Organic microcontaminants in tomato crops irrigated with reclaimed water grown under field conditions: occurrence, uptake and health risk assessment

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ABSTRACT

In many regions reuse of reclaimed water (RW) is a necessity for irrigation. Presence of organic microcontaminants (OMCs) in RW and their translocation to plants may represent a risk of human exposure. Nevertheless, information available about real field crops is scarce and focused on a limited number of compounds. The novelty of this work relies on the application of a wider-scope analytical approach based on a multi-analyte target analysis (60 compounds) and a suspect screening (1300 compounds). This methodology was applied to real field-grown tomato crops irrigated with RW. The study revealed the presence of 17 OMCs in leaves (0.04 - 32 ng g^{-1}), and 8 in fruits (0.01 - 1.1 ng g^{-1}); 5 of them not reported before in real field samples. A health-risk assessment, based on the toxicological threshold concern (TTC) concept, showed that RW irrigation applied under the conditions given do not pose any threat to humans.

KEY WORDS

Organic microcontaminants

Plant uptake

Reclaimed water reuse

Health risk assessment

LC-MS target/suspect analysis

INTRODUCTION

The lack of fresh water resources for agriculture in arid and semiarid regions is a worldwide problem that needs to be addressed in the 21st century. Factors such as climate change and increasing population have led to severe droughts in areas where intensive agriculture is the main economic activity. The reuse of reclaimed water (RW) for agriculture irrigation seems to be an excellent approach to deal with water scarcity,[1–5] since it not only promotes efficient water usage, but also has other advantages such as reducing the application of fertilizers and avoiding the discharge of waste into natural water bodies, thus contributing towards the preservation of the environment.[6]

In Europe, the Mediterranean area is heavily influenced by low and irregular rainfall, a fact that has worsened water shortages leading to a lower water supply for agricultural purposes mainly during peak water demand periods. Nowadays, countries such as Cyprus, France, Greece, Italy, Portugal and Spain, have adopted regulations regarding the reuse of RW for crop irrigation due to the increasing application of this practice.[7] So much so, in Spain, the 10.8% of the RW is reused, being the 71% of it destined to agriculture.[7] In most cases, the national regulations include specific threshold values for either microbiological (e.g. *E. coli*, intestinal nematodes) and physical-chemical parameters (e.g. total suspended solids, turbidity) for any restricted use,[8] being more strict for agricultural uses. The European Commission has recently launched a proposal for a regulation on minimum requirements for water reuse, which includes recommendations based on a health and environmental risk management framework for future water reuse legislation.[9] Again, only microbiological and physical-chemical parameters have been considered. However, in the last decade, the presence of organic microcontaminants (OMCs) in RW, which are not completely removed during the treatments,[10] have been pointed out as a potential risk. It has been demonstrated that intensive use of RW in agriculture leads to their accumulation in agricultural soils[11,12] and their subsequent uptake by plant roots, in some cases being able to translocate to aerial parts of plants such as leaves and fruits through the vascular plant system.[2,13–15] However, some knowledge gaps and the lack of reliable data still prevent to make definite conclusions about their risk posed to humans and the environment.

Numerous studies have shown translocation of OMCs to edible parts of crops in simulated or controlled conditions.[13–21] Nevertheless, little is known about their occurrence and accumulation in real field crops exposed to RW irrigation for long time periods. Recently, Picó et al.[14] have evaluated the accumulation of OMCs in agricultural soils and crops irrigated with treated wastewater, finding up to 6 pharmaceuticals in different crops as cabbage, green beans or eggplants. Also Riemenscheneider et al.[5] reported the translocation in real field samples of 12 micropollutants and metabolites to different plant organs such as roots, stems, leaves and fruits of 10 different vegetables irrigated with river water mixed with effluent from a wastewater treatment plant (WWTP). In another study, Wu et al.[2] monitored the accumulation of 19 OMCs in 8 vegetables irrigated with RW showing a detection frequency of 64% at concentrations in the range of 0.01-3.87 ng g^{-1}, dry weight, (d.w.).

However, most of the reported studies analyze a low number of compounds or are focused on certain pharmaceutical classes. In order to obtain a comprehensive evaluation of the impact of OMCs in the food chain, it is necessary to apply multi-analyte/class methodologies able to provide qualitative information for a wide range of compounds, given the large number of OMCs reported in RW. Therefore, in addition to wider target methods, non-target screening methodologies based on high resolution mass spectrometry (HRMS) should be applied, leading to the identification of substances outside the limited scope of the target analysis.[12,14] This approach should contribute towards improving data available regarding the occurrence/accumulation of OMCs in final products intended for human consumption to ensure safe use of RWW in terms of health risk assessment.

Finally, the reported accumulation of OMCs in crops is in general low and no risk for public health is expected to be associated to the until now, few known individual compounds in crops grown under real field conditions.[22,23] However, further work needs to be carried out to assess the risk of not previously evaluated compounds that are present in the edible tissues of plants grown under long-term and continuous exposition to these microcontaminants.[24] This data will be valuable to study the risk associated with mixtures of OMCs in end-products in future works.

The goal of this work was to increase the current information about the translocation of OMCs derived from reuse by providing reliable data on their occurrence and fate in real tomato crops (leaves and fruits) after long-term exposure to RW irrigation practices under field conditions. Field-grown tomato plants were cultivated in agricultural soils previously analyzed[12] and irrigated with RW for more than 10 years without soil substitution. With this aim in mind, a combined strategy based on a multi-analyte target analysis (including 60 compounds considered as contaminants of emerging concern) together with a suspect screening methodology (covering a list of 1300 potential contaminants) was applied. A simple and quick QuEChERS-based method was used for sample preparation and liquid chromatography coupled to low and high resolution mass spectrometry, were selected. A health-risk assessment approach was also applied to evaluate human exposure of the RW-derived OMCs in tomato fruits.

MATERIALS AND METHODS

Chemicals and Reagents. A total of 60 OMCs (mainly pharmaceuticals from a variety of therapeutic classes) (Table S1) were analyzed due to their frequent identification in

WWTP effluents.[10] All reference standards (purity > 98%) were acquired from Sigma-Aldrich (Steinheim, Germany). Methanol (MeOH), acetonitrile (ACN), water, formic acid and acetic acid (LC-MS grade) were obtained from Sigma-Aldrich. Ultrapure water for LC-MS/MS analysis was produced using a Milli-Q water purification system from Millipore (Darmstadt, Germany). For the QuEChERS extraction method, anhydrous magnesium sulfate ($MgSO_4$) and sodium acetate (NaOAc) were purchased from Sigma Aldrich (all purity > 98%). Octadecyl-silyl-modified silica gel (C18) and primary-secondary amine (PSA) were acquired from Supelco (Bellefonte, PA, USA).

Stock standard solutions of each compound were prepared at 1000-2000 mg L^{-1} in MeOH. Multi-compound working solutions were prepared at a concentration of 10 mg L^{-1} in MeOH by diluting the individual stock solutions. All standard solutions were stored in amber glass vials at -20°C. Matrix matched calibration solutions were daily prepared and used for quantification purposes. Two surrogate standards, carbamazepine-d_{10} and ^{13}C-caffeine, were used to check the extraction efficiency.

Sample Collection. To study the occurrence and distribution of OMCs in the plant system, three greenhouses were selected (GH1, GH2 and GH3; intensive production; 13000–25000 m^2), in which two different tomato varieties, ramyle (GH1, GH2) and retinto (GH3) were grown. A fourth greenhouse dedicated to the experimental soilless culture (SP1) of the cherry tomato variety, which was grown in pots filled with perlite substrate, was also included in the study. All greenhouses were located in Almeria province (Spain) and had been irrigated with RW for no less than ten years without soil replacement. The RW was provided by a regeneration plant facility which treats municipal wastewater secondary effluents by filtration (sand and anthracite filters) and chlorination (NaClO). Treated water fulfilled the requirements of water quality according to the Spanish regulation for water reuse.[8] Drip irrigation was employed in all greenhouses. Four sampling events during the commercial tomato campaign took place from January (full plant growth) to May 2016 (removal of tomato plants). In each sampling event, tomatoes at a mature stage of growth and leaves of tomato plant samples (500 g in each case) of similar size were taken from different parts of the greenhouse following a W sampling route. The subsamples were chopped and mixed to form a homogeneous composite sample and were kept in the dark at -20°C until their analysis. Three replicates of each sample were extracted for quantification purposes. RW was analyzed coinciding with the first sampling of tomato fruits and leaves.

Sample Extraction. The extraction of OMCs in tomato fruit and leaves was carried out by a modification of the QuEChERS acetate extraction method previously published by our group.[13] Briefly, a portion of 10 g of plant material were placed into a 50-mL polypropylene centrifuge tube. After that, 10 mL of 1% acetic acid in ACN and 20 µL of the extraction quality control solution were added to the sample and the tube was shaken for 5 min and centrifuged at 3500 rpm (2054x*g*) for 5 min. Following the extraction procedure, a clean-up step was carried out. An aliquot of 5 mL of the upper organic layer was transferred to a 15-mL centrifuge tube containing 750 mg of anhydrous $MgSO_4$, 125 mg of primary-secondary amine (PSA) and 125 mg of C18. Then the tube was shaken for 30 s in a vortex and centrifuged at 3500 rpm for 5 min. Following this, the extract (4 mL) was transferred to screw-cap vials adding 10 µL of ACN at 1% of formic acid per milliliter of extract. Prior to injection into the LC-MS/MS system, 100 µL of the extract was evaporated and reconstituted in 100 µL of ACN:H_2O (10:90, *v/v*).

Liquid Chromatography-Mass Spectrometry. *LC-MS/MS Target Analysis.* The HPLC system (Agilent Series 1200, Agilent Technologies, Palo Alto, CA, USA) consisted of a binary pump, a degasser and an autosampler. Chromatographic separation was accomplished using a XDB C18 (50 x 4.6 mm, 1.8 µm particle size) column (Agilent

Technologies). Mobile phases were 0.1% formic acid in MilliQ water (solvent A) and ACN (solvent B). The gradient used ranged from 10% to 100% of solvent B: initially it was kept at 10% for 1 min, increased from 10% to 50% over 3 min and from 50% to 100% over 10 min; kept at 100% for 4 min and finally returned to its initial conditions. The total analysis run time was 18 min. The injection volume was 10 μL and the flow rate was set to 0.4 mL min^{-1}. The column outlet system was connected to a hybrid triple quadrupole-linear ion trap-mass spectrometer 5500 QTRAP® (Sciex Instruments, Foster City, CA, USA) equipped with an ESI source (TurboIon Spray) operating with positive and negative polarities. The ionization settings used were: ionspray voltage, 5000 V; curtain gas, 25 (arbitrary units); GS1, 50 psi, GS2, 40 psi; and a temperature, 500 °C. Nitrogen was used as a nebulizer, curtain and collision gas. The multiple reaction monitoring (MRM) mode was chosen for the analysis of the target compounds. To increase the sensitivity for the acquisition performance, the schedule MRM™ algorithm was applied with a retention time window of 40 s per transition. The optimal mass spectrometric parameters for each compound are summarized in Table S2. Sciex Analyst version 1.6.2 software was applied for data acquisition and processing, and MultiQuant 3.0.1 software for data quantification.

LC-QTOF-MS/MS Suspect screening analysis. Chromatographic separation was performed using a HPLC (Agilent 1260 Infinity system) equipped with a Poroshell 120 EC-C18 (50 x 4.6 mm, 2.7 μm particle size) analytical column (Agilent Technologies). 0.1% formic acid in ultrapure water (solvent A) and ACN (solvent B) were used as mobile phases. The injection volume was 20 μL and the flow rate was 0.5 mL min^{-1}. The gradient used ranged from 10% to 100% of solvent B: initially it was kept constant at 10% for 2 min, then increased linearly from 10% to 100% for 9 min and finally it remained constant for 4 min before being returning to initial conditions. The total analysis run time was 22 min. The LC system was coupled to a QTOF mass analyzer Triple TOF 5600+ (Sciex Instruments), with a DuoSpray^{TM} ion source consisting of an electrospray (ESI) interface for sample injection and an atmospheric-pressure chemical ionization interface (APCI) for calibrant delivery. Samples were analyzed in ESI+ and ESI− modes. The ESI source parameters were: ionspray voltage, 4500 V; curtain gas, 25 (arbitrary units); GS1, 60 psi; GS2, 60 psi; and temperature, 575°C. Nitrogen served as a nebulizer, curtain and collision gas. The equipment worked via TOF MS survey scan (resolving power of 30000) with an accumulation time of 250 ms followed by four IDA (Information Dependent Acquisition) TOF MS/MS scans with an accumulation time of 100 ms. The IDA feature allows the performance of MS/MS acquisitions simultaneously with the MS acquisition. The *m/z* range was from 100 to 2000. IDA criteria considered dynamic background subtraction. Collision energy of 30 eV with a ± 15 eV spread was applied for MS/MS fragmentation. Diverse Sciex software (Analyst TF 1.5, PeakView™ 2.2 and MasterView 1.1) was used to record and process LC-QTOF-MS/MS data. A suspect list containing 1300 OMCs commonly found in WWTP effluents was made before sample processing. The settings considered for a final confirmation of the compounds were: a) a mass accuracy error for the precursor ion < 5 ppm; b) an isotope ratio difference < 10%; c) a MS/MS spectra fit ≥ 80% when the acquired spectra was compared with the MS/MS spectra of the standard; and d) a difference of ± 0.1 min in the retention time (RT) when it was compared with the standard in matrix.

Method Validation. Concerning the quantitative method for tomato fruits and leaves, the present methodology was validated assessing trueness (in terms of recoveries), precision (expressed as relative standard deviation, RSD), linearity and limits of quantification (LOQs). For method validation, tomato leaves and fruits not irrigated with RW were used as blank matrices. Triplicate analyses of samples spiked at 0.5 ng g^{-1} were used to calculate the recoveries. Satisfactory mean recovery values were considered in the range 70-120% with an associated precision RSDs ≤ 20%. The linearity was studied by matrix-matched standard calibration curves at six concentration levels ranging from 0.01 to 10 ng g^{-1}. Linearity was considered as acceptable when the determination coefficients (R^2) were ≥ 0.990. The LOQs were set as the lowest acceptable concentration in the matrix-matched calibration curve which yielded the signal-to-noise (S/N) ratio closer to 10 for the quantification transition (SRM 1). The quantification of the analytes present in the samples was carried out by matrix-matched calibration curves of all validated compounds. OMCs quantified in real samples fulfilled the requirements for recoveries, precision and linearity (Table S3).

Regarding RW, the sample collected was analyzed per triplicate by direct injection following the methodology reported elsewhere,[10] which was previously validated for the analysis of 115 OMCs in WWTP effluents.

Health-risk Assessment. The health risk associated with presence of OMCs in tomato fruits was estimated using the threshold of toxicological concern (TTC) approach. This is useful for assessing the risk involved with substances present in food at low concentrations and for which toxicity data is still scarce.[25] TTC has previously been applied to the risk assessment of OMCs in crops.[3,20] In this study, an average body weight of 70 kg for adults and 12 kg for toddlers was considered for the estimation of daily consumption. The TTC values and compounds classification were estimated based on the well-known Cramer classification tree. The Cramer method mainly utilizes chemical structures and evaluates the total human intake to establish priorities for testing.[26] This protocol considers a number of factors related to the presence of the chemical component under study or the frequency of ingestion, including: a) different metabolic pathways for either activation or deactivation of the chemicals under study; b) partial presence of a target substance in a variety of standard foods and their metabolites; c) toxicity data for each substance; and d) the level of exposure to humans via oral ingestion. This information is then managed to obtain the TTC value of each compound in terms of µg-ng kg^{-1} body weight (b.w.) day^{-1}.[27] For the OMCs translocation that were not reported before, we considered as minimum tolerated exposure of each OMC as equals to the TCC value given for the parent compound (Houeto et al, 2012; Munro et al., 1996; Stanard et al., 2015).

TCC values and compound classification were estimated using ToxTree software (ToxTree v.3.1.0, by JRC Computational Toxicology and Modeling and developed by Ideaconsult Ltd, Sofia, Bulgaria). The TTC values for all the compounds under study were determined for the highest CEC concentrations of all greenhouses (SP1, GH1, GH2, GH3) obtained in each sampling event (S1 - S4). Statistical analysis of all the samples and repeated measurements in pairs ($p < 0.05$) were performed using ANOVA analysis.

RESULTS AND DISCUSSION

Method validation results. The proposed methodology was validated in tomato fruits and leaves of tomato plant for a total of 60 OMCs. The validation results are presented in Table S3.A total of 48 out of 60 compounds (80%) in fruit and 31 (51%) in leaves showed acceptable recoveries in the 70-120% range with RSD ≤ 20%. Tomato results are in line with the previous method validation of the same compounds in other vegetable matrices such as lettuce, radish and strawberry.[13] However, the number of successfully recovered OMCs in leaves was lower than in fruits probably due to the complexity of this matrix. The high content of chlorophylls and pigments may suppress OMCs extraction efficiency in leaves case. In general, very low RSD values under 10% were found in the majority of the cases regardless the recovery value. Solely for loratadine in leaves it was obtained a

RSD out of the acceptable values. This demonstrates the repeatability of the method. All compounds presented good linearity with R^2 values higher than 0.991 and LOQs ranged from 0.01 to 2 ng g^{-1}; showing more than the 50% of the analytes low LOQs below 0.1 ng g^{-1} in both commodities. In spite of some OMCs such as clotrimazole, fenoprofen or sulfapyridine do not fulfill the acceptable criteria for validation; they were maintained in the method for qualitative purposes. Only those OMCs for which the method could be fully validated adopting the criteria aforementioned were quantified in real samples.

OMCs in Irrigation Water. An analysis of the irrigation water was carried out at the beginning of the study to obtain an overview of the potential exposure of the crops to the tested OMCs. As can be observed in Table S4, up to 51 OMCs could be identified at concentration values ranging from 15 to 14424 ng L^{-1}. The metabolites of dipyrone, 4-FAA and 4-AAA (14424 and 5396 ng L^{-1}, respectively), the diuretics hydrochlorothiazide and furosemide (2758 and 1694 ng L^{-1}, respectively), and the beta-blocker atenolol (1279 ng L^{-1}), were detected at the highest concentrations. It was expected that OMC concentrations in RW would vary throughout the study. However, overall these results are in line with previous monitoring studies carried out on urban WWTP effluents from Almeria[10,13] and can be considered as representative of the type/concentration of compounds usually present in the RW. The presence of 35 of these compounds has also been previously reported by our group in soil and soilless perlite substrate samples taken from the greenhouses monitored, which show average concentrations in the range 0.14 - 99 ng g^{-1}, d.w. (Table S4). Although the presence of OMCs in irrigation water and soils cannot be directly related to their occurrence in plant tissues due to the diverse factors involved in plant uptake, it can be assumed that their availability to be taken up by roots and translocate to edible parts is feasible when RW is used in irrigation.

Occurrence of OMCs in Tomato Plant Leaves. Greater knowledge about the occurrence of OMCs in vegetables irrigated with RW under field conditions is key to evaluating the quality of crops and determining potential consequences of reusing RW in agriculture irrigation. Moreover, the analysis of non-edible parts of the tomato crop, such as leaves, which may be used as sustenance for livestock feeding, is also important since it could represent another pathway for human exposure to OMCs. In this study, a total of 60 target compounds (Table S1) were monitored in real samples of tomato and tomato plant leaves to evaluate their distribution throughout the plant-fruit system.

The average concentration levels of OMCs found in leaf samples during the four sampling events are shown in Table 1. Up to 17 CECs were detected in leaves with average concentrations ranging from 0.04 to 32 ng g^{-1} wet weight (w.w.). The compounds that eventually reached the higher concentrations were the metabolites of dypirone, 4-AAA and 4-FAA (11 and 32 ng $g^{-1,}$ respectively), the anticonvulsant drug carbamazepine (8.9 ng g^{-1}), its metabolite carbamazepine epoxide (8.1 ng g^{-1}) and the antidepressant venlafaxine (4.0 ng g^{-1}). Regarding the frequency of detection, only 7 OMCs were found in all samples, namely caffeine, paraxanthine, carbamazepine, carbamazepine epoxide, hydrochlorothiazide, mepivacaine and venlafaxine; evidencing their higher capability of uptake and translocation within the plant. Nevertheless, their concentrations did not increase during the sampling period; a fact that could demonstrate stable accumulation despite constant irrigation with RW. Another group of OMCs were detected at very low concentrations (<LOQ) and/or showed low frequency of detection. This was the case for acetaminophen, antipyrin, diazepam, propranolol and the antibiotic trimethoprim.

In addition to the target analysis, samples were retrospectively analyzed by the acquired LC-QTOF-MS/MS sample information. The strategy allowed the identification of 3 other OMCs: flecainide, lidocaine and tramadol (Figures S1-S3). These compounds were also found in the irrigation water and soil samples (Table S4).[12] Almost all of them were identified in every sampling event, showing uptake from soil to leaf plant tissues. As the methodology could not be validated for these analytes, estimated concentration values had to be calculated (Table 1).

In general, no significant differences considering concentration levels were found between the different tomatoes produced in the greenhouses. This suggests there is no correlation between plant uptake and the tomato plant variety.

Table 1. Average OMC concentrations (ng g^{-1}, w.w.) quantified in tomato plant leaves

	SP1[a]				GH1[b]				GH2				GH3			
Compound	**S1[c]**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
4-AAA	<LOQ[d]	-	0.5	<LOQ	<LOQ	-	-	<LOQ	0.4	-	<LOQ	<LOQ	<LOQ	<LOQ	12	<LOQ
4-FAA	8	n.d.[e]	32	13	n.d.	n.d.	7	5	<LOQ	n.d.	4	3	<LOQ	3	4	2
Acetaminophen	n.d.	n.d.	<LOQ	2	n.d.	n.d.	n.d.	2	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	3
Antipyrine	<LOQ	<LOQ	1	<LOQ	<LOQ	<LOQ	0.7	n.d.	<LOQ	<LOQ	0.6	n.d.	<LOQ	<LOQ	<LOQ	n.d.
Caffeine	0.5	1	0.7	0.5	0.5	1	0.4	<LOQ	0.4	1	0.5	<LOQ	0.5	1	0.5	<LOQ
Carbamazepine	5	5	5	2	3	6	5	2	2	9	3	6	6	4	7	4
Carbamazepine epox	3	3	2	3	3	2	0.7	4	2	2	0.5	8	4	2	1	8
Flecainide[f]	n.d.	2	n.d.	0.9	n.d.	2	4	4	2	4	4	4	3	2	3	4
Diazepam	<LOQ	<LOQ	0.06	0.04	<LOQ	<LOQ	<LOQ	0.01	<LOQ	<LOQ	n.d.	<LOQ	<LOQ	n.d.	<LOQ	<LOQ
Hydrochlorothiazide	1	1	1	0.6	1	1	2	1	0.9	1	0.9	0.6	1	0.6	1	0.6
Lidocaine[f]	1	2	10	8	3	8	6	11	3	5	6	6	7	6	4	8
Mepivacaine	0.6	0.5	0.5	0.3	0.8	0.7	0.8	0.9	0.6	1	0.6	1	1	0.6	0.3	0.8
Paraxanthine	0.2	0.6	0.4	0.3	0.2	0.6	<LOQ	<LOQ	0.2	0.5	0.3	0.2	0.2	0.3	<LOQ	<LOQ
Propranolol	<LOQ	<LOQ	<LOQ	<LOQ	0.3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.4	<LOQ	<LOQ	<LOQ
Tramadol[f]	1	2	1	4	0.8	2	2	3	0.6	3	2	3	0.2	3	3	3
Trimethoprim	n.d.	n.d.	2	n.d.	n.d.	n.d.	0.7	n.d.	n.d.	n.d.	2	n.d.	n.d.	n.d.	n.d.	n.d.
Venlafaxine	2	1	2	0.7	2	2	2	3	2	4	3	4	4	2	2	4

[a]SP: soiless perlite culture; [b]GH: greenhouse; [c]S: sampling event; [d]<LOQ: concentration below the limit of quantification; [e]n.d.: not detected; [f]Estimated OMC concentrations quantified by LC-QTOF-MS/MS

Results obtained in the field study concerning translocation of selected OMCs via plant roots to other plant tissues, confirm previous results reported in studies under controlled conditions. For instance, Martínez-Piernas et al.[13] reported the accumulation of diverse analytes such as 4-AAA, 4-FAA, caffeine, carbamazepine, carbamazepine epoxide, hydrochlorothiazide, lincomycin, mepivacaine and venlafaxine, among others, in lettuce and leaves of radish when RW was used as irrigation water. Wu et al.[16] compared the concentrations found for a group of OMCs such as acetaminophen, caffeine, carbamazepine and diazepam in roots and leaves of lettuce, spinach, cucumber and pepper irrigated with spiked water. The metabolism and plant uptake of diazepam has also been evaluated by Carter et al.[17] in radish and silverbeet cultivated with spiked soil. The antibiotic trimethoprim has been reported by Dodgen et al.[18] as being translocated to lettuce, carrot and tomato leaves in an experiment carried out under controlled conditions of temperature and humidity. Other studies have investigated the impact of soil composition in OMCs' plant uptake in leafy crops when they were cultivated with spiked water, observing correlations between soil characteristics and root uptake.[19,20]

However, very few studies have analyzed real field samples exposed to OMCs. Wu et al.[2] described the translocation of caffeine and carbamazepine within the different plant organs in vegetables irrigated with RW and cultivated under field conditions. In addition, Riemenschneider et al.[5] observed the accumulation of caffeine, carbamazepine, carbamazepine epoxide and hydrochlorothiazide in different vegetables and agricultural plant tissues. In another study, levels of lincomycin were reported up to 20 µg kg^{-1} (d.w.) in leafy vegetables such as rape, celery and coriander grown in soil amended with manure.[28]

Occurrence of OMCs in Tomato Fruit. Concentrations of OMCs were found to a lesser extent in tomato fruits, these generally being 10 times lower in fruit compared to leaves (Figure 1). A total of 12 OMCs were detected in tomato samples. However, only 8 compounds could be quantified in at least one sample (Table 2). In general, the compounds that showed higher frequencies of detection and concentrations in leaves were also present in tomatoes, showing mobility through the plant transpiration stream up to fruits. The highest concentration was observed for caffeine (1.1 ng g^{-1}), followed by the metabolite 4-AAA (0.4 ng g^{-1}), then carbamazepine (0.23 ng g^{-1}), hydrochlorothiazide (0.15 ng g^{-1}), venlafaxine (0.15 ng g^{-1}), mepivacaine (0.09 ng g^{-1}) and carbamazepine epoxide (0.07 ng g^{-1}). 4-FAA, acetaminophen, acetanilide and paraxanthine were identified at concentrations below the LOQ in at least one sample.

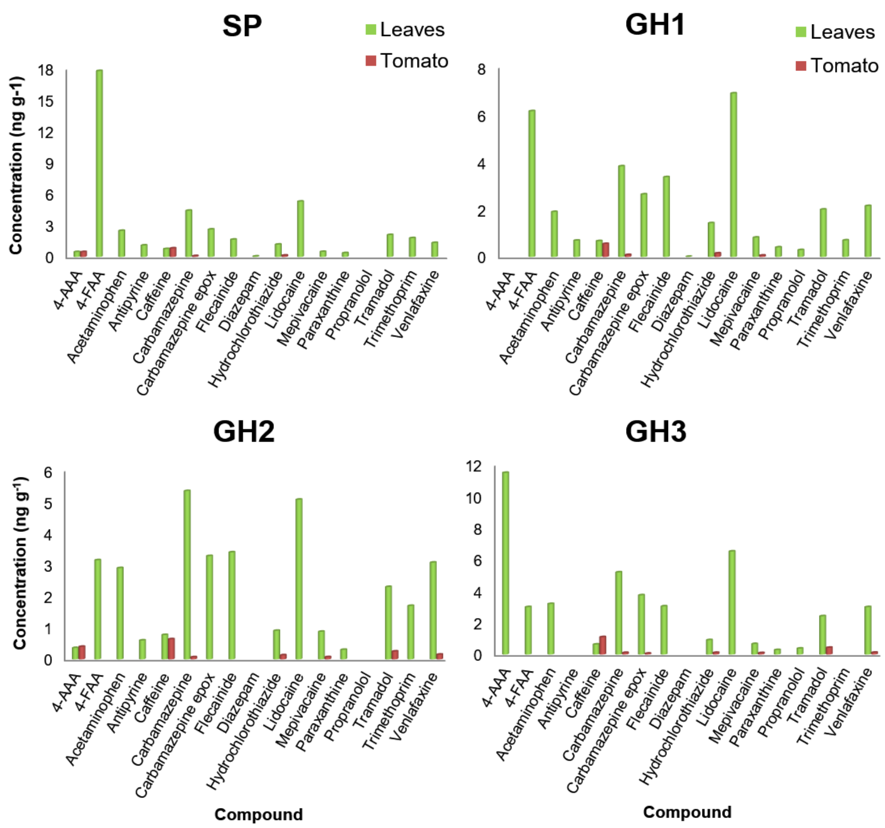

Figure 1. Average OMC concentrations found in leaves (green) and tomatoes (red) in each sampling site during the four sampling events.

The retrospective analysis of tomato fruit samples revealed the presence of a previously detected analyte in leaves by the same approach: tramadol (Figure S3). It is an opioid analgesic generally used for moderate and severe pain. Tramadol was found in tomatoes from two different greenhouses. Estimated concentrations of this compound are shown in Table 2.

No remarkable differences were found between the concentrations observed for cherry (SP1), ramyle (GH1, GH2) or retinto (GH3) tomato varieties. This fact evidences that despite the higher size of the last two types and the different agricultural practices (soilless culture for cherry and real soils for the rest), OMC accumulation was similar in all cases.

Table 2. Average OMC concentrations (ng g^{-1} , w.w.) quantified in tomato fruit samples

	SP1[a]				GH1[b]				GH2				GH3			
Compound	**S1[c]**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
4-AAA	-	<LOQ[d]	-	0.4	-	-	-	<LOQ	<LOQ	-	-	0.4	-	-	-	-
Caffeine	<LOQ	0.4	<LOQ	<LOQ	<LOQ	0.3	0.8	<LOQ	<LOQ	0.8	0.5	<LOQ	<LOQ	1	<LOQ	<LOQ
Carbamazepine	0.2	0.01	0.01	<LOQ	0.2	0.01	0.03	0.1	0.05	<LOQ	0.06	0.1	0.05	0.07	0.1	0.2
Carbamazepine epox	<LOQ	<LOQ	<LOQ	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.05	<LOQ	<LOQ	0.07
Hydrochlorothiazide	-	-	0.1	0.1	-	-	<LOQ	0.2	-	<LOQ	<LOQ	0.1	-	0.1	<LOQ	0.1
Mepivacaine	<LOQ	-	-	-	<LOQ	-	<LOQ	0.06	<LOQ	-	<LOQ	0.07	-	<LOQ	<LOQ	0.1
Tramadol [a]	-	-	-	-	-	-	-	-	-	0.2	-	-	-	0.1	-	0.7
Venlafaxine	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	<LOQ	<LOQ	<LOQ	0.1	<LOQ	<LOQ	<LOQ	<LOQ	0.1

[a]SP: soiless perlite culture; [b]GH: greenhouse; [c]S: sampling event; [d]<LOQ: concentration below the limit of quantification; [e]estimated OMC concentrations quantified by LC-QTOF-MS/MS

As was observed in leaves, caffeine and carbamazepine were detected in all greenhouses in every sampling event. Their plant uptake and translocation to the edible parts of vegetables is well described in literature.[2,13,29] Some studies have already reported them in tomato crops cultivated under field and controlled conditions.[5,21] Also metabolites such as carbamazepine epoxide have been identified in tomatoes when plants were irrigated with water spiked with carbamazepine under experimental conditions.[30] Hydrochlorothiazide has been reported in other vegetable tissues such as roots and leaves of parsley cultivated under field conditions[5] evidencing its high capability of translocation through the plant system. OMCs such as 4-AAA, 4-FAA, mepivacaine and venlafaxine, which were quantified in leaves, were also translocated to fruits and identified in certain sampling events. This group of OMCs has been found in the edible parts of lettuce and radish cultivated under controlled conditions submitted to RW irrigation.[13] Considering that 4-AAA and 4-FAA have exhibited toxicity,[31] it is important to monitor their occurrence and to evaluate their repercussions on human exposure.

To our knowledge, 4-AAA, mepivacaine, paraxanthine, tramadol and venlafaxine have not previously been identified either in plant tissues or edible parts of real field samples, which highlights the importance of applying wide-scope analytical methods for the evaluation of reuse of RWW in agriculture under different conditions and crops, and the potential of HRMS for the identification of non reported substances in environmental analysis. These results contribute to cover the gap of knowledge regarding the possible OMCs that can be present in edible parts of crops. This will help future studies dealing with the evaluation of the environmental and human risks associated with mixtures of analytes.

Accumulation in Plant Tissues and Properties of Compounds. It is well-known that OMCs' uptake by roots is accessible for those compounds that are dissolved in the solution of the soil pore water. In general, neutral and cationic species in the soil solution are susceptible to uptake by roots and subsequently translocate to the aboveground parts of plants by the transpiration stream.[16,20,32] On the other hand, anions are considered less transported to aerial parts due to their accumulation in cell roots by mechanisms such as ion-trapping.[32] The translocation of OMCs from roots to other plant organs is possible due to their capability of moving through the transpiration streams. This mobility depends on diverse analyte physical-chemical properties such as lipophilicity (K_{ow}), pK_a or the type of crop, among others.[3,33]

The results found in this study revealed that the OMC concentration values detected in tomato leaves were significantly higher (up to ten times in some cases, Table 1) than those found in tomatoes (Table 2). This behavior has been already reported in several studies.[2,13,20,33] This issue is explained by the greater water flow to leaves, leading to higher accumulation of OMCs in leafy parts than in fruits.

In Table S5, the diverse lipophilic coefficients (log K_{ow} for neutral compounds and log D_{ow} for ions), pK_a and molecular charge (soil pore solution pH = 7.5) of the OMCs identified in this work are shown. In general, moderate to strong bases ($pKa \geq 7$), in its cationic species or partially ionized (flecainide, hydrochlorothiazide, lidocaine, mepivacaine, propranolol, trimethoprim and venlafaxine); weak bases ($pK_a < 6$) in neutral form (4-AAA, 4-FAA, antipyrine, caffeine, carbamazepine, carbamazepine epoxide, diazepam and paraxanthine) and a very weak acid ($pK_a > 7.5$) in its neutral form (acetaminophen) were detected. The fact that these analytes are neutral or cations for a wide range of pH values explains their good distribution through the transpiration streams ($\sim 5.5 < pH < \sim 7.5$), being able to cross membranes, reaching leaves and fruits.[20] Although some compounds were partially ionized, they were translocated via the transpiration-derived mass flow subsequently being found in leaves, and in case of mepivacaine and hydrochlorothiazide, in both leaves and fruits. No OMC in anionic form was detected in either leaves or tomato. This is in accordance with the aforementioned reasons about the expected low translocation of anions through the vascular system, making its distribution less possible through plant streams.

As shown in Table S5, log K_{ow} and log D_{ow} of the OMCs identified, ranged from low to medium lipophilic values (-0.63 < log K_{ow}, D_{ow} < 3.08), demonstrating that the OMCs observed have different affinities to lipid tissues. According to Miller et al.[33] non-ionized compounds with -1 < log K_{ow} < 5 are expected to translocate from roots to other plant tissues, which is consistent with the results obtained for all the neutral compounds identified in this study (Table S5).

Human Exposure and Health-risk Assessment Analysis. Tomato is one of the most important crops around the world, with global production currently around 130 million tons, of which 88 million is destined for the fresh market and 42 million processed. Considering the intensive consumption of tomato worldwide, the evaluation of human OMC exposure when RW is used as irrigation is of particular interest, even more when this assessment focuses on real samples submitted to long-time RW irrigation.

In this study, an assessment of human exposure for each analyte quantified in samples was carried out by the estimation of the daily tomato consumption required to reach TTC levels in adults (average 70 kg) and toddlers (12 kg). All daily intakes were calculated taking into account the worst-case scenario possible. To this aim, a single sampling event with the highest value for TTC estimations was taking into account to provide the most conservative considerations out of this study.

Regarding toxicological effects, substances were classified as follows. ‘Class I’ for chemicals with simple structure and known metabolic pathways leading to innocuous end products showing a low order of oral toxicity. Class II contains substances that are intermediate. Very few compounds are included in this category, which is not very well characterized and even questionable.[34] They have less innocuous structures than those in Class I but they do not contain potentially toxic structural features. Class III contains substances with complex chemical structures that provide no strong initial presumption of safety and indeed may produce a significant toxicity effect, some of them being genotoxic compounds. Examples of Class III are a number of pharmaceuticals and other common used stimulants including, carbamazepine, caffeine, bezafibrate, clofibric acid, ketoprofen, naproxen, and metoprolol.[20] TTC levels of these pharmaceuticals typically reach values of around 1500 ng kg^{-1} b.w. day^{-1}, while the TTC for genotoxic chemicals is only 2.5 ng kg^{-1} b.w. day^{-1} or 0.15 µg $person^{-1}$ day^{-1}.[27,34] Nevertheless, it is important to remark that the consumption of a substance above the estimated TTC level would not imply that there is a toxicological risk. It may even point out a demand for specific toxicity analysis of the compound.

Some analytes quantified in tomato samples in this study are classified in Cramer Class III (4-AAA, caffeine, carbamazepine, hydrochlorothiazide and mepivacaine). Regular TTC values for these substances range from 1500 to 1800 ng kg^{-1} b.w. day^{-1}.[35] Venlafaxine and tramadol are categorized as chronic toxic, being their TTC value commonly set in 240 ng kg^{-1} b.w. day^{-1}.[36] Carbamazepine epoxide has potential genotoxic carcinogenicity. Therefore, TTC reported values are between 1.5 and 2.5 ng kg^{-1} b.w. day^{-1}.[37]

As can be observed in Table 3, the OMC concentrations found require an adult and toddler consumption of tens to hundreds of kg to reach the TTC values in most cases.

Considering a reasonable tomato daily consumption (according to FAP the average is 0.13 kg of tomatoes per adult per day,[38] depending on the dietary habits and country), these results do not bring along a health risk for the consumers.

As carbamazepine epoxide exhibit genotoxic carcinogenicity, it presented the lowest daily consumption of tomatoes per toddler and adult (400 g and 2.5 kg, respectively) to reach the TTC, despite its low concentration in the samples. These results are in agreement with the low amount intake of carrots to reach the estimated TTC reported by Malchi et al.[20]

The results of this presented study clearly indicate that the estimated TTC values do not pose a health risk for any of the substances at the concentrations found. This contributes towards the safe usage of RW for tomato irrigation under the conditions presented even when the worst conditions were taking into account.

Table 3. Health-risk assessment based on TTC levels of the OMCs quantified in tomato samples.

Sampling	**S1[a]**	**S2**	**S3**	**S4**
Maximum OMC concentration (ng g^{-1}, w.w.) detected in tomato samples				
4-AAA	<LOQ[b]	<LOQ	<LOQ	0.4
Caffeine	<LOQ	1	0.8	<LOQ
Carbamazepine	0.2	0.07	0.1	0.2
Carbamazepine epoxide	0.05	<LOQ	<LOQ	0.07
Hydrochlorothiazide	<LOQ	<LOQ	0.1	0.2
Mepivacaine	<LOQ	<LOQ	<LOQ	0.1
Tramadol	<LOQ	0.2	<LOQ	0.7
Venlafaxine	<LOQ	<LOQ	0.1	0.1
Daily consumption of tomatoes (kg) per adult (70 kg) to reach the TTC values				
4-AAA[c]	-	-	-	315
Caffeine[c]	-	114	150	-
Carbamazepine[c]	548	1800	1260	600
Carbamazepine epoxide[d]	3.5	-	-	2.5
Hydrochlorothiazide[c]	-	-	840	840
Mepivacaine[c]	-	-	-	1400
Tramadol[d]	-	67	-	22
Venlafaxine[d]	-	-	112	140
Daily consumption of tomatoes (kg) per toddler (12 kg) to reach the TTC values				
4-AAA[c]	-	-	-	54

Caffeine[c]	-	20	26	-
Carbamazepine[c]	94	309	216	103
Carbamazepine epoxide[d]	0.6	-	-	0.4
Hydrochlorothiazide[c]	-	-	144	144
Mepivacaine[c]	-	-	-	240
Tramadol[d]	-	67	-	4
Venlafaxine[d]	-	-	19	24

[a]S: sampling event; [b]<LOQ: concentration below the limit of quantification; [c]compound classified according to Munro *et al.* 1996;[35] [d]compound classified according to Houeto *et al.* 2012.[37]

Nevertheless, more studies are needed including the evaluation of exposure to other hazards such as synergistic effects due to the addition of concentrations, mixtures of compounds and the formation of metabolites and transformation products that may be more toxic than the original compound. More information about OMCs identified in real crop samples, agricultural procedures and the consideration of sensitive population groups, should also be evaluated to conclude that reuse of RW in agriculture is a safe approach.

ABBREVIATIONS USED

4-AAA: 4-acetyl-aminoantipyrine

4-FAA: 4-formyl-aminoantipyrine

APCI: Atmospheric pressure chemical ionization

ESI: Electrospray

GH: Greenhouse

IDA: Information dependent acquisition

LOQ: Limit of quantification

OMCs: Organic microcontaminants

PSA: Primary-secondary amine

RSD: Relative standard deviation

RW: Reclaimed water

SP: Soilless perlite culture

TTC: Toxicological threshold concern

ASSOCIATED CONTENT

Supporting Information

Information about experimental details: list of target analytes, LC-MS/MS details; analytical method validation in tomato fruit and leaves information, OMCs found in RW and agricultural soils, physico-chemical properties of compounds detected in samples and extracted ion chromatograms and MS/MS spectra of the identified compounds by suspect screening strategy (PDF).

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