standard (Figure S11B).

309 In the case of CLA 764 (C₃₈H₆₉NO₁₄, [M+H]⁺ m/z 764.4790), two different structures could be
310 proposed: the hydroxylation of the parent compound (14-OH-CLA) and the CLA-N-oxide
311 formation. Based on the differences observed with the spectrum reported for CLA-N-oxide by
312 Tian et al. [20] and considering that the acquired spectrum (Figure S12) scored 71% for 14-OH-
313 CLA using ChemSpider, the latter structure was the proposed and CLA 764 was included in
314 confidence level 2b.

315

316 3.1.5 DIP TP

317 The structure of DIP 267 corresponds to the 4-brominated derivate of phenazone (Figure S13):
318 4-bromoantipyrine (C₁₁H₁₁BrN₂O, [M+H]⁺ m/z 267.0127) (Figure S14A). The similarities of the
319 acquired MS² with the compound tentatively identified in literature [36] and a score of 72% on
320 ChemSpider were the reasons for purchasing the analytical standard. Finally, its RT could be
321 confirmed (Figure S14B).

322

323 3.1.6 HCT TP

324 The HCT 293 (C₇H₆ClN₃O₄S₂, [M-H]⁻ m/z 293.9415) structure corresponded to the
325 dehydrogenation of HCT (Figure S15), which resulted in an additional double bond located in
326 the benzothiadiazine ring to form a tertiary amine. The acquired spectrum (Figure S16A) was
327 checked against MassBank Europe and ChemSpider, and both results indicated a good match with
328 chlorothiazide (91% and 78% of score, respectively). Analysis of the corresponding analytical
329 standard confirmed the identity of this TP (Figure S16B).

330

331 3.1.7 IRB TPs

332 Up to 5 TPs of IRB (Figure S17) could be identified, which were formed by oxidation reactions
333 of the parent compound. The main reactions observed were: i) hydroxylation in the alkyl chain

334 (IRB 445 A), ii) oxidation of the double bond of the imidazolone ring (IRB 445 B, IRB 447) and
335 iii) oxidation of both positions (IRB 461 A).

336 The hydroxylation of the alkyl chain was observed for IRB 445 A ($C_{25}H_{28}N_6O_2$, $[M+H]^+$ m/z
337 445.2346, RT 7.03 min) (Figure S18). The acquired MS² spectrum of this TP also showed the
338 breakage of the molecule observed for IRB, yielding a coincident ion at m/z 207.0917 ($C_{14}H_{10}N_2$)
339 corresponding to the tetraazolic biphenyl moiety; and a second fragment at m/z 211.1441
340 ($C_{11}H_{18}N_2O_2$) instead of the ion at m/z 195.1492 ($C_{11}H_{18}N_2O$) present in IRB spectrum. This
341 indicates that the hydroxylation occurred in this part of the structure. Although some authors
342 propose the hydroxylation of the alkyl chain as the most probable structure [37], the absence of
343 characteristic ion fragments does not allow to confirm this hypothesis. Therefore, IRB 445 was
344 classified in level 3.

345 For IRB 447 ($C_{25}H_{30}N_6O_2$, $[M+H]^+$ m/z 447.2503), the ESI+ fragments at m/z 252.1244, and
346 196.1331 (Figure S19A), would indicate the oxidation of the imidazolone ring and its subsequent
347 opening. The fragments at m/z 305.1659 and 211.1452 in the ESI- MS² spectrum (Figure S19B)
348 also confirmed the formation of the amide group. After obtaining a 91% of score in ChemSpider
349 and comparing the RT with that of the analytical standard, the proposed structure for IRB 447
350 was confirmed.

351 IRB 445 B ($C_{25}H_{28}N_6O_2$, $[M+H]^+$ m/z 445.2346, RT 8.84 min) presented a fragmentation pattern
352 similar to that of IRB 447 in ESI+, with two coincident ions (m/z 196.1332 and 168.1383) while
353 other three ions were found with a difference of 2 Da (m/z 250.1087, 233.0822 and 205.0760,
354 Figure S20). This mass difference indicated a related structure with an extra unsaturation
355 (RDB=15), probably located between the carbon in alpha with the biphenyl group and the nitrogen
356 atom, which is supported by the presence of fragments at m/z 361.1771 and m/z 250.1087.
357 However, due to the lack of conclusive information, IRB 445 B was included in level 2b as
358 probable structure.

359 The MS² spectrum of IRB 461 A ($C_{25}H_{28}N_6O_3$, $[M+H]^+$ m/z 461.2295, RT 7.35 min) showed
360 common ions with IRB 447 (m/z 252.1244, 235.0978, 207.0917 and 84.0808, Figure S21A). But
361 presence of one additional oxygen atom and an extra unsaturation, suggested the presence of an
362 additional carbonyl group. Presence of product ion at m/z 99.0441 in ESI+ was decisive to ensure
363 the presence of two carbonyl groups in the alkyl chain (Figure S21A) [37]. The spectrum in ESI-
364 was also compatible with the proposed structure by the structural allocation of product ion at m/z
365 207.1139, which presumably could be formed by the rearrangement of the imidazole ring
366 producing the loss of H₂O (Figure S21B). However, and although the similarities found with the
367 ESI+ spectrum reported in literature [37] were consistent, IRB 461 A was included in confidence
368 level 3.

369 Finally, IRB 461 B isomer (RT 7.61 min) presented fragment ions similar to IRB 461 A, at m/z
370 252.1244, 235.0992, 207.0917 and 180.0808 (Figure S22A). However, the characteristic product

371 ions of IRB 461 A at m/z 210.1125 and 99.0441, which supported the presence of the two carbonyl
372 moieties in the alkyl chain, were not found in IRB 461 B. This suggested that the extra oxygen
373 and the unsaturation should be in another position of the molecule. The associated molecular
374 formulae of product ions found at m/z 102.0913 in ESI+ and at m/z 124.0404 and 81.0346 in ESI-
375 (Figure S22B), pointed out that the oxidation may occur in the cyclopentane ring. However, the
376 structural assignment of the ESI- product ion at m/z 220.0768 ($C_{15}H_{11}NO$) was not possible under
377 this premise. Thus, IRB 461 B was kept in identification level 4. Although a structure could not
378 be proposed in this case, MS² information provided sufficient keys to correlate this TP with
379 sartans. To our knowledge, this TP has not been reported before.

380

381 3.1.8 TEL TP

382 The structure proposed for TEL 439 ($C_{27}H_{26}N_4O_2$ ($[M+H]^+$, m/z 439.2128) was obtained using
383 the in silico prediction tool EAWAG-BBD Pathway Prediction System [27]. TEL 439 would be
384 formed by an initial dihydroxylation of the biphenyl carboxylic moiety of TEL, followed by a
385 meta-cleavage pathway that would produce a para-phenyl acid derivate (Figure S23). Degradation
386 of various alkylphenols has been reported to occur by bacterial biotransformation in sediments
387 and sludge via the proposed meta-cleavage route [38]. The spectral information of parent TEL
388 439 was compared to internal and external databases and no match was found. However, MS²
389 spectrum of parent TEL showed a common product ion with TEL 439 at m/z 276.1369 (Figure
390 S24, Table 1). The investigated product ions revealed an initial loss of H₂O (m/z 421.2023) and
391 subsequent losses of CO (m/z 393.2074) and CO₂ (m/z 365.1761), which support the presence of
392 a carboxylic acid group (Figure 2). Presence of product ion at m/z 289.1448 would indicate a loss
393 of the propyl chain and the phenyl acid groups, while characteristic ion at m/z 276.1369 would
394 correspond to both imidazolone core groups (shared with TEL). Although the mass fragments
395 obtained support the assignment of the proposed structure, the lack of additional evidence led to
396 the inclusion of TEL 439 in level 3. To our best knowledge, TEL 439 has not been previously
397 reported.

398

399 3.1.9 TRA TP

400 Up to 5 different structures included in the suspect list (Table S1), belonging not only to TRA
401 but also to VEN TPs, matched with the molecular formula of TRA 250 ($C_{15}H_{23}NO_2$, $[M+H]^+$ m/z
402 250.1801). O-desmethyltramadol and O-desmethylvenlafaxine were discarded based on the RTs
403 of the corresponding analytical standards. N,N-didesmethylvenlafaxine and N,O-
404 didesmethylvenlafaxine proposals were not considered as their MS² spectra did not match when
405 compared in Mass Bank Europe. Characteristic fragment ions of TRA (Figure S25) were observed
406 at m/z 159.0804, 121.0648 and 91.0542 (Figure S26A). MS² spectrum scored 98% and 78% with
407 N-desmethyltramadol in MassBank Europe and ChemSpider, respectively. It also matched with

408 literature reported [19]. Finally, TRA 250 was confirmed with the analytical standard (Figure
409 S26B).

410

411 *3.2 Occurrence of TPs in agricultural samples*

412 The occurrence and distribution of the 18 TPs in RW, soil, plant leaves and tomato fruits were
413 evaluated throughout the different sampling events at the selected sampling points (SP, GH1,
414 GH2, GH3). Table 2 shows the average chromatographic peak areas of the TPs in the samples in
415 which they were detected. Table 3 depicts the average concentrations of the confirmed TPs. The
416 concentrations of the parent compounds can be found elsewhere [23,24]. Up to 5 TPs were found
417 in RW, while 15 TPs could be determined in agricultural soils and perlite. Regarding tomato
418 plants, only 2 TPs were detected in leaves and none of them were found in tomato fruits (Table
419 2, Figure 3). SP substrate contained by far the highest number of compounds (14 TPs), followed
420 by agricultural soils from GH2 (8 TPs), GH3 (3 TPs), and GH1 (1 TP). This finding was closely
421 related to the presence of parent compounds previously reported in these matrices (Table 2)
422 [23,24]. Although perlite showed the highest number of TPs and some of them presented certain
423 accumulation or persistence, this fact could not be associated with a higher availability and
424 translocation of such TPs in the plant. This behavior agrees with the results obtained in previous
425 studies evaluating the occurrence of the parent pharmaceuticals. The data demonstrated that there
426 was not any difference in the detected concentrations or the detection frequency of the CECs in
427 leaves and tomato samples from SP when compared with the samples from typical GHs [23,24].

428 ATE 268, CLA 764, IRB 445 A, IRB 447, and IRB 461 A were the only five TPs identified in
429 RW. Although ATE and ATE 268 had been reported in wastewater [8] and ATE 268 is recognized
430 as the main transformation product of ATE biodegradation in soil under controlled conditions
431 [21]. Nevertheless, none of the two compounds was detected in any of the real soil samples
432 analysed. The same occurred for IRB 445 A and CLA 764. The latter was only present at low
433 abundance in a single soil sample. However, two additional IRB TPs, IRB 447 and IRB 461 A,
434 were found in perlite in both sampling events, with IRB 447 showing slight accumulation between
435 samplings. Other IRB TPs, IRB 445 B and IRB 461 B (not present in the RW) could be detected
436 in SP in both samplings. However, despite the high occurrence of IRB TPs in perlite, none of
437 them could be detected in GH soil samples, except for IRB 445 B which was present in a single
438 sample. The sorption of IRB in soil has been described [39], however, its behavior is still unclear
439 and only a limited number of reports have examined its fate. Some authors have reported that IRB
440 potential to migrate is moderate or low in a subsurface water environment [16]. This fact, together
441 with its high dissipation half-life reported in soil, can limit the formation of TPs at detectable
442 concentrations, which agrees with the results obtained in this work.

443 AZI TPs (AZI 374, AZI 434, and AZI 592) were found in various soil samples from different
444 sampling points presenting persistence in most cases. AZI 374 was only detected in SP while AZI

445 592 was also observed in soil samples from GH2. It must be noticed that AZI 434 was present in
446 all perlite and soil samples, except for the first sampling carried out in GH3, showing a higher
447 detection frequency than that observed for the parent AZI [23]. AZI 434 and AZI 592 have already
448 been tentatively identified in real RW intended for agricultural purposes [14]. Noticeably, these
449 TPs maintain intact the macrocyclic lactone ring and, thus, they may still show residual
450 antimicrobial activity [40]. Other compounds that may have toxicological implications on the
451 spread of antibiotic resistance are CLA 590 and CLA 764, as they preserve the antimicrobial ring
452 and the tertiary amine group of the desosamine moiety [40]. A study carried out by Baumann et
453 al. revealed a comparable environmental risk of CLA 764 (tentatively identified as 14-OH-CLA)
454 than parent CLA due to their similar concentrations found in surface waters [41]. However, in
455 this work, CLA 764 was only detected in the samples in which the parent compound was
456 quantified at the highest concentrations [23]. Although data indicates less occurrence of CLA TPs
457 compared to AZI TPs, their presence should not be underestimated due to the potential
458 ecotoxicological implications. Evidence about the risk associated with the presence of antibiotics
459 and their TPs in agricultural systems have been studied by Tadić et al. The authors reported that
460 only the 54 % of the total variation in antibiotic resistant genes abundance could be explained by
461 the detected antimicrobials in vegetables irrigated with RW [15]. Therefore, further insight about
462 antibiotic TPs occurrence and their possible activity should be investigated, including regular
463 monitoring.

464 CIT related TPs, CIT 325, CIT 339, and CIT 343 were detected only in samples from SP (both
465 samplings except for CIT 343) and GH2 (first sampling). An estimated environmental risk
466 assessment in aquatic environments carried out by Beretsou et al. (2016) reported that no
467 individual risk is expected for CIT 343 at a semi-quantified concentration in wastewater of 0.01
468 $\mu\text{g L}^{-1}$.

469 DIP 267 was detected in perlite substrate in both sampling events showing an accumulation
470 tendency. This phenazone halogenated derivate, commonly used as a contrast agent for brain scan,
471 has been identified during chlorination of antipyrine under controlled conditions when bromide
472 was present in the degradation solution [36]. However, no environmental risk assessment or
473 hazard information were found for DIP 267.

474 The presence of TEL 439 was observed in all the GH and SP soil samples, with a notably higher
475 intensity in the latter. This behavior is in agreement with the high concentrations also observed in
476 SP for the parent TEL [23]. Because of the high $\log K_{O/W} = 7.7$ of TEL, its presence at high
477 concentrations in sewage sludge used as a soil amendment, as well as a very high bioaccumulation
478 in roots, have been reported [42]. To our knowledge, no previous information about TEL TPs has
479 been found. However, the high frequency of detection observed in this work would require further
480 studies to evaluate the presence and fate of TEL TPs to assess their contribution to overall

481 agricultural risks, where the presence of TEL can be abundant due to both reuse practices and use
482 of sewage sludge.

483 At last, two TPs were detected in leaves, HCT 293 and TRA 250, confirmed as chlorothiazide
484 and N-desmethyltramadol, respectively. HCT 293 was detected in leaves from SP and GH 2 (third
485 and fourth samplings) and SP substrate (last sampling), showing comparable concentrations than
486 those found for parent HCT in leaves (Table 3) [24]. Interestingly, this TP was not detected in
487 soil samples from GH 2, which would indicate that its formation could be related to the
488 biotransformation/uptake of the parent HCT in plant tissues. The formation of HCT 293 from the
489 parent compound has already been reported by abiotic and biotic reactions such as ozonation,
490 photodegradation, hydrolysis, and biotransformation in river sediments [43,44]. Regarding
491 environmental hazardous implications, an ecotoxicological evaluation with bacterial
492 bioluminescence revealed that HCT 293 did not pose increased effects in comparison with HCT
493 [44]. On the contrary, the occurrence of TRA 250 did not follow a clear trend. It was found in the
494 first sampling of SP, but surprisingly, it was not present in the second sampling. Furthermore, this
495 TP was observed in the leaves from GH3 (fourth sampling) without being detected in soil samples
496 from the same site. This would indicate a probable formation by biotic transformations of TRA
497 in plant tissues. In line with this, and according to Kostanjevecki et al. (2019), TRA 250 has been
498 detected as a microbial biodegradation product of TRA in activated sludge culture and no
499 significant toxic effects were found in algal bioassay.

500 To our knowledge, occurrence data of the 18 TPs investigated in this work have not been
501 reported in real field agricultural samples. However, in terms of agricultural systems, there is a
502 knowledge gap regarding CEC TPs due to the high variety of compounds that can be present and
503 the scarce information available about real-field samples at environmental concentrations. For
504 instance, effects on microorganisms, terrestrial wildlife and plant stress inducers, spread of
505 antibiotic resistance, toxicological synergistic effects related to mixtures, transformation of parent
506 compounds in plant metabolism, introduction into the food chain and human low-level exposure,
507 among others, are practically unknown for the reuse of RW in agriculture [17]. From this point
508 of view, the application of HRMS analytical strategies able to detect and identify compounds not
509 previously reported in agricultural environments is of high importance to fill in the knowledge
510 gaps in risk assessment associated with reuse practices.

511

512 **4. Conclusions**

513 The originality of this study is based on the first application of a retrospective suspect screening
514 focused on 262 TPs from 20 parent CECs in actual agricultural environments. Four real-field
515 agricultural systems (SP, GH1, GH2, GH3) irrigated with RW for more than 15 years were
516 investigated in search of pharmaceutical TPs. A thorough investigation of TP fragmentation
517 patterns together with a comparison with spectral libraries and literature evidence were decisive

518 for the structural assignment and classification of up to 18 TPs from 9 CECs. The developed
519 analytical strategy has been successfully applied for the tentative identification with high
520 confidence of 12 TPs, which led to the confirmation of 7 TPs. 2 TPs were tentatively identified
521 for the first time. Occurrence and environmental impact of the 18 TPs were evaluated. SP (perlite
522 matrix) showed the highest number of compounds (15 TPs), followed by agricultural soils from
523 GH1, GH2 and GH3 (8 TPs), RW (4 TPs) and plant leaves (2 TPs). Remarkably, none of the
524 investigated TPs was found in tomato fruit samples. Although perlite substrate accumulated the
525 highest number of TPs, no significant and specific availability of TPs for plants was observed.
526 Up to 6 TPs showed persistence between sampling events in perlite/soil samples and occurrence
527 of AZI 434 and TEL 439 was found to be almost ubiquitous in these matrices. In general, no clear
528 trend showing uptakes from soils/perlite to leaf plant tissues was detected. HCT 293 and TRA
529 250 were the only two compounds identified in leaves. To our knowledge, no previous data is
530 available regarding the TPs evaluated in this work in agricultural environments. This study
531 stresses the wide variety of CEC TPs derived from reuse practices that can be present in
532 agricultural systems as well as the need for specific knowledge to evaluate TP environmental
533 impact, including the possible spread of antibiotic resistance in agricultural environments
534 submitted to RW irrigation.

535

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540

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710

711 **Figure captions**

712 **Figure 1.** Sampling events carried out for each matrix at all sampling sites (SP, GH1-3)

713 **Figure 2.** ESI+ MS² spectrum of TEL 439 in a perlite sample and proposed fragmentation

714 **Figure 3.** Total number of TPs found in each sampling site/matrix

1 **Assessment of the presence of transformation products of**
2 **pharmaceuticals ~~contaminants of emerging concern~~ in agricultural**
3 **environments irrigated with reclaimed water by wide-scope LC-**
4 **QTOF-MS suspect screening**

5

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17

18 **Abstract**

19 The transformation that ~~contaminants of emerging concern (CECs) pharmaceuticals~~ can ~~suffer~~
20 undergo during the water reclamation cycle, or by biotic/abiotic reactions when reclaimed water
21 (RW) is used for irrigation, can lead to the presence of transformation products (TPs) in
22 agricultural environments. However, data on TPs in real crops are scarce. Herein, a suspect
23 screening approach was applied for the comprehensive investigation of 262 potential TPs,
24 associated ~~to~~ with 20 prioritised ~~CECs pharmaceuticals~~ found in real tomato crops exposed to
25 long-term RW irrigation. The occurrence and fate of the TPs was evaluated by the retrospective
26 analysis of RW, soil, leave and tomato samples from 4 intensive production greenhouses. Sample
27 analysis was accomplished by liquid chromatography coupled to quadrupole-time-of-flight mass
28 spectrometry (LC-QTOF-MS). Up to 18 TPs were tentatively identified, with being of which 2 TPs
29 of them were not previously reported. 7 TPs were finally confirmed with analytical standards. 5
30 TPs were determined in RW, 15 TPs in soil and 2 TPs in leaves. Remarkably, the investigated
31 TPs were not found in tomato fruits. These ~~evidences results~~ shed light on the variety of TPs that
32 can be found in the water reuse cycle and contribute to ~~assess the assessment of~~ the global risks
33 of wastewater reuse and the safety of the vegetable and fruit production system ~~the safety of the~~
34 ~~produced vegetables and fruits~~.

35

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37 **Keywords:** Water reuse, transformation products, contaminants of emerging concern, LC-
38 QTOF-MS, suspect screening

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43 1. Introduction

44 Reuse of reclaimed water (RW) for agricultural purposes is one of the solutions to reduce and
45 mitigate water stress in arid and semi-arid regions. In these areas, where the lack of water is a
46 widespread problem, and it is particularly important where agriculture represents the main
47 economic activity. Consequently, water reuse in agriculture ~~this strategy is of great interest since~~
48 contributes to an efficient water usage and the preservation of the environment [1]. ~~Due its~~
49 ~~inherent benefits~~ In this sense, the European Union (EU) is ~~facing the water scarcity problem by~~
50 promoting a circular economy strategy through urban wastewater reuse as a reliable alternative
51 water source for agricultural irrigation [2]. ~~Thus, In order to harmonize the different regulations~~
52 ~~of the European countries,~~ the EU European Parliament and the Council ~~launched the~~ Regulation
53 (EU) 2020/741 [3] on minimum requirements for water reuse (including agriculture irrigation),
54 establishes a common framework based on ~~considering only~~ physico-chemical and
55 microbiological parameters ~~to ensure the safe use of RW for agricultural purposes.~~ However,
56 reference levels for contaminants of emerging concern (CECs), term which also includes
57 pharmaceuticals, are not defined in this document.

58 One of the possible risks derived from water ~~Although~~ reuse practices is ~~seems to be effective~~
59 ~~dealing with water shortages, due to~~ the presence of contaminants of emerging concern (CECs)
60 in RW and their release into agricultural systems. ~~is a matter of current concern.~~ CECs are defined
61 ~~as chemicals of widespread human use, which may pose unwanted ecological effects on living~~
62 ~~organisms as a result of their persistence and distribution in the environment [4].~~ However,
63 Water monitoring approaches legislation only focus on a set of 45 priority substances (PS) and
64 priority hazardous substances (PHS) at European EU level (Directive 2013/39/EU [4]) only
65 focuses on a set of 45 priority substances (PS) and priority hazardous substances (PHS).
66 Additionally, the so-called EU 2020 Watch List proposes the monitoring of 19 compounds ~~are~~
67 ~~included in the so-called Watch List that was recently published by the European Commission in~~
68 ~~2020~~ for their future consideration as possible PS [5]. ~~Despite this, the joint efforts to prepare a~~
69 ~~regulation, experts on the field have pointed out that~~ current European legislation is still
70 insufficient to manage the risks derived from wastewater reuse in agriculture due to the
71 occurrence of CECs and their TPs ~~posed by CECs in RW~~ and their possible plant/fruit uptake in
72 the agricultural reuse context [6].

73 One of the main knowledge gaps in addressing general risk assessment of water reuse ~~to assess~~
74 ~~the global risk of reuse~~ is the determination of the levels of pharmaceutical ~~fate of transformation~~
75 products (TPs) in the water-soil-plant nexus derived from CECs. These TPs can be generated by
76 different biotic and abiotic processes reactions in ~~during~~ wastewater treatments processes for
77 ~~water reclamation~~ [7,8] and by parent compound biotransformation ~~carried out by environmental~~
78 ~~organisms~~ [9]. In addition, some TPs can be present in RW at ~~similar~~ concentration levels similar
79 to those of their parent CECs pharmaceuticals [10]. Available data indicate that, in most cases,

80 TPs are as toxic as or less toxic than their parents [11]. Nevertheless, some TPs ~~and can may~~
81 ~~pose have even increased eco-toxic~~ increased environmental risks than parent compounds: i) if
82 ~~they are formed at >10% the concentration of the parent compounds, ii) if they show higher~~
83 ~~persistence and mobility than their parents, and iii) if they exhibit toxicity due to the preservation~~
84 ~~of the bioactive moiety or result in a different and more bioactive action than parents [11,12]-. A~~
85 ~~recent study has reported that TPs of sulfamethoxazole, trimethoprim, diclofenac, tetracycline,~~
86 ~~and ibuprofen, which were generated during wastewater treatments, exhibited higher toxicity to~~
87 ~~aquatic microorganisms than parent compounds [13]. Besides, r~~Recently, works pointed out that
88 ~~the potential synergistic effects of parent antimicrobial-antibiotics and their TPs cannot be~~
89 ~~obviated, including their role in antibiotic resistance spread in agricultural ecosystems derived~~
90 ~~TPs have been addressed as precursors on the spread of antibiotic resistance in RW intended for~~
91 ~~reuse practices~~ [14,15]. The negative effects on the composition of soil microbial community due
92 ~~to the presence of CECs have also been reported [16], but effects associated with their TPs at~~
93 ~~environmental concentrations still requires further investigation. In plants, the highest risk is~~
94 ~~related to the possible uptake of CECs and TPs and the subsequent health risks when consuming~~
95 ~~the fruit or final product, which are still under discussion [15,17]. Considering these facts,~~
96 ~~additional investigation of TPs and their fate in the water reuse cycle is needed.~~

97 ~~Scientific research has tackled~~Research efforts have mainly been focused on the ~~exclusive~~
98 identification of ~~pharmaceutical TPs from pharmaceutical CECs~~ in the water-soil-plant nexus
99 ~~reuse cycle from different perspectives, which include~~ the study of the TPs generated ~~during in~~
100 secondary and tertiary wastewater treatments [18,19], ~~after as well as by~~ CEC degradation in soils,
101 and ~~as a consequence of~~ in-plant biotransformation [20–22]. The mechanisms explaining the
102 formation of TPs ~~have not been~~ ~~are still not~~ thoroughly described ~~yet~~. ~~Consequently, s~~Studies
103 dealing with the identification of TPs in agro-ecosystems are usually performed under controlled
104 conditions. ~~Thus, S~~Single or ~~cocktails mixtures~~ of a few parent CECs are ~~used to spike added to~~
105 the investigated medium at ~~much~~considerably higher concentrations than those ~~environmentally~~
106 expected ~~in a real -agroecosystem~~. These strategies are essential to identify ~~new TP their~~ structures
107 and predict their ~~potential~~ ecotoxicological ~~risks-effects~~ under the ~~specified-evaluated~~ conditions.
108 However, the number and nature of TPs reported in real-~~field~~ agricultural environments is still
109 scarce [15,23–25], ~~and, t~~To our best knowledge, ~~studies dealing~~ ~~no~~ wide-scope search of TPs ~~at in~~
110 real ~~environmental concentrations has been performed in~~ agricultural ecosystems ~~have not been~~
111 ~~reported so far~~. Due to the high number of unknown TPs that can be present in
112 ~~environmental agricultural~~ -compartments, their identification is a considerably complex and
113 difficult task. In this sense, liquid chromatography (LC) coupled to high-resolution mass
114 spectrometry (HRMS) has demonstrated its potential and capabilities as a powerful analytical tool
115 for the detection of unknown compounds at trace levels by the application of suspect screening
116 methodologies and retrospective analysis [26].

117 The present study aims to ~~investigate contribute to the current knowledge about~~ the presence of
118 ~~pharmaceutical~~ TPs ~~due to the use of RW derived from water reuse practices~~ in agricultural
119 ~~environments~~ irrigation in real field crops. The selected agricultural systems consisted of plastic-
120 ~~based greenhouses devoted to the intensive production of tomato and showing a long-term~~
121 ~~exposition to RW irrigation. For this purpose, the identification of TPs in real field agricultural~~
122 ~~samples was performed~~ Samples from these greenhouses, including RW, soil, plant leaves and
123 ~~tomato fruits, were studied in detail. A retrospective analysis to search and identify TPs from~~
124 ~~prioritized pharmaceuticals was performed by using the application of a~~ LC-HRMS ~~data~~
125 ~~workflow using a and a~~ suspect screening approach. ~~The occurrence and fate of TPs from~~
126 ~~prioritized CECs were studied in RW, soil, plant leaves and tomato fruits from crops cultivated~~
127 ~~in greenhouses devoted to the intensive production of tomato and showing a long-term exposition~~
128 ~~to RW irrigation.~~

130 2. Materials and methods

131 2.1 Chemicals and reagents

132 Ultrapure water, acetonitrile (MeCN), glacial acetic acid and formic acid (LC-MS grade) were
133 purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was produced using a
134 Milli-Q water purification system from Millipore (Darmstadt, Germany). PTFE syringe filters (25
135 mm diameter, 0.22 µm pore size) were from SinerLab Group (Madrid, Spain). A total of 7
136 analytical reference standards were acquired for confirmatory purposes, namely atenolol acid
137 (CAS 56392-14-4, Toronto Research Chemicals, North York, Canada), 4-bromoantipyrine (CAS
138 5426-65-3, Sigma-Aldrich), chlorothiazide (CAS 58-94-6, TRC), citalopram amide (CAS 64372-
139 56-1, TRC), de(cladinosyl)clarithromycin (CAS 118058-74-5, TRC), N-desmethyltramadol
140 (CAS 1018989-94-0, LGC Standards (Middlesex, United Kingdom) and SR-49498 (CAS
141 748812-53-5, TRC) with purity > 98%). Individual stock solutions of each compound were
142 prepared in concentrations ranging from 1000 to 2000 mg L⁻¹ in MeOH. All standard solutions
143 were stored in amber glass vials at -20°C. Working solutions were prepared at appropriate
144 concentrations in MeCN:H₂O (10:90, v/v).

146 2.2 Sample collection

147 RW was supplied by a private regeneration plant facility, which treats municipal wastewater
148 secondary effluents by filtration (sand and anthracite filters) and chlorination (NaClO). This plant
149 provides RW to greenhouses (GH) of Almería province (Spain). Among them, 4 GHs devoted to
150 intensive production of tomato (13000–25000 m²) were selected and sampled due to RW has been
151 used for irrigation for more than 10 years. One of the GHs was dedicated to an experimental
152 soilless culture of ~~cherry~~ tomato (*Solanum lycopersicum* var. *cerasiforme*~~cherry variety~~) grown
153 in pots filled with perlite substrate (SP), while the other 3 produced tomato ramyle and retinto

154 varieties (*Solanum lycopersicum* L.) ground in real agricultural soils (GH1-3). Physical-
155 chemical characterization of the sampled soil can be found elsewhere [23]. Figure 1 shows the
156 sampling timeline followed for each matrix in the four sampling sites (GH1, GH2, GH3 and SP).
157 For soil, two sampling events were scheduled in consecutive years coinciding with the end of the
158 tomato cultivation (May 2016 and May 2017). In every sampling event, 500 g of soil were taken
159 (composite sample, five soil cores, zig-zag sampling ~~W-distribution in the GH~~, depth 10-15 cm
160 close to the plant root). The subsamples were mixed to conform the homogeneous composite
161 sample which was sieved, freeze dried until constant weight, grinded and kept in the dark at -
162 20°C until analysis. For plant material (plant leaves and tomato fruit), up to 4 sampling events
163 were fixed in different periods throughout a commercial tomato campaign (from January 2016 to
164 May 2016). In each sampling event, tomatoes at mature stage of growth and leaves of similar size
165 (500 g each) were taken from different plants of the greenhouse following a W-zig-zag sampling
166 route. The subsamples were chopped and mixed to form a homogeneous composite sample and
167 were kept in the dark at -20°C until analysis. Regarding RW, only a single RW sample was taken
168 in November 2015.

169 170 2.3. Sample preparation

171 Sample extraction (soil, leaves and tomato) was carried out using two different versions of ~~the~~
172 QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) acetate methodology,
173 which were previously published by our group [23,24]. For leaves and tomatoes, a portion of 10
174 g of plant material were placed into a 50-mL polypropylene centrifuge tube. Then, 10 mL of 1%
175 acetic acid in MeCN and 20 µL of the extraction quality control solution (100 µg/L) were added
176 and the tube was shaken (5 min). After that, 6 g of anhydrous MgSO₄ and 1.5 g of NaOAc were
177 added and the tube was shaken again (5 min) and centrifuged at 3500 rpm (2054xg, 5 min).
178 Following this, a dispersive solid-phase extraction (d-SPE), used as clean-up step, was carried
179 out. To this purpose, 5 mL of the upper organic layer were placed into a 15-mL centrifuge tube
180 containing 750 mg of anhydrous MgSO₄, 125 mg of primary-secondary amine (PSA) and 125 mg
181 of C18. Then, the tube was vortexed (30 s) and centrifuged at 3500 rpm (5 min). Finally, 4 mL of
182 extract were transferred to screw-cap vials where 40 µL of MeCN at 1% of formic acid were
183 added. Prior to injection into the HPLC-QTOF-MS system, 100 µL of the extract were evaporated
184 until dryness and reconstituted in 100 µL of MeCN:H₂O (10:90, v/v).

185 In case of soil samples, 1 g of freeze-dried sample was weighed in a 50-ml polypropylene tube.
186 After that, 4 mL of Milli-Q H₂O were added, then shaken in a vortex (30 s) and left for 15 min
187 for rehydration. Afterwards, 10 mL of 1% acetic acid in MeCN and 20 µL of the extraction quality
188 control solution were added and the tube was shaken (5 min). Following this, 5 g of anhydrous
189 MgSO₄ and 1.5 g of NaOAc were added and the tube was shaken again (5 min) and centrifuged
190 (3500 rpm, 2054g, 5 min). In this case, the d-SPE consisted in a mixture of 750 mg of MgSO₄

191 and 125 mg of C18. Then, the protocol followed the same steps as for plant material described
192 before. (Text S1), which were previously published by our group. RW samples was~~were~~ filtered
193 and 100 μL of MeCN were added to an aliquot of 900 μL previously to direct injection in the
194 HPLC-QTOF system, as it is described in [23].

196 2.34. LC-QTOF-MS analysis

197 Analysis of samples was carried out using a LC Agilent 1260 Infinity system (Agilent
198 Technologies, Foster City, CA, USA) equipped with a Poroshell 120 EC-C18 analytical column
199 (50 x 4.6 mm, 2.7 μm particle size, Agilent Technologies) operated at a flow rate of 0.5 mL min^{-1}
200 and using an injection volume of 20 μL . Ultrapure water (0.1% formic acid, eluent A) and MeCN
201 (eluent B) were used as mobile phases. The gradient used ranged from 10% to 100% of solvent
202 B: initially it was kept constant at 10% for 2 min, then increased linearly from 10% to 100% for
203 9 min and finally it remained constant for 4 min before returning to the initial conditions. The
204 total analysis run time was 22 min. The LC system was connected to a QTOF mass analyzer
205 Triple TOF 5600+ from Sciex Instruments (Foster City, CA, USA) with a DuoSprayTM ion source
206 consisting of an electrospray (ESI) interface for sample injection and an atmospheric-pressure
207 chemical ionization interface (APCI) for calibrant solution delivery. Samples were analyzed in
208 both ESI⁺ and ESI⁻ modes. The ESI source parameters were: ionspray voltage, 4500 V; curtain
209 gas, 25 (arbitrary units); GS1, 60 psi; GS2, 60 psi; and temperature, 575°C. The acquisition was
210 made via TOF MS survey scan (resolving power of 30000) with an accumulation time of 250 ms
211 followed by four IDA (Information Dependent Acquisition) TOF MS/MS scans with an
212 accumulation time of 100 ms. The m/z scan range for both TOF MS and IDA was 50-1000.
213 Dynamic background subtraction was considered for IDA experiments. For MS² fragmentation,
214 a collision energy of 30 eV with a ± 15 eV spread was applied. Analyst TF 1.5 software was used
215 for data acquisition, and Sciex OS 1.5 for data processing (both ~~from~~ Sciex).

217 2.45. Prioritization of CECs and TPs suspect screening list

218 The previous analysis of the agricultural samples (RW, soil, leaves, tomato), retrospectively
219 evaluated in this work, revealed the presence of 57 CECs (pharmaceuticals and some of their
220 major and more frequently reported TPs) [23,24]. To investigate the presence TPs, a previous
221 selection of the parent analytes was carried out based on the following criteria: (i) compounds
222 found at high concentration; (ii) analytes most frequently detected in the different commodities;
223 (iii) drugs suspected to have an environmental/human toxic impact, according to literature
224 evidences; and (iv) those included in current regulations [4,5]. Based on these criteria, 20 parent
225 CECs were selected: amitriptyline (AMI), atenolol (ATE), azithromycin (AZI), caffeine (CAF),
226 cetirizine (CET), citalopram (CIT), clarithromycin (CLA), dipyrone (DIP), flecainide (FLE),
227 fluoxetine (FLU), hydrochlorothiazide (HCT), irbesartan (IRB), lamotrigine (LAM), lidocaine

228 (LID), mepivacaine (MEP), propranolol (PRP), telmisartan (TEL), tramadol (TRA), trimethoprim
229 (TRI) and venlafaxine (VEN).

230 Once the selection was performed, a literature search focused on their relevant and previously
231 reported TPs was carried out, minded diverse formation sources such as wastewater treatments
232 and biological processes. For certain CECs, reported TPs were not found in literature, and thus,
233 the *in silico* prediction tool EAWAG-BBD Pathway Prediction System [27] was used to obtain
234 possible TP structures. The suspect list finally developed contained 262 TPs and is shown in Table
235 S1. 8 TPs from the list had already been analysed in our laboratory [23,24]. Accordingly, their
236 retention time (RT) were included in the TP processing list to exclude them from possible isomer
237 candidates and were not considered as suspect TPs onwards. This was the case of paraxanthine
238 (CAF TP); N-desmethylcitalopram (CIT TP); 4-acetylaminoantipyrine, 4-formylaminoantipyrine
239 and antipyrine (DIP TPs); o-desmethyltramadol and tramadol-N-oxide (TRA TPs); and o-
240 desmethylvenlafaxine (VEN TP) (Table S1).

241

242 2.56. Suspect screening workflow

243 The analysis of HRMS data was entirely performed by Sciex OS software. The suspect list was
244 processed using the $[M+H]^+$ and $[M-H]^-$ adducts in samples analyzed by ESI+ and ESI-,
245 respectively. The criteria considered to obtain tentative candidates included an absolute intensity
246 threshold of 1000 cps, a S/N ratio >10, a tolerance of 5 ppm mass accuracy and an isotope ratio
247 difference (IRD) <10%. Only chromatographic peaks with an intensity response ten times higher
248 than that of the procedural blanks [28] were further studied. The algebraic calculation of the best
249 formula to fit accurate masses of the product ions was performed by the Sciex OS software tool,
250 Formula Finder, and fragment alignment was also verified. Acquired MS² spectra were primarily
251 compared with two spectral libraries, the internal library All-in one HRMS (Sciex) and the [open-
252 access database of mass spectra](#) ~~open library~~ MassBank [Europe database \(NORMAN network\)](#)
253 [29]. In case of match with libraries, a score >80% was set for the tentative assignment of
254 candidates. Additionally, the *in silico* fragmentation tool ChemSpider database [30] (integrated
255 into the Sciex OS software) was checked to enhance spectra interpretation. A minimum score of
256 70% was considered for candidates. TPs for which no match was found in libraries or using the
257 *in silico* fragmentation tool, the criteria adopted for their further investigation as candidates was
258 the presence of at least two product ions with a mass accuracy error <5 ppm. Furthermore, their
259 acquired MS² spectra were checked with literature [evidence](#) ~~evidence~~, when available. ~~In order~~
260 ~~to~~ [To](#) use the chromatographic retention behavior of the TPs to help with their structural
261 elucidation, an in-house RT prediction model was applied using a linear correlation of the
262 measured RTs and the estimated log $K_{O/W}$ values ($RT=0.8363 \times \log K_{O/W} + 4.2853$, $R^2=0.4705$) of
263 120 analytical standards analyzed in the same conditions. A window of ± 2 min was considered.
264 TP log $K_{O/W}$ were estimated by the EPI Suit ~~software~~ [31]. Due to the rough RT estimation, the

265 prediction approach was not considered as exclusion criteria for structure allocation, due to the
266 reliability requirements and the inherent limitations of the method. TPs tentatively identified were
267 grouped according to the identification confidence levels proposed by Schymanski et al. [32]. The
268 concentration of the TPs confirmed with the analytical standard (identification level 1), was
269 estimated by external standard calibration curves. Experimental limits of quantification (LOQs)
270 were set as the lowest acceptable concentration in the calibration curve which yielded a signal-
271 to-noise (S/N) ratio close to 10 with a mass error < 5 ppm and an IRD <10%. Spatial The location
272 and temporal occurrence of TPs tentatively identified were evaluated in samples.

273

274 3. Results and discussion

275 3.1. Tentative identification of TPs

276 The identification of TPs in agro-ecosystems presents some analytical difficulties,
277 including which include: (i) the complexity of the matrices; (ii) the frequent lack of high-quality
278 MS² spectra, mainly due to their low TP concentrations in ~~the~~ samples; (iii) the lack of MS²
279 spectra for many of these TPs in libraries; and (iv) the lack of commercially available analytical
280 standards for final structure confirmation of the structure with RT. For these reasons, it is often
281 necessary to perform carry out the structural elucidation of ~~the~~ TPs based on a laborious and
282 detailed observation of the spectra, which in many cases leads to a tentative identification.

283

284 The suspect screening strategy applied in this study yielded a first list of 44 TP candidates in
285 RW, soil, leaves and tomato samples. After comparing the acquired spectra with spectral
286 databases and literature, and scrutinizing the fragmentation pattern of each compound, a total of
287 18 TPs could be tentatively identified in the samples. ~~12-6~~ TPs were grouped in the high
288 confidence level 2 and, eventually, 7 TPs could be confirmed with the RT of their analytical
289 standards. Table 1 shows the proposed structures, identification levels (according to Schymanski
290 et al [32]), and the chromatographic, and spectral information and a summary. Table S2
291 summarizes of the criteria considered for structure allocation in each case. A thorough discussion
292 devoted to structure assignment of the identified TPs has been performed and is shown below.

293

294 3.1.1 ATE ~~268~~TP

295 The proposed structure for this TP, formation of the only detected ATE TP, ATE 268 acid with
296 molecular formula $([M+H]^+ - C_{14}H_{21}NO_4 - ([M+H]^+ - m/z 268.1543))_2$ involves the hydrolysis of the
297 amide group of ~~ATE~~ the parent compound (Figure S1A), which results in the formation of an acid.
298 The acquired MS² spectrum of ATE 268 (Figure S1B) showed the same similar fragmentation
299 pattern as ATE (Figure S1A), presenting a coincident characteristic fragment at m/z 145.0648 and
300 ions with a difference of 1 Da with the parent compound at m/z 226.1074, 191.0703 and 165.0546,
301 which would correspond to N-dealkylation, neutral loss of H₂O plus NH₃ and a subsequent

302 dealkylation, respectively (Figure S1B). Although its predicted RT difference was 1.63 min,
303 aforementioned spectral evidences, similarities and matched with the reported spectrum in
304 literature [21]. A score of 92% with ATE acid in MassBank Europe database supported the
305 purchase of the analytical standard and its RT. Finally, this TP was finally confirmed with the
306 reference standard (Figure S1C).

308 3.1.2 AZI TPs

309 Up to three AZI TPs (Figure S2) were identified in the samples. AZI 592 ($C_{30}H_{57}NO_{10}$, $[M+H]^+$,
310 m/z 592.4055) and AZI 434 ($C_{22}H_{43}NO_7$, $[M+H]^+$, m/z 434.3112) kept the macrolactone ring intact
311 and were formed by the consecutive enzymatic removal of the desosamine (Figure S43) and
312 cladinose (Figure S54) moieties, reported in biotransformation reactions [2]. Both compounds
313 were included at identification level 2b, due to a 92 % of score with the structure proposed for
314 AZI 434 in ChemSpider, and the match of three diagnostic fragments of AZI 592 with the most
315 intense ions found in the literature [18]. AZI 374 ($C_{19}H_{35}NO_6$, $[M+H]^+$, m/z 374.2534) represents
316 a further step in the AZI degradation process, which mainly results in the opening of the macrolide
317 ring and subsequent losses and oxidative reactions in the resulting structure. MS² information
318 (Figure S35) coincided well with two different isomers [18], AZI, AZI 374 B and 374 C in Table
319 S1, but not sufficient spectral evidence was found for an unambiguous assignment. For this
320 reason, AZI 374 was included in identification level 3.

324 3.1.3. CIT TPs

325 The TPs CIT 339 ($C_{20}H_{19}FN_2O_2$, $[M+H]^+$ m/z 339.1503) and CIT 325 ($C_{19}H_{17}FN_2O_2$, $[M+H]^+$
326 m/z 325.1346) identified were formed by an oxidation reaction of CIT (Figure S6) in the furan
327 ring and further N-demethylation (Figures S7 and S8, respectively). Detection of the neutral loss
328 of CO from the product ion at m/z 276.0819 was indicative of the presence of a carbonyl group
329 in the furan ring, which produced 3-oxo-citalopram, a CIT human metabolite, as confirmed by
330 Beretsou et al [33]. The subsequent N-demethylation was supported by the loss of the NH_2CH_3
331 group observed in CIT 325 [34]. The full agreement of the MS² spectra of both compounds with
332 those reported in the literature placed them in the identification level 2b.

333 CIT 343 ($C_{20}H_{23}FN_2O_2$, $[M+H]^+$ m/z 343.1816) was confirmed as CIT amide. This TP is formed
334 by hydrolysis reaction of the nitrile group of CIT to yield a primary amide. This proposal was
335 supported by the presence of the product ion corresponding to the loss of the amide moiety at m/z
336 237.1074 (Figure S9A) and similarities of the MS² fragmentation pattern found in literature
337 [33,34]. Lastly, structure confirmation was carried out by reference standard analysis (Figure
338 S9B).

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3.1.4. CLA TPs

CLA 590 (C₃₀H₅₅NO₁₀, [M+H]⁺ m/z 590.3898) would be formed by cleavage of the cladinose group of CLA (Figure S10), typical of macrolide antibiotics, which led to the formation of a hydroxy derivative. The CLA 590 MS² spectrum (Figure S11A) matched with the fragmentation pattern reported in the literature [20,35] and by the ChemSpider database (85% of score). Its RT was finally confirmed with the analytical standard (Figure S11B).

In the case of CLA 764 (C₃₈H₆₉NO₁₄, [M+H]⁺ m/z 764.4790), two different structures could be proposed: the hydroxylation of the parent compound (14-OH-CLA) and the CLA-N-oxide formation. Based on the differences observed with the spectrum reported for CLA-N-oxide by Tian et al. [20] and considering that the acquired spectrum (Figure S12) scored 71% for 14-OH-CLA using ChemSpider, the latter structure was the proposed and CLA 764 was included in confidence level 2b.

3.1.5 DIP TP

The structure of DIP 267 corresponds to the 4-brominated derivate of phenazone (Figure S13): 4-bromoantipyrine (C₁₁H₁₁BrN₂O, [M+H]⁺ m/z 267.0127) (Figure S14A). The similarities of the acquired MS² with the compound tentatively identified in the literature [36] and a score of 72% on ChemSpider were the reasons for purchasing the analytical standard. Finally, its RT could be confirmed (Figure S14B).

3.1.6 HCT TP

The HCT 293 (C₇H₆ClN₃O₄S₂, [M-H]⁻ m/z 293.9415) structure corresponded to the dehydrogenation of HCT (Figure S15), which resulted in an additional double bound located in the benzothiadiazine ring to form a tertiary amine. The acquired spectrum (Figure S16A) was checked against MassBank Europe and ChemSpider, and both results indicated a good match with chlorothiazide (91% and 78% of score, respectively). Analysis of the corresponding analytical standard confirmed the identity of this TP (Figure S16B).

3.1.7 IRB TPs

Up to 5 TPs of IRB (Figure S17) could be identified, which were formed by oxidation reactions of the parent compound. The main reactions observed were: i) hydroxylation in the alkyl chain

376 [\(IRB 445 A\)](#), ii) oxidation of the double bond of the imidazolone ring ([IRB 445 B](#), [IRB 447](#)) and
377 [iii\) oxidation of both positions \(IRB 461 A\)](#).

378 [The hydroxylation of the alkyl chain was observed for IRB 445 A \(C₂₅H₂₈N₆O₂, \[M+H\]⁺ *m/z* 445.2346, RT 7.03 min\) \(Figure S18\). The acquired MS² spectrum of this TP also showed the](#)
379 [breakage of the molecule observed for IRB, yielding a coincident ion at *m/z* 207.0917 \(C₁₄H₁₀N₂\)](#)
380 [corresponding to the tetraazolic biphenyl moiety;](#) and a second fragment at *m/z* 211.1441
381 [\(C₁₁H₁₈N₂O₂\);](#) instead of the ion at *m/z* 195.1492 (C₁₁H₁₈N₂O) present in IRB spectrum. **This;**
382 **which confirm** indicates that the hydroxylation occurs in this part of the structure. Although
383 [some authors propose the hydroxylation of the alkyl chain as the most probable structure \[37\], the](#)
384 [absence of characteristic ion fragments does not permit](#) to confirm this hypothesis.
385 **Therefore,** and IRB 445 was classified in level 3.

387 [For IRB 447 \(C₂₅H₃₀N₆O₂, \[M+H\]⁺ *m/z* 447.2503\), the ESI+ fragments at *m/z* 252.1244, and](#)
388 [196.1331 \(Figure S19A\), would indicate the oxidation of the imidazolone ring and its subsequent](#)
389 [opening. The fragments at *m/z* 305.1659 and 211.1452 in the ESI- MS² spectrum \(Figure S19B\)](#)
390 [also confirmed the formation of the amide group. After obtaining a 91% of score with-in](#)
391 [ChemSpider and comparing the RT with that of the analytical standard, the proposed structure for](#)
392 [IRB 447 was confirmed.](#)

393 [IRB 445 B \(C₂₅H₂₈N₆O₂, \[M+H\]⁺ *m/z* 445.2346, RT 8.84 min\) presented a fragmentation pattern](#)
394 [similar to that of IRB 447 in ESI+, with two coincident ions \(*m/z* 196.1332 and 168.1383\) while](#)
395 [other three ions were found with a difference of 2 Da \(*m/z* 250.1087, 233.0822 and 205.0760,](#)
396 [Figure S20\). This mass difference indicated a related structure with an extra unsaturation](#)
397 [\(RDB=15\), probably located between the carbon in alpha with the biphenyl group and the nitrogen](#)
398 [atom, which is supported by the presence of fragments at *m/z* 361.1771 and *m/z* 250.1087.](#)
399 [However, due to the lack of conclusive information, IRB 445 B was included in level 2b as](#)
400 [probable structure.](#)

401 [The MS² spectrum of IRB 461 A \(C₂₅H₂₈N₆O₃, \[M+H\]⁺ *m/z* 461.2295, RT 7.35 min\) showed](#)
402 [common ions with IRB 447 \(*m/z* 252.1244, 235.0978, 207.0917 and 84.0808, Figure S21A\). But](#)
403 [presence of one additional oxygen atom and an extra unsaturation, suggested the presence of an](#)
404 [additional carbonyl group. Presence of the product ion at *m/z* 99.0441 in ESI+ was decisive to](#)
405 [ensure the presence of two carbonyl groups in the alkyl chain \(Figure S21A\) \[37\]. The spectrum](#)
406 [in ESI- was also compatible with the proposed structure by the structural allocation of product](#)
407 [ion at *m/z* 207.1139, which presumably could be formed by the rearrangement of the imidazole](#)
408 [ring producing the loss of H₂O \(Figure S21B\). However, and although the similarities found with](#)
409 [the ESI+ spectrum reported in literature \[37\] were consistent, IRB 461 A was included in](#)
410 [confidence level 3.](#)

411 [Finally, IRB 461 B isomer \(RT 7.61 min\) presented fragment ions similar to IRB 461 A, at *m/z*](#)
412 [252.1244, 235.0992, 207.0917 and 180.0808 \(Figure S22A\). However, the characteristic product](#)

413 ions of IRB 461 A at m/z 210.1125 and 99.0441, which supported the presence of the two carbonyl
414 moieties in the alkyl chain, were not found in IRB 461 B. This suggested that the extra oxygen
415 and the unsaturation should occupy be in another position ~~in~~ of the molecule. The associated
416 molecular formulae of product ions found at m/z 102.0913 in ESI+ and at m/z 124.0404 and
417 81.0346 in ESI- (Figure S22B), pointed out that the oxidation may occur in the cyclopentane ring.
418 However, the structural assignment of the ESI- product ion at m/z 220.0768 ($C_{15}H_{11}NO$) was not
419 possible under this premise. Thus, IRB 461 B was kept in identification level 4. Although a
420 structure could not be proposed in this case, MS² information provided sufficient keys to correlate
421 this TP with sartans. To our knowledge, this TP has not been reported before.

422 423 3.1.8 TEL TP

424 The structure proposed for TEL 439 ($C_{27}H_{26}N_4O_2$ ($[M+H]^+$, m/z 439.2128) was obtained using
425 the in silico prediction tool EAWAG-BBD Pathway Prediction System [27]. TEL 439 would be
426 formed by an initial dihydroxylation of the biphenyl carboxylic moiety of TEL, followed by a
427 meta-cleavage pathway that would produce a para-phenyl acid derivate (Figure S23). Degradation
428 of various alkylphenols has been reported to occur by bacterial biotransformation in sediments
429 and sludge via the proposed meta-cleavage route [38]. The spectral information of parent TEL
430 439 was compared to internal and external databases and no match was found. However, MS²
431 spectrum of parent TEL showed a common product ion with TEL 439 at m/z 276.1369 (Figure
432 S24, Table 1). The investigated product ions revealed an initial loss of H₂O (m/z 421.2023) and
433 subsequent losses of CO (m/z 393.2074) and CO₂ (m/z 365.1761), which support the presence of
434 a carboxylic acid group (Figure 2). ~~The p~~Presence of product ion at m/z 289.1448 would indicate
435 a loss of the propyl chain and the phenyl acid groups, while characteristic ion at m/z 276.1369
436 would correspond to both imidazolone core groups (shared with TEL). Although the mass
437 fragments obtained support the assignment of the proposed structure, the lack of additional
438 evidence led to the inclusion of TEL 439 in level 3. To our best knowledge, TEL 439 has not been
439 previously reported.

440 441 3.1.9 TRA TP

442 Up to 5 different structures included in the suspect list (Table S1), belonging not only to TRA
443 but also to VEN TPs, matched with the molecular formula of TRA 250 ($C_{15}H_{23}NO_2$, $[M+H]^+$ m/z
444 250.1801). O-desmethyltramadol and O-desmethylvenlafaxine were discarded based on the RTs
445 of the corresponding analytical standards. N,N-didesmethylvenlafaxine and N,O-
446 didesmethylvenlafaxine proposals were not considered as their MS² spectra did not match when
447 compared in Mass Bank Europe. Characteristic fragment ions of TRA (Figure S25) were observed
448 at m/z 159.0804, 121.0648 and 91.0542 (Figure S26A). MS² spectrum scored 98% and 78% with
449 N-desmethyltramadol in MassBank Europe and ChemSpider, respectively. It also **matched** with

450 [literature reported \[19\]. Finally, TRA 250 was confirmed with the analytical standard \(Figure](#)
451 [S26B\).](#)

453 3.1.1 ATE 268

454 ~~The proposed structure for this TP, ATE acid with molecular formula $C_{14}H_{21}NO_4$ ($[M+H]^+$, m/z~~
455 ~~268.1543) involves the hydrolysis of the amide group of ATE, which results in the formation of~~
456 ~~an acid. The acquired MS² spectrum of ATE 268 showed the same fragmentation pattern as ATE~~
457 ~~(Figure S1A), presenting a coincident characteristic fragment at m/z 145.0648 and ions with a~~
458 ~~difference of 1 Da with the parent compound at m/z 226.1074, 191.0703 and 165.0546, which~~
459 ~~would correspond to N-dealkylation, neutral loss of H₂O plus NH₃ and a subsequent dealkylation,~~
460 ~~respectively (Figure S1B). Although its predicted RT difference was 1.63 min, aforementioned~~
461 ~~spectral evidences, similarities with literature [17] and a score of 92% with ATE acid in~~
462 ~~MassBank database supported the purchase of the analytical standard. Finally, this TP was~~
463 ~~confirmed with the reference standard (Figure S1C).~~

465 3.1.2 AZI 374

466 ~~Three different structures included in the suspect list (AZI 374 A, AZI 374 B and AZI 374 C,~~
467 ~~Table S1) could match with the molecular formula $C_{19}H_{35}NO_6$ ($[M+H]^+$, m/z 374.2534). The three~~
468 ~~proposals correspond to the macrolide ring opening from AZI (Figure S2) and the subsequent~~
469 ~~oxidation to the hydroxylated isomers. The proposed structure for 374 A was discarded due to~~
470 ~~differences in the acquired spectrum and the spectra reported in literature [14]. Four product ions~~
471 ~~described for the isomers B and C (m/z 272.1856, 216.1594, 198.1489 and 159.1016) matched~~
472 ~~with the acquired product ions (Figure S3). However, because of the similarities in the MS²~~
473 ~~spectra, it was not possible to allocate a unique structure [14]. The reported retention behavior~~
474 ~~was not enough to select one of the two proposed structures. The RT prediction model showed a~~
475 ~~difference of 1.84 min for structure B and 0.56 min for C, pointing to structure C as the most~~
476 ~~probable. However, due to limitations of the model, this information was not considered~~
477 ~~conclusive enough to propose this structure. Thus, this TP remained at identification level 3.~~

479 3.1.3 AZI 434 and AZI 592

480 ~~The proposed structures for these TPs kept the macrolactone ring intact. It has been reported that~~
481 ~~they are formed by the enzymatic removal of desosamine (AZI 592; $C_{30}H_{57}NO_{10}$, $[M+H]^+$, m/z~~
482 ~~592.4055) and cladinose moieties (AZI 434; $C_{22}H_{43}NO_7$, $[M+H]^+$, m/z 434.3112) in~~
483 ~~biotransformation reactions [14]. For AZI 434, no MS² information was found in literature.~~
484 ~~Although the acquired spectra were of low quality, up to four product ions could be molecularly~~
485 ~~assigned (Figure S4), corresponding to a neutral loss of water (m/z 416.3007) and further~~
486 ~~transformations that involved the macrolide ring opening, due to the breakage of the C-N bond~~

487 (m/z 318.2275), and successive losses of the remaining parts of the molecule (m/z 300.2169 and
488 186.1489). In addition, the comparison with ChemSpider database, which showed a 92% of score
489 with the proposed structure, and the low difference with the predicted RT (0.26 min), supported
490 the inclusion of AZI 434 at the identification level 2b.

491 —Regarding AZI 592 (Figure S5), three diagnostic fragments could be extracted, which matched
492 with the three most intense ions found in literature [14]. Among them, the most intense product
493 ion coincided with the extraction mass of AZI 434 (m/z 434.3112) and the presence of its
494 subsequent neutral loss of water (m/z 416.3007), which demonstrates the correlation of both
495 structures. Thus, AZI 592 was also considered in identification level 2b.

496 497 3.1.4 CIT 325 and CIT 339

498 Two different but related structures (Table S1) could be suggested for CIT 325 ($C_{19}H_{17}FN_2O_2$,
499 $[M+H]^+$ m/z 325.1346) and CIT 339 ($C_{20}H_{19}FN_2O_2$, $[M+H]^+$ m/z 339.1503). They match with an
500 oxidation that could take place in the tetrahydrofuran ring or in the alkyl chain. The presence of
501 the carbonyl group is justified by the increase in the number of rings and double bonds (RDB)
502 (one more than CIT, Figure S6). Furthermore, identical characteristic product ions were found at
503 m/z 258.0714 and 248.0870 for both TPs, which would correspond to the neutral loss of H_2O and
504 the subsequent loss of CO from the product ion at m/z 276.0819, thus indicating the presence of
505 a carbonyl group (Figures S7, S8) [29]. According to Beretsou et al. [30], the presence of the
506 diagnostic fragment at m/z 109.0048 (not detected in the by products showing oxidation in the
507 tetrahydrofuran ring) indicated the possible position of the carbonyl moiety in the alkyl chain.
508 However, as the precise allocation of the group is not possible, both compounds were grouped in
509 identification level 3.

510 511 3.1.5 CIT 343

512 The associated structure to CIT 343 (CIT amide, $C_{20}H_{23}FN_2O_2$, $[M+H]^+$ m/z 343.1816)
513 corresponds to the hydrolysis of the nitrile group of CIT to yield a primary amide group. This
514 proposal was supported by the typical loss of $CHNO$ observed (m/z 237.1074) in the MS^2
515 spectrum, which corresponded to the cleavage of the amide moiety (Figure S9A). Since this
516 fragmentation pattern was also reported in literature [29,30], the analytical standard of CIT amide
517 was purchased and this TP could be confirmed (Figure S9B).

518 519 3.1.6 CLA 590

520 The MS^2 spectrum of CLA 590 corresponded to de(cladinosyl)CLA ($C_{30}H_{35}NO_{10}$, $[M+H]^+$ m/z
521 590.3898), formed by the cleavage of the cladinose group, typical of macrolide antibiotics, which
522 led to the formation of a hydroxy derivative. The spectrum showed a fragmentation pattern
523 common with CLA (Figure S10) and previously reported for this compound [16,31]. The ion at

~~m/z 558.3637 comes from demethylation and subsequent neutral loss of water in the lactone ring (Figure S11A). The fragment at m/z 158.1176 corresponded to the oxidation product of the desosamine moiety, typical of macrolide TPs, and its demethylated subproduct yielded the fragment at m/z 116.1070. The identity of CLA 590 was also supported with the ChemSpider tool (score of 85%), and the predicted RT (difference with the expected RT of 0.03 min). Finally, it was confirmed with the analytical standard (Figure S11B).~~

~~3.1.7 CLA 764~~

~~Two different structures of CLA by products could match with CLA 764, the hydroxylation of CLA (14-OH CLA) and CLA N-oxide ($C_{38}H_{69}NO_{14}$, $[M+H]^+$ m/z 764.4790). Mined literature showed two main product ions for both structures: m/z 606 and m/z 158 in low resolution. These ions corresponded to the loss of cladinose group and to the fragment of the desosamine ring [32], being the most intense fragments present in our acquired spectrum (m/z 606.3848 and 158.1176, Figure S12). CLA N-oxide was primary discarded because the fragments at low m/z were different from those of the TP tentatively identified by Tian et al. [16]. Since this comparison resulted rough because no direct contrast of 14-OH CLA HRMS spectra was found, the retention behavior of both TPs was evaluated. 14-OH CLA eluted before CLA, whereas CLA N-oxide eluted after CLA [14,16]. According to this, and based on our experimental data, CLA 764 retention behavior fitted with 14-OH CLA. Furthermore, a score of 71% was obtained in ChemSpider. Consequently, CLA 764 was tentatively identified as 14-OH CLA at a confidence level 2b.~~

~~3.1.8 DIP 267~~

~~The proposed structure for this TP corresponds to the halogenated derivate in position 4 of phenazone (Figure S13): 4-bromoantipyrene ($C_{11}H_{11}BrN_2O$, $[M+H]^+$ m/z 267.0127). The acquired spectrum shows firstly a demethylation (m/z 251.9893) and subsequent losses corresponding to bromide (m/z 188.0944) and to the N-methylene-ethanamine bromated fragment (m/z 135.9756) (Figure S14A). Due to the assignation of the product ions in ChemSpider (score 72%) and the similarity of the acquired MS^2 with the tentative identified compound in literature [33], the analytical standard was purchased (Figure S14B) and DIP 267 was confirmed.~~

~~3.1.9 HCT 293~~

~~The proposed structure for HCT 293, which is chlorothiazide ($C_7H_6ClN_3O_4S_2$, $[M-H]^-$ m/z 293.9415), would correspond to the dehydrogenation of HCT (Figure S15) resulting in an additional double bond located in the benzothiadiazine ring to form a tertiary amine. This TP showed characteristic ions at m/z 213.9609 and 178.9921, which would be correlated with a loss of one of the sulfonamide groups and a subsequent loss of chlorine (Figure S16A). The acquired~~

spectrum was checked against the MassBank [Europe](#) and ChemSpider indicating a match with chlorothiazide (91% and 78%, respectively). Furthermore, its RT differed in 0.3 min from that expected. The analysis of the corresponding standard confirmed the identity of this TP (Figure S16B).

3.1.10 IRB 445 A

Two different isobaric compounds were found in samples with the molecular formula $C_{25}H_{28}N_6O_2$ ($[M+H]^+$ m/z 445.2346) at 7.03 min and 8.84 min (IRB 445 A and IRB 445 B, respectively). Three structures could match with this formula according to Carpinteiro et al. (2019) and it represents the oxidation of the parent compound to form alcohol and amide derivatives (Table S1). The acquired MS²-spectrum of IRB 445 A presented a fragmentation pattern similar to that of IRB (Figure S17), showing the breakage of the molecule to yield two characteristic ions at m/z 207.0917 ($C_{14}H_{10}N_2$), corresponding to the tetraazolic biphenyl moiety after losing N_2 , and at m/z 195.1492 ($C_{11}H_{18}N_2O$), which matches with the remaining part of the molecule. In the case of IRB 445 A, the ion at m/z 195.1492 was not observed, instead, m/z 211.1441 ($C_{11}H_{18}N_2O_2$) was detected, thus indicating the hydroxylation in this part of the structure (Figure S18). Although some authors propose the hydroxylation of the alkyl chain as the most probable structure [34], the absence of ion fragments does not permit to confirm this hypothesis, and IRB 445 was classified in level 3.

3.1.11 IRB 447 and IRB 445 B

Three different structures were found in literature that could match with the molecular formula of IRB 447, $C_{25}H_{30}N_6O_2$ ($[M+H]^+$ m/z 447.2503, Table S1). Diagnosis of ESI⁺ fragments at m/z 252.1244, 235.0978 and 207.0917 (Figure S19A) would indicate the oxidation of the imidazolone ring and its subsequent opening. However, this fragmentation pattern was compatible with hydroxylation in the carbonyl group, which would lead to the formation of an acid, or with hydroxylation in the carbon connected to the alkyl chain, which would result in the formation of an amide. To clarify this point, the ESI-MS²-spectrum was evaluated (Figure S19B). The presence of the fragments at m/z 305.1659 and 211.1452 were the keys to ensure the formation of the amide group, specifically the ion at m/z 211.1452, which implied the presence of the two amide groups. Due to the match of the MS²-spectra with the analytical standard reported in literature [34] and the 91% of score obtained in ChemSpider, this compound was finally confirmed (Figure S19C-D).

IRB 445 B ($C_{25}H_{28}N_6O_2$, $[M+H]^+$ m/z 445.2346) presented a fragmentation pattern similar to that of IRB 447 in ESI⁺. Two coincident ions were found at m/z 196.1332 and 168.1383, whilst other three ions presented a difference of 2 Da at m/z 250.1087, 233.0822 and 205.0760 (Figure S20). This fact would indicate a related structure with an extra unsaturation (RDB=15). The

598 double bond was expected to be located among the carbon in alpha with the biphenyl group and
599 the nitrogen atom, which is supported by the presence of fragments at m/z 361.1771 and m/z
600 250.1087. Due to the lack of additional evidences, IRB 445 B was included in level 2b as probable
601 structure.

602 603 3.1.12 IRB 461 A and IRB 461 B

604 Two chromatographic peaks could be identified at 7.35 min (IRB 461 A) and 7.61 min (IRB 461
605 B) when the masses m/z 461.2295 (ESI⁺, [M+H]⁺) and m/z 459.2145 (ESI⁻, [M-H]⁻),
606 corresponding to the molecular formula C₂₅H₂₈N₆O₃, were extracted. The MS² spectrum of IRB
607 461 A in ESI⁺ shares common ions with IRB 447 at m/z 252.1244, 235.0978, 207.0917 and
608 84.0808 (Figure S21A). The molecular formula of IRB 461 A differs from IRB 447 in one
609 additional oxygen and an unsaturation more (RDB=15), which suggests the presence of an extra
610 carbonyl group. Therefore, IRB 461 could be formed by the hydroxylation of IRB 447 and further
611 oxidation to form a ketone or an aldehyde in the alkyl chain. The presence of the product ion at
612 m/z 99.0441 in ESI⁺ was decisive to ensure the presence of two carbonyl groups in the alkyl chain
613 (Figure S21A). This fact has been already reported [34], although their exact position could not
614 be addressed. Furthermore, the spectrum in ESI⁻ was also compatible with the proposed structure
615 by the structural allocation of product ion at m/z 207.1139, which presumably could be formed
616 by the rearrangement of the imidazole ring producing the loss of H₂O (Figure S21B). Although
617 the similarities found with the ESI⁺ spectrum reported in literature [34] were consistent, IRB 461
618 A was still included in confidence level 3.

619 IRB 461 B presented an ESI⁺ product ion spectrum similar to that of IRB 461 A, with identic
620 ions at m/z 252.1244, 235.0992, 207.0917 and 180.0808 (Figure S22A), indicating they showed
621 correlated structures. However, characteristic IRB 461 A product ions at m/z 210.1125 and
622 99.0441, which supported the two carbonyl moieties in the alkyl chain, were not found for IRB
623 461 B. Based on this, the extra oxygen and the unsaturation could be located in other parts of the
624 molecule. The associated molecular formulae of product ions found in ESI⁺ at m/z 102.0913 and
625 ESI⁻ at m/z 124.0404 and 81.0346 (Figure S22B), pointed out that the oxidation may occur in the
626 cyclopentane ring. However, the structural assignment of the ESI⁻ product ion at m/z 220.0768
627 (C₁₅H₁₁NO) was not possible under this premise. Thus, IRB 461 B was kept in identification level
628 4. Although a structure could not be proposed in this case, MS² information provided sufficient
629 keys to correlate this TP with sartans. To our knowledge, this TP has not been reported before.

630 631 3.1.13 TEL 439

632 The structure proposed for TEL 439 with molecular formula C₂₇H₂₆N₄O₂ ([M+H]⁺, m/z 439.2128)
633 was obtained using the *in silico* prediction tool EAWAG-BBD Pathway Prediction System, which
634 predicts transformations based on biotransformation rules [24]. TEL 439 would be formed by a

635 primarily dihydroxylation of the biphenyl carboxylic moiety of TEL, followed by a *meta*-cleavage
636 pathway producing a *para*-phenyl acid derivate (Figure S23). Degradation of various
637 alkylphenols has been reported to occur by bacterial biotransformation in sediments and sludge
638 via the proposed *meta*-cleavage route [35]. The spectral information of parent TEL 439 was
639 compared to internal and external databases and no match was found. However, MS²-spectrum of
640 parent TEL showed a common product ion with TEL 439 at m/z 276.1369 (Figure S24, Table 1).
641 The investigated product ions of TEL 439 revealed an initial loss of H₂O (m/z 421.2023) and
642 subsequent losses of CO (m/z 393.2074) and CO₂ (m/z 365.1761), facts that support the presence
643 of a carboxylic acid group (Figure 2). The presence of product ion at m/z 289.1448 would indicate
644 a loss of the propyl chain and the phenyl acid groups, while characteristic ion at m/z 276.1369
645 would correspond to both imidazolone core groups (shared with TEL). Although the mass
646 fragments obtained support the assignment of the proposed structure, the lack of additional
647 evidences led to the inclusion of TEL 439 in level 3. To our best knowledge, TEL 439 has not
648 been previously reported.

649 650 3.1.12 TRA 250

651 Up to 5 different structures included in the suspect list (Table S1), belonging not only to TRA but
652 also to VEN TPs, matched with the molecular formula of TRA 250, C₁₅H₂₃NO₂ ([M+H]⁺ m/z
653 250.1801). O-desmethyltramadol and O-desmethylvenlafaxine were discarded based on the RTs
654 of the corresponding analytical standards. N,N-didesmethylvenlafaxine and N,O-
655 didesmethylvenlafaxine proposals were not considered as their MS²-spectra did not show any
656 match in the Mass Bank database. Furthermore, the presence of characteristic fragments of TRA
657 (Figure S25) corresponding to a reduction of the hexyl ring (m/z 159.0804) that rearranges into a
658 single aromatic 7-carbon structure (m/z 121.0648 and 91.0542), and m/z 189.1274 indicated the
659 presence of a full hexyl ring (Figure S26A). The acquired spectra matched with the MS²-of the
660 analytical standard of N-desmethyltramadol reported by Zimmermann et al. [15]. Besides, scores
661 of 98% and 78% were obtained with the same compound in MassBank Europe and ChemSpider,
662 respectively. Finally, TRA 250 was identified as N-desmethyltramadol with the corresponding
663 analytical standard (Figure S26B).

664 665 3.2 Occurrence of TPs in agricultural samples *and environmental relevance*

666 The occurrence and distribution of the 18 TPs ~~were evaluated~~ in RW, soil, plant leaves and
667 tomato fruits ~~was were evaluated throughout along~~ the different sampling events ~~and at the~~
668 selected sampling points (SP, GH1, GH2, GH3). Table 23 shows ~~The~~ the average
669 chromatographic peak areas of the TPs in the ~~different~~ samples in which they were detected ~~are~~
670 shown in Table 32. Table 33 depicts the average semi-quantified concentrations of the confirmed
671 TPs. The concentrations of the parent compounds can be found elsewhere [23,24]. Up to 5 TPs

672 were found in RW, ~~whilst~~ while 15 TPs could be determined in agricultural soils and perlite.
673 Regarding tomato plants, only 2 TPs were detected in leaves and none of them ~~were~~ found in
674 tomato fruits (Table 2, Figure 3). SP substrate contained by far the highest number of compounds
675 (14 TPs), followed by agricultural soils from GH2 (8 TPs), GH3 (3 TPs), and GH1 (1 TP). This
676 finding was closely related to the presence of parent compounds previously reported in these
677 matrices (Table 2) [23,24]. Although perlite showed the highest number of TPs and some of them
678 presented certain accumulation or persistence, this fact could not be associated with a higher
679 availability and translocation of such TPs in the plant. This behavior agrees with the results
680 obtained in previous studies evaluating the occurrence of the parent ~~CECs~~ pharmaceuticals. The
681 data demonstrated that there was not any difference in the detected concentrations or the detection
682 frequency of the CECs in leaves and tomato samples from SP when compared with the samples
683 from typical GHs [23,24].

684 ATE 268, CLA 764, IRB 445 A, IRB 447, and IRB 461 A were the only five TPs identified in
685 RW. Although ATE and ATE 268 had been reported in wastewater [8] and ATE 268 is recognized
686 as the main transformation product of ATE biodegradation in soil under controlled conditions
687 [21]. ~~Nevertheless, the presence of both~~ none of the two compounds was ~~not~~ detected in any of
688 the real soil samples analyzed. The same ~~fact was observed~~ occurred for IRB 445 A and CLA
689 764, ~~and~~ †The latter was only present at low abundance in a single soil sample. However, two
690 additional IRB TPs, IRB 447 and IRB 461 A, were found in perlite in both sampling events, with
691 IRB 447 showing slight accumulation between samplings. Other IRB TPs, IRB 445 B and IRB
692 461 B (not present in the RW) could be detected in SP in both samplings. However, despite the
693 high occurrence of IRB TPs in perlite, none of them could be detected in GH soil samples, ~~with~~
694 ~~the exception of~~ except for IRB 445 B, which was present in a single sample. The sorption of IRB
695 in soil has been described [39], however, its behavior is still unclear and only a limited number
696 of reports have examined its fate. Some authors have reported that IRB potential to migrate is
697 moderate or low in a subsurface water environment ~~is moderate or low~~ [16]. This fact, together
698 with ~~its~~ the high dissipation half-life reported in soil, can limit the formation of ~~this~~ TPs at
699 detectable concentrations, which agrees with the results obtained in ~~of~~ this work.

700 AZI TPs (AZI 374, AZI 434, and AZI 592) were found in various soil samples from different
701 sampling points presenting persistence ~~with the time~~ in most ~~of the~~ cases. AZI 374 was only
702 detected in SP while AZI 592 was also observed in soil samples from GH2. It must be noticed
703 that AZI 434 was present in all perlite and soil samples, except for the first sampling carried out
704 in GH3, showing a higher detection frequency than that observed for the parent AZI [23]. ~~–~~ AZI
705 434 and AZI 592 have already been tentatively identified in real RW intended for agricultural
706 purposes [14]. Noticeably, these TPs maintain intact the macrocyclic lactone ring and, ~~–~~ thus, they
707 may still show ~~certain~~ residual antimicrobial activity [40]. ~~–~~ This fact is remarkable since an
708 undesirable ecotoxicological impact may be produced by the presence of antibiotic resistance in

709 ~~agricultural environments [14]. Further insight about their occurrence and possible activity should~~
710 ~~be investigated, including regular monitoring.~~ Other compounds that ~~may could~~ have
711 toxicological implications on the spread of antibiotic resistance are CLA 590 and CLA 764, as
712 they preserve the antimicrobial ring and the tertiary amine group of the desosamine moiety,
713 ~~exhibiting bactericidal activity similar to the parent compound~~ [40]. ~~A study carried out by~~
714 ~~Baumann et al. revealed a comparable environmental risk of CLA 764 (tentatively identified as~~
715 ~~14-OH-CLA) than parent CLA due to their similar concentrations found in surface waters [41].~~
716 ~~However, in this work, CLA 764 was only detected in the samples in which the parent compound~~
717 ~~was quantified at the highest concentrations [23].~~ Although data indicates less occurrence of CLA
718 TPs compared to AZI TPs, their presence should not be underestimated due to the ~~similar potential~~
719 ecotoxicological implications. ~~Evidence about the risk associated with the presence of antibiotics~~
720 ~~and their TPs in agricultural systems have been studied by Tadić et al. The authors reported that~~
721 ~~only the 54 % of the total variation in antibiotic resistant genes abundance could be explained by~~
722 ~~the detected antimicrobials in vegetables irrigated with RW [15]. Therefore, further insight about~~
723 ~~antibiotic TPs occurrence and their possible activity should be investigated, including regular~~
724 ~~monitoring.~~

725 CIT related TPs, CIT 325, CIT 339, and CIT 343 were detected only in samples from SP (both
726 samplings except for CIT 343) and GH2 (first sampling). An estimated environmental risk
727 assessment in aquatic environments carried out by Beretsou et al. (2016) reported that no
728 individual risk is expected for CIT 343 at a semi-quantified concentration in wastewater of 0.01
729 $\mu\text{g L}^{-1}$. ~~However, other study developed by Osawa et al. (2019) found that CIT 343 showed~~
730 ~~positive results in two of the three *in silico* carcinogenicity prediction models applied.~~

731 DIP 267 was detected in perlite substrate in both sampling events showing an accumulation
732 tendency. This phenazone halogenated derivate, ~~is~~ commonly used as ~~a contrast agent for brain~~
733 ~~scan brain scan contrast agent, ner and~~ has been identified during chlorination of antipyrine under
734 controlled conditions, when bromide was present in the degradation solution [36]. However, no
735 ~~ecotoxicological details environmental risk assessment~~ or hazard information were found for DIP
736 ~~267 in real environmental samples.~~

737 The presence of TEL 439 was observed in all the GH and SP soil samples, with a notably higher
738 intensity in the latter. ~~This behavior is, are~~ in agreement with the ~~higher~~ concentrations also
739 observed in SP for ~~the parent~~ TEL [23]. Because of the high $\log K_{OW} = 7.7$ of TEL, its presence
740 at high concentrations in sewage sludge used as a soil amendment, as well as a very high
741 bioaccumulation in roots, have been reported [42]. To our knowledge, no previous information
742 about TEL TPs has been found. However, the high frequency of detection observed in this work
743 would require further studies ~~to evaluate of~~ the presence and ~~fate of TEL TPs to assess their~~
744 ~~contribution to overall agricultural risks potential ecotoxicological effects in agricultural~~

745 **environments**, where the presence of TEL can be abundant due to both reuse practices and use of
746 sewage sludge.

747 At ~~lastlast,~~ two TPs were detected in leaves, HCT 293 and TRA 250, confirmed as
748 chlorothiazide and N-desmethyltramadol, respectively. HCT 293 was detected in leaves from SP
749 and GH 2 (third and fourth samplings) and SP substrate (last sampling), **showing comparable**
750 **concentrations than those found for parent HCT in leaves (Table 3)** [24]. Interestingly, this TP
751 was not detected in soil samples from GH 2, which would indicate that its formation could be
752 related to the biotransformation/uptake of the parent HCT in plant tissues. The formation of HCT
753 293 from the parent compound has already been reported by abiotic and biotic reactions such as
754 ozonation, photodegradation, hydrolysis, and biotransformation in river sediments [43,44].
755 Regarding environmental hazardous implications, an ecotoxicological evaluation with bacterial
756 bioluminescence revealed that HCT 293 did not pose increased effects in comparison with HCT
757 [44]. On the contrary, the occurrence of TRA 250 did not follow a clear trend. It was found in the
758 first sampling of SP, but surprisingly, it was not present in the second sampling. Furthermore, this
759 TP was observed in the leaves from GH3 (fourth sampling) without being detected in soil samples
760 from the same site. This would indicate a probable formation by biotic transformations of TRA
761 in plant tissues. In line with this, and according to Kostanjevecki et al. (2019), TRA 250 has been
762 detected as a microbial biodegradation **product** of TRA in activated sludge culture and no
763 significant toxic effects were found in algal bioassay.

764 ~~Since the majority of the tentatively identified TPs in this study have been only investigated in~~
765 ~~aquatic environments, all potential ecotoxicological evidences are referred to this media.~~ To our
766 knowledge, occurrence data of the 18 TPs investigated in this work have not been reported in real
767 field agricultural samples. However, **in terms of when talking about** agricultural systems, there is
768 a knowledge gap regarding CEC TPs due to the high variety of compounds that can be present
769 and the scarce information available about real-field samples at environmental concentrations.
770 For instance, effects on microorganisms, terrestrial wildlife and plant stress inducers, spread of
771 antibiotic resistance, toxicological synergistic effects related to mixtures, transformation of parent
772 compounds in plant metabolism, introduction into the food chain and human low-level exposure,
773 among others, are practically unknown for **the** reuse of RW in agriculture [17]. From this point
774 of view, the application of HRMS analytical strategies able to detect and identify compounds not
775 previously reported in agricultural environments is of high importance to fill in the **knowledge**
776 gaps in **ecotoxicological knowledge risk assessment associated with reuse practices**.

777 778 **4. Conclusions**

779 The originality of this study is based on the first application of a retrospective suspect screening
780 focused on 262 TPs from 20 parent CECs in actual agricultural environments. Four real-field
781 agricultural systems (SP, GH1, GH2, GH3) irrigated with RW for more than 15 years were

782 investigated in search of ~~CEC-pharmaceutical~~ TPs. A thorough investigation of TP fragmentation
783 patterns together with a comparison with spectral libraries and literature evidences were decisive
784 for the structural assignment and classification of up to 18 TPs from 9 CECs. The developed
785 analytical strategy has been successfully applied for the tentative identification with high
786 confidence of 12 TPs, which led to the confirmation of 7 TPs. 2 TPs were tentatively identified
787 for the first time. Occurrence and environmental impact of the 18 TPs were evaluated. SP (perlite
788 matrix) showed the highest number of compounds (15 TPs), followed by agricultural soils from
789 GH1, GH2 and GH3 (8 TPs), RW (4 TPs) and plant leaves (2 TPs). Remarkably, none of the
790 investigated TPs was found in tomato fruit samples. Although perlite substrate accumulated the
791 highest number of TPs, no significant and specific availability of TPs for plants was observed.
792 Up to 6 TPs showed persistence between sampling events in perlite/soil samples and occurrence
793 of AZI 434 and TEL 439 was found to be almost ubiquitous in these matrices. In general, no clear
794 trend showing uptakes s from soils/perlite to leaf plant tissues was detected. HCT 293 and TRA
795 250 were the only two compounds identified in leaves. To our knowledge, no previous data is
796 available regarding the TPs evaluated in this work in agricultural environments. This study
797 stresses the wide variety of CEC TPs derived from reuse practices that can be present in
798 agricultural systems as well as ~~and~~ the need for specific knowledge to evaluate TP ~~ecotoxicological~~
799 ~~effects~~ environmental impact, including the possible spread of antibiotic resistance in agricultural
800 environments submitted to RW irrigation.

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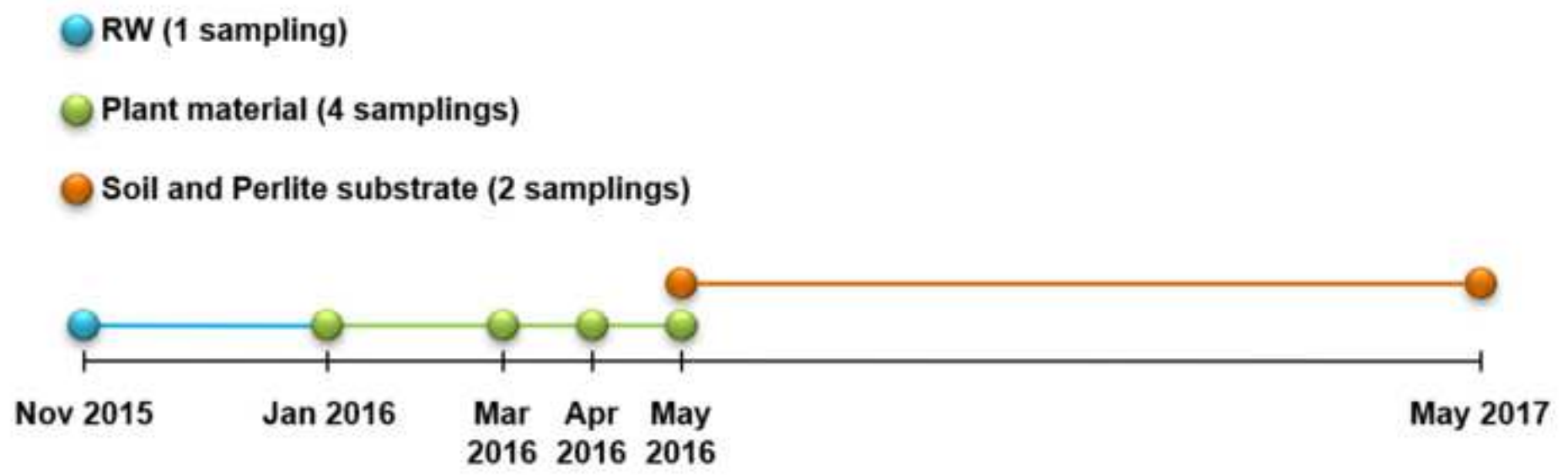
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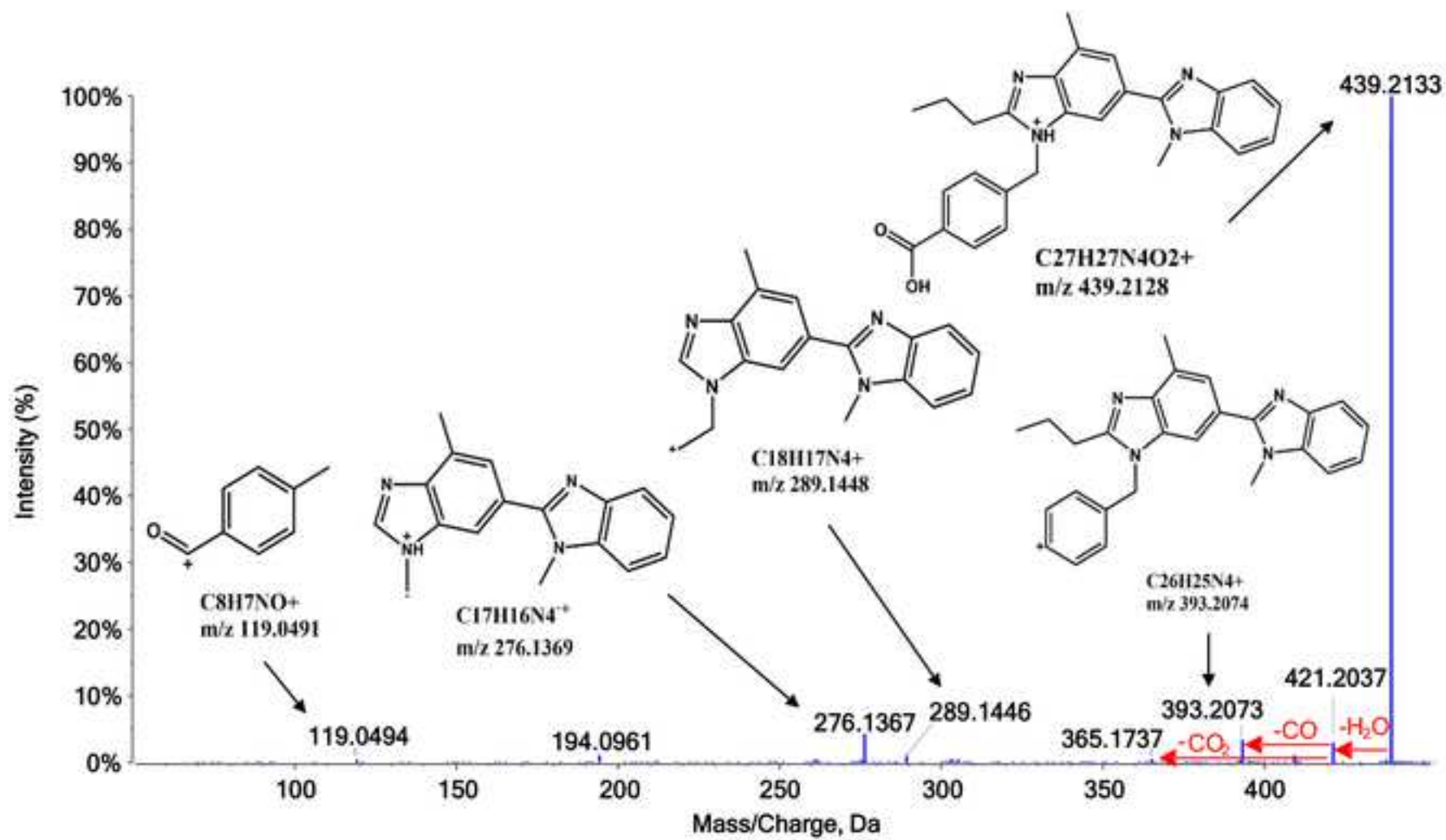
977 **Figure captions**

978 **Figure 1.** Sampling events carried out for each matrix at all sampling sites (SP, GH1-3)

979 **Figure 2.** ESI+ MS² spectrum of TEL 439 in a perlite sample and proposed fragmentation

980 **Figure 3.** Total number of TPs found in each sampling site/matrix





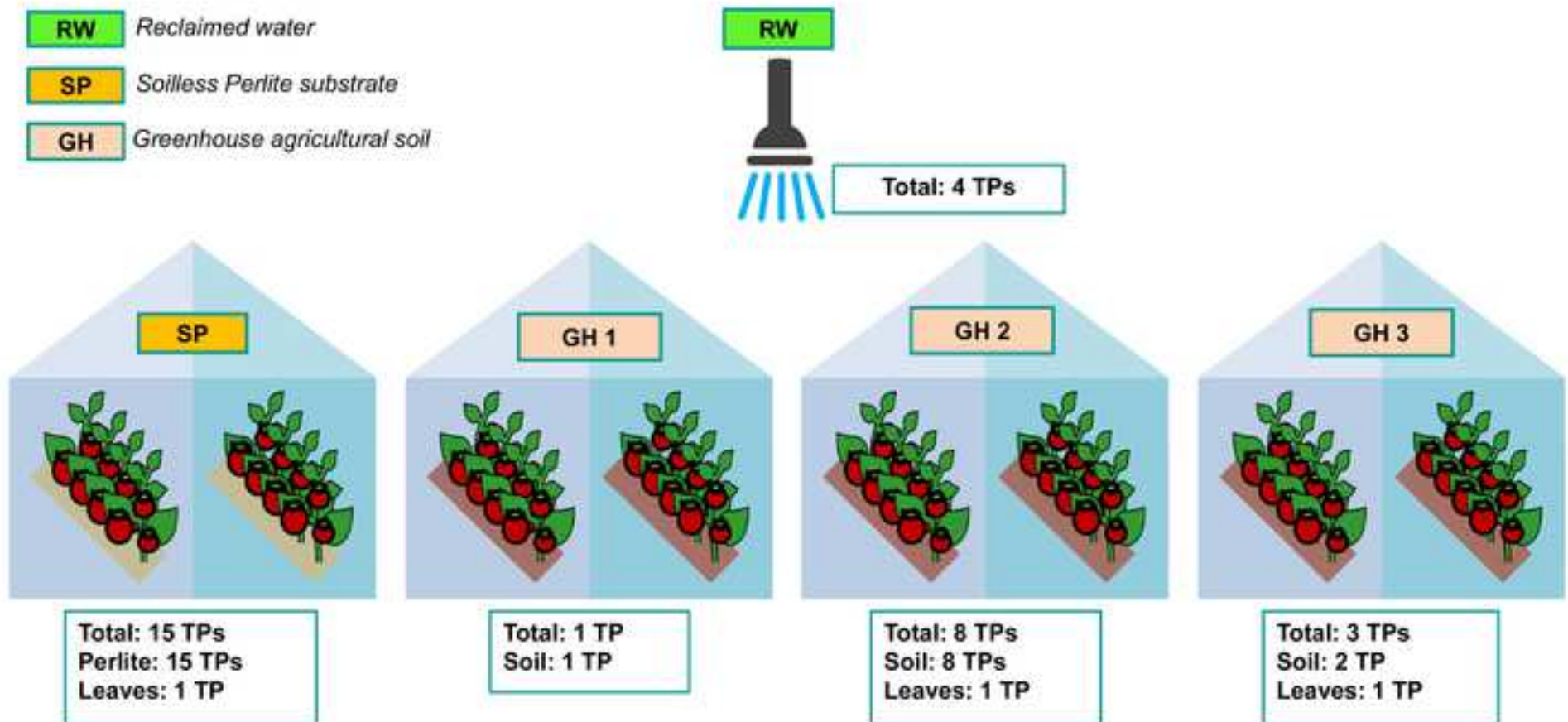
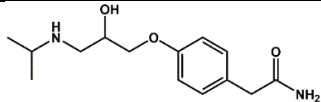
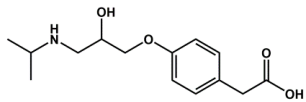
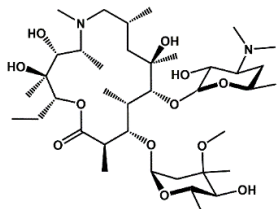
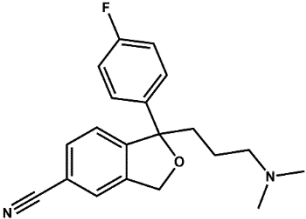
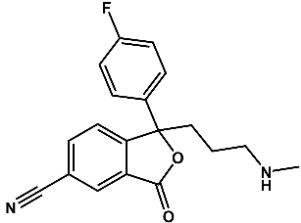
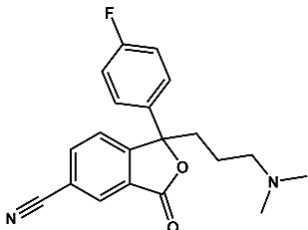
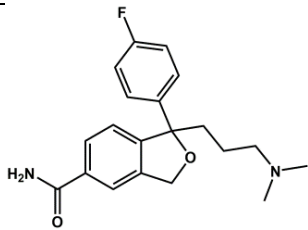
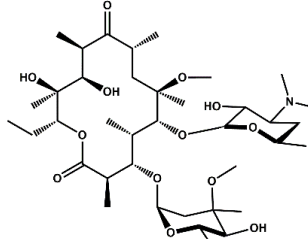
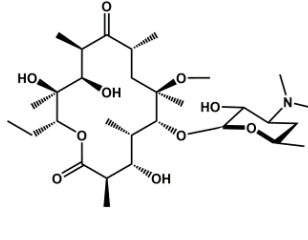
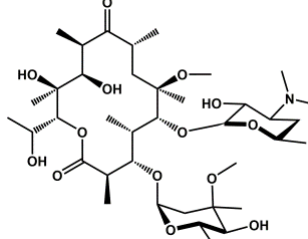
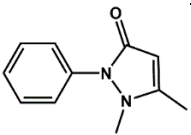
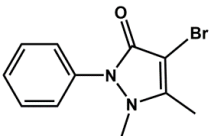
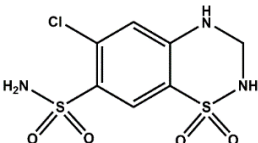
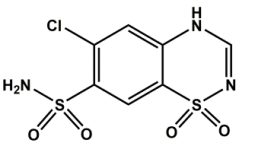
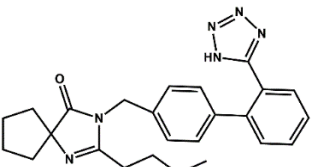


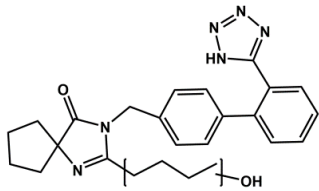
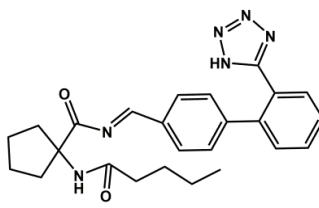
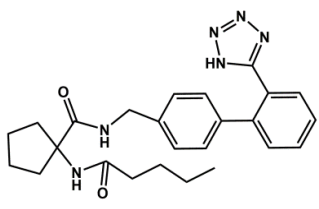
Table 1. List of parent pharmaceuticals (in bold) and TPs tentatively identified in samples. Chromatographic, spectral information, identification level and criteria considered for structure allocation.

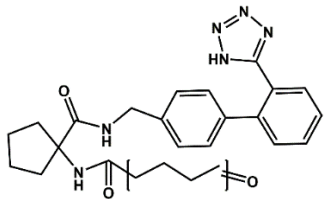
Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (<i>m/z</i>)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref
ATE		1.88	C₁₄H₂₂N₂O₃	267.1703	0.7	5	1.9	1		
			C ₁₁ H ₁₆ N ₂ O ₃	225.1234	1.0	5				
			C ₁₁ H ₁₁ NO ₂	190.0863	1.8	7				
			C ₁₀ H ₁₁ NO ₂	178.0863	-7.6	6				
			C ₁₀ H ₈ O	145.0648	7.6	7				
			C ₇ H ₆ O	107.0491	4.3	5				
ATE 268 Atenolol acid		4.40	C₁₄H₂₁NO₄	268.1543	-0.7	5	2	1	MassBank Europe score: 92% Literature: 4 PI matched Predicted RT difference: 1.63 min RT match with analytical standard	[1,2]
			C ₁₁ H ₁₅ NO ₄	226.1074	-2.1	5				
			C ₁₁ H ₁₀ O ₃	191.0703	-0.4	7				
			C ₉ H ₈ O ₃	165.0546	-8.6	6				
			C ₁₀ H ₈ O	145.0648	-4.8	7				
			C ₆ H ₁₃ NO	116.1070	6.1	1				
			C ₃ H ₇ NO	74.0600	0.8	1				
AZI		6.08	C₃₈H₇₂N₂O₁₂	749.5158	-0.9	4	8.9	1		
			C ₃₀ H ₅₈ N ₂ O ₉	591.4215	-1.4	3				
			C ₃₀ H ₅₆ N ₂ O ₈	573.4109	0.3	4				
			C ₂₂ H ₄₃ NO ₇	434.3112	-2.8	2				
			C ₈ H ₁₅ NO ₂	158.1176	2.2	2				
			C ₆ H ₁₃ NO	116.1070	7.8	1				

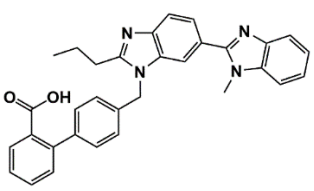
Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (m/z)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref
CIT		7.22	C₂₀H₂₁FN₂O	325.1711	-0.4	11	2.4	1		
			C ₂₀ H ₁₉ FN ₂	307.1605	-1.0	12				
			C ₁₈ H ₁₄ FNO	280.1132	-1.9	12				
			C ₁₈ H ₁₂ FN	262.1027	-1.0	13				
			C ₁₇ H ₁₀ FN	247.0792	-1.9	13.5				
			C ₁₆ H ₈ FN	234.0714	-1.9	13				
			C ₁₅ H ₈ FN	221.0635	-3.3	12.5				
			C ₁₂ H ₇ N	166.0651	-3.2	10				
C ₇ H ₅ F	109.0448	7.3	5							
CIT 325		6.97	C₁₉H₁₇FN₂O₂	325.1346	-0.3	12	5	2b	Literature: 8 PI match Predicted RT difference: 0.57min	[5]
			C ₁₉ H ₁₅ FN ₂ O	307.1241	-6.9	13				
			C ₁₈ H ₁₀ FNO	276.0819	-4.1	14				
			C ₁₈ H ₈ FN	258.0714	0.2	15				
			C ₁₈ H ₉ NO	256.0757	-4.3	15				
			C ₁₇ H ₁₀ FN	248.0870	-4.9	13				
			C ₁₄ H ₁₀ FNO	228.0819	-1.4	10				
			C ₁₁ H ₉ FN ₂ O ₂	221.0721	-0.8	8				
C ₁₀ H ₅ NO ₂	172.0393	-7.6	9							
CIT 339		7.05	C₂₀H₁₉FN₂O₂	339.1503	0.1	12	0.9	2b	Literature: 6 PI match Predicted RT difference: 0.47min	[5,6]
			C ₂₀ H ₁₇ FN ₂ O	321.1398	-5.5	13.5				
			C ₁₈ H ₁₂ FNO ₂	294.0925	-2.3	13				
			C ₁₈ H ₁₀ FNO	276.0819	1.7	14				
			C ₁₈ H ₈ FN	258.0714	2.9	15				
			C ₁₇ H ₁₀ FN	248.0870	4.4	13				
			C ₁₀ H ₅ NO ₂	172.0393	-4.7	9				

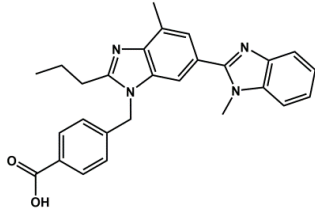
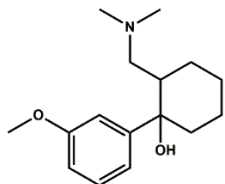
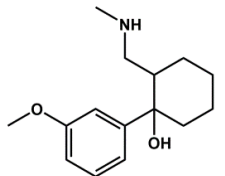
Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (<i>m/z</i>)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref
CIT 343 Citalopram amide		6.1	C₂₀H₂₃FN₂O₂ C ₂₀ H ₂₁ FN ₂ O C ₁₈ H ₁₄ FNO C ₁₇ H ₁₃ F C ₈ H ₇ FO ₂ C ₇ H ₅ F	343.1816 325.1711 280.1132 237.1074 155.0503 109.0448	-0.8 -7.9 8.9 4.6 -10.2 0.9	10 11 12 11 5 5	3.1	1	Literature: 5 PI match Predicted RT difference: -0.64min RT match with analytical standard	[5,6]
CLA		7.72	C₃₈H₆₉NO₁₃ C ₃₀ H ₅₅ NO ₁₀ C ₂₉ H ₅₁ NO ₉ C ₁₆ H ₂₉ NO ₅ C ₈ H ₁₅ NO ₂ C ₆ H ₁₃ NO	748.4841 590.3899 558.3637 316.2118 158.1176 116.1070	-0.5 -3.9 -3.7 -4.6 0.9 3.5	5 4 5 3 2 1	1.7	1		
CLA 590 De(cladinosyl) Clarithromycin		6.73	C₃₀H₅₅NO₁₀ C ₂₉ H ₅₁ NO ₉ C ₈ H ₁₅ NO ₂ C ₆ H ₁₃ NO	590.3898 558.3637 158.1176 116.1070	-1.8 3.3 -1 -6.8	4 5 2 1	2.4	1	Literature: 3 PI match ChemSpider score: 85% Predicted RT difference: -0.03 min RT match with analytical standard	[4,7,8]
CLA 764		6.79	C₃₈H₆₉NO₁₄ C ₃₀ H ₅₅ NO ₁₁ C ₂₉ H ₅₁ NO ₁₀ C ₈ H ₁₅ NO ₂ C ₆ H ₁₃ NO	764.4790 606.3848 574.3586 158.1176 116.1070	0.2 -3.8 3.4 -6 4.4	5 4 5 2 1	5.8	2b	ChemSpider score: 71% Predicted RT difference: 1.13 min	[4]

Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (m/z)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref
DIP		5.85	C₁₁H₁₂N₂O C ₁₀ H ₁₀ N ₂ O C ₁₀ H ₁₂ N ₂ C ₉ H ₁₀ N ₂ C ₉ H ₇ N C ₇ H ₅ N	189.1022 174.0788 161.1073 147.0917 130.0651 104.0495	-1.7 -3.2 -4.5 -1.9 -1.7 3.1	7 7.5 6 6 7 6	0.7	1		
DIP 267 4-Bromoantipyrene		6.82	C₁₁H₁₁BrN₂O C ₁₀ H ₉ BrN ₂ O C ₁₁ H ₁₂ N ₂ O C ₁₀ H ₈ N ₂ O C ₃ H ₆ BrN	267.0127 251.9893 188.0944 173.0709 135.9756	-3 4.5 -0.1 -8.3 -9.8	7 7.5 7.5 8 1	8.9	1	Literature: 4 PI match ChemSpider score: 72% Predicted RT difference: 1.71 min RT match with analytical standard	[4,9]
HCT ^f		4.50	C₇H₈ClN₃O₄S₂ C ₆ H ₇ ClN ₂ O ₄ S ₂ C ₆ H ₇ ClN ₂ O ₂ S	295.9572 268.9463 204.9844	-1.0 2.6 2.9	5 4 4	3.1	1		
HCT 293 ^f Chlorothiazide		3.91	C₇H₆ClN₃O₄S₂ C ₇ H ₅ ClN ₂ O ₂ S C ₇ H ₃ ClN ₂ O ₂ S C ₇ H ₄ N ₂ O ₂ S	293.9415 214.9687 213.9609 178.9921	-2 -14.7 -4.3 1.8	4 6 6.5 7	5.4	1	MassBank Europe score: 91% Literature: 2 PI matched ChemSpider score: 78.4% Predicted RT difference: -0.3 min RT match with analytical standard	[2,4,10]
IRB		7.90	C₂₅H₂₈N₆O C ₂₅ H ₂₇ N ₃ O C ₁₄ H ₁₀ N ₂ C ₁₁ H ₁₈ N ₂ O C ₁₃ H ₉ N	429.2397 386.2227 207.0917 195.1492 180.0808	-1.1 -1.0 1.1 0.1 -0.4	15 14 11 4 10	0.3	1		

Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (m/z)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref
IRB 445 A		7.03	C₂₅H₂₈N₆O₂	445.2346	-1.7	15	0.6	3	Literature: 3 PI matched	[11]
			C ₂₅ H ₂₆ N ₆ O	427.2241	-4.9	16				
			C ₂₅ H ₂₈ N ₄ O ₂	417.2285	0.2	14				
			C ₂₅ H ₂₇ N ₃ O ₂	402.2176	9.7	14				
			C ₁₁ H ₁₈ N ₂ O ₂	211.1441	9.1	4				
			C ₁₄ H ₁₀ N ₂	207.0917	2.1	11				
IRB 445 B		8.84	C₂₅H₂₈N₆O₂	445.2346	-0.6	15	3.2	2b	Literature: 4 PI matched Predicted RT difference: 2.06 min	[11]
			C ₂₅ H ₂₈ N ₄ O ₂	417.2285	-7.7	14				
			C ₂₀ H ₂₀ N ₆ O	361.1771	2.7	14				
			C ₁₄ H ₁₁ N ₅	250.1087	-0.1	12				
			C ₁₄ H ₈ N ₄	233.0822	7.4	13				
			C ₁₄ H ₁₀ N ₂ O	223.0866	4.5	11				
			C ₁₄ H ₁₁ N ₃	222.1026	6.0	11				
			C ₁₄ H ₈ N ₂	205.0760	1.3	12				
			C ₁₁ H ₁₇ NO ₂	196.1332	-0.5	4				
			C ₁₀ H ₁₇ NO	168.1383	2.4	3				
IRB 447 SR-49498		8.34	C₂₅H₃₀N₆O₂	447.2503	0.2	14	5.8	1	ChemSpider score: 91% Literature: 7 PI matched ESI+, 4 PI ESI- Predicted RT difference: 1.84 min RT match with analytical standard	[4,11]
			C ₂₅ H ₂₈ N ₆ O	429.2397	0.6	15				
			C ₁₃ H ₁₈ N ₆ O ₂	291.1564	-8.6	8				
			C ₁₄ H ₁₃ N ₅	252.1244	2.1	11				
			C ₁₄ H ₁₀ N ₄	235.0978	-0.1	12				
			C ₁₄ H ₁₀ N ₂	207.0917	-2.3	11				
			C ₁₁ H ₁₇ NO ₂	196.1332	-0.5	4				
			C ₁₀ H ₁₇ NO	168.1383	1.8	3				
			C ₅ H ₉ N	84.0808	5.0	2				
			C₂₅H₃₀N₆O₂^f	445.2357^f	-3.1^f	14^f	1.5^f			
			C ₂₀ H ₂₂ N ₂ O	305.1659	-2.1	11				
			C ₁₁ H ₂₀ N ₂ O ₂	211.1452	-5.7	3				
			C ₁₃ H ₁₀	165.0710	-2.9	9				

Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (<i>m/z</i>)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref		
IRB 461 A		7.35	C₂₅H₂₈N₆O₃	461.2295	-0.7	15	7.4	3	Literature: 3 PI matched	[11]		
			C ₂₅ H ₂₆ N ₆ O ₂	443.2190	-2.7	16						
			C ₂₅ H ₂₃ N ₅ O ₂	425.1846	0.2	17.5						
			C ₂₂ H ₂₁ N ₅ O ₃	403.1639	-2.5	15.5						
			C ₁₄ H ₁₃ N ₅	252.1244	-4.6	11						
			C ₁₄ H ₁₀ N ₄	235.0978	-5.2	12						
			C ₁₁ H ₁₅ NO ₃	210.1125	-2.7	5						
			C ₁₄ H ₁₀ N ₂	207.0917	2.5	11						
			C ₁₀ H ₁₅ NO ₂	182.1176	3.5	4						
			C ₁₃ H ₉ N	180.0808	2.4	10						
			C ₈ H ₁₂ N ₄	164.1056	3.4	5.5						
			C ₅ H ₆ O ₂	99.0441	8.5	3						
			C ₅ H ₉ N	84.0808	12.2	2						
			C₂₅H₂₈N₆O₃^f	459.2145^f	-3.4^f	15^f	7.2^f					
			C ₂₃ H ₂₄ N ₆ O ₂	416.1966	-0.8	14.5						
			C ₂₀ H ₂₂ N ₂ O	305.1659	3.5	11						
			C ₁₁ H ₁₆ N ₂ O ₂	207.1139	1.0	5						
			C ₁₄ H ₁₁ N	192.0819	-2.5	10						
			C ₁₃ H ₁₀	165.0710	6.2	9						

Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (m/z)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref	
IRB 461 B	No structure proposal	7.61	C₂₅H₂₈N₆O₃	461.2295	1.3	15	1.2	4	-	[11]	
			C ₂₀ H ₁₇ N ₅ O ₂	360.1455	-1.9	15					
			C ₂₀ H ₁₄ N ₄ O ₂	343.1190	2.8	16					
			C ₂₀ H ₁₇ N ₃ O ₂	332.1394	4.7	14					
			C ₁₄ H ₁₃ N ₅	252.1244	-3.1	11					
			C ₁₄ H ₁₀ N ₄	235.0992	-2.6	12					
			C ₁₃ H ₁₀ N ₄	223.0978	-2.3	11					
			C ₁₄ H ₁₀ N ₂	207.0917	-2.8	11					
			C ₁₄ H ₇ N	190.0651	-9.1	12					
			C ₁₃ H ₉ N	180.0808	9.5	10					
			C ₅ H ₁₁ NO	102.0913	3.5	1					
			C₂₅H₂₈N₆O₃^f	459.2150^f	-2.2^f	15^f	1.7^f				
			C ₂₀ H ₁₇ N ₅ O ₂	358.1309	-3.2	15					
			C ₂₀ H ₁₇ N ₃ O ₂	330.1248	-2.4	14					
			C ₂₀ H ₁₆ N ₂ O ₂	315.1139	-8.3	14					
			C ₁₄ H ₁₃ N ₅	250.1098	-4.5	11					
			C ₁₅ H ₁₁ NO	220.0768	-3.1	11					
			C ₁₄ H ₁₁ N	192.0819	-10.6	10					
			C ₁₄ H ₁₂	179.0866	2.7	9					
			C ₆ H ₇ NO ₂	124.0404	-1.6	4					
C ₅ H ₆ O	81.0346	-18.7	3								
TEL		7.70	C₃₃H₃₀N₄O₂	515.2441	-0.5	21	1.2	1			
			C ₃₃ H ₂₈ N ₄ O	497.2336	-1.8	22					
			C ₁₉ H ₂₀ N ₄	305.1761	0.7	12					
			C ₁₉ H ₁₆ N ₂ O	289.1335	1.8	13					
			C ₁₇ H ₁₆ N ₄	276.1369	-1.5	12.5					
			C ₁₄ H ₁₀ O ₂	211.0754	2.1	10					

Compound	Structure	RT ^a (min)	Molecular formula	Theoretical [M+H] ⁺ (m/z)	Error (ppm)	RDB ^b	IRD ^c (%)	IL ^d	Criteria	Ref
TEL 439		6.50	C₂₇H₂₆N₄O₂ C ₂₇ H ₂₄ N ₄ O C ₂₅ H ₂₀ N ₄ O ₂ C ₂₆ H ₂₄ N ₄ C ₂₄ H ₂₀ N ₄ C ₁₈ H ₁₆ N ₄ C ₁₇ H ₁₆ N ₄ C ₁₆ H ₁₂ N ₄ C ₁₄ H ₁₁ N C ₈ H ₆ O	439.2128 421.2023 409.1659 393.2074 365.1761 289.1448 276.1369 261.1135 194.0964 119.0491	-0.8 3.4 1.5 -0.2 6.5 -0.6 -1.6 -4.1 -1.7 -2.0	17 18 18 17 17 13 12.5 13 10 6	3.1	3	MS/MS Elucidation Predicted RT difference: -3.64min	
TRA		5.90	C₁₆H₂₅NO₂ C ₁₆ H ₂₃ NO C ₃ H ₇ N	264.1958 246.1852 58.0648	-0.8 -0.6 -15.2	5 6 0	2.6	1		
TRA 250 N- Desmethyltramadol		6.0	C₁₅H₂₃NO₂ C ₁₅ H ₂₁ NO C ₁₃ H ₁₆ O C ₁₁ H ₁₀ O C ₈ H ₈ O C ₇ H ₆	250.1801 232.1696 189.1274 159.0804 121.0648 91.0542	-2.2 0.5 -5.8 2.9 0.1 4.1	5 6 6 7 5 5	8.9	1	MassBank Europe score: 98% ChemSpider score: 77% Literature: 3 PI matched Predicted RT difference: -0.63min RT match with analytical standard	[2,4,12]

^a Retention time, ^b Ring and double bonds, ^c Isotope ratio difference, ^d Identification level, ^e Data correspondent to the [M-H]⁻ adduct from ESI⁻ analysis.

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	SP					GH 1		GH 2				GH 3		
	RW	Perlite		Leaves		Soil		Soil		Leaves		Soil		Leaves
		S1	S2	S3	S4	S1	S2	S1	S2	S3	S4	S1	S2	S4
IRB 461A	5.04E+04	ND	5.27E+04	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IRB 461B	ND	5.22E+04	9.30E+04	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TEL 439	ND	1.05E+05	6.22E+04	ND	ND	ND	3.91E+03	ND	1.58E+04	ND	ND	4.41E+04	4.09E+03	ND
TRA 250	ND	1.14E+06	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.63E+05

Abbreviations: SP, soilless perlite culture; GH, greenhouse; RW, reclaimed water; S, sampling event; ND, not detected. In bold, TPs for which the parent compound was also determined in this sample.

Table 3. Average concentrations of TPs confirmed in samples.

			SP				GH 1		GH 2				GH 3			
	LOQ (ng/g)	RW (ng/L)	<i>Perlite</i> (ng/g)		<i>Leaves</i> (ng/g)		<i>Soil</i> (ng/g)		<i>Soil</i> (ng/g)		<i>Leaves</i> (ng/g)		<i>Soil</i> (ng/g)		<i>Leaves</i> (ng/g)	
			S1	S2	S3	S4	S1	S2	S1	S2	S3	S4	S1	S2	S4	
ATE 268	1	8023	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CIT 343	0.1	ND	0.4	ND	ND	ND	ND	ND	0.5	ND	ND	ND	ND	ND	ND	ND
CLA 590	0.1	ND	2.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DIP 267	1	ND	2.9	6.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HTC 293	0.5	ND	ND	1.9	1.3	1.1	ND	ND	ND	ND	0.6	1.7	ND	ND	ND	ND
IRB 447	0.1	883	6.2	14.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TRA 250	1	ND	18.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.5

Abbreviations: SP, soilless perlite culture; GH, greenhouse; LOQ, limit of quantification; RW, reclaimed water; S, sampling event; ND, not detected.



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Ana Belén Martínez-Piernas: Conceptualization, Methodology, Data curation, Investigation, Writing - original draft, Writing - review & editing. **Patricia Plaza-Bolaños:** Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **Ana Agüera:** Supervision, Writing - review & editing, Project administration, Funding acquisition.