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## Application of a fast and sensitive method for the determination of contaminants of emerging concern in wastewater using a quick, easy, cheap, effective, rugged and safe extraction and liquid chromatography coupled to mass spectrometry
### --Manuscript Draft--

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| **Abstract:** | The inefficiency of wastewater treatment plants (WWTPs) to remove contaminants of emerging concern (CECs) leads to their continuous release to the environment. Consequently, CECs are present at low concentrations in the treated wastewater (TWW), producing unpredicted and unwanted effects on living organisms as they are discharged into water receiving bodies. This work presents a fast and reliable method for the determination of CECs in treated wastewater (TWW) based on the innovative application of a QuEChERS (quick, easy, cheap, effective, rugged and safe) method for water extraction and determination by sensitive liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry (LC-QqLIT-MS/MS). The scope of the proposed QuEChERS-based method allows the monitoring of 107 CECs, including pharmaceuticals (58), antibiotics (16) and pesticides (33). The proposed method was successfully validated in urban TWW at two concentration levels (50 and 500 ng L $^{-1}$ ) and it is a feasible alternative to conventional and time-consuming solid-phase extraction (SPE) methodologies. 89% of the CECs presented mean recovery values in the 70-120% range with relative standard deviations (RSDs) always <20% (intra and inter-day precision), and limits of quantification (LOQs) in the range 5-500 ng L $^{-1}$ (89% of the compounds showed an LOQ ≤50 ng L $^{-1}$ ). The applicability of the method was demonstrated by the analysis of urban TWW samples (7 sampling events). In total, 35 CECs (23 pharmaceuticals, 2 antibiotics and 10 pesticides) were detected in the monitored samples with concentrations ranging from 5 to 677 ng L $^{-1}$ . |

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**Highlights**

- A QuEChERS-based method has been validated for the determination of 107 CECs in TWW.
- QuEChERS-based methods demonstrate their suitability to analyze CECs in wastewater.
- Recovery ranged from 70-120 % for 89% of the target CECs at 50 and 500 ng L$^{-1}$.
- LOQs were ≤50 ng L$^{-1}$ for 89% of the target CECs.
- Analysis of treated wastewater samples using the validated method (35 CECs detected).

1      **Application of a fast and sensitive** ~~QuEChERS-based extraction~~

2      **method for the determination of contaminants of emerging concern in**

3      **wastewater _using a quick, easy, cheap, effective, rugged and safe-based_**

4      **_extraction and_**~~by~~ **liquid chromatography coupled to mass spectrometry**

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## Abstract

The inefficiency of wastewater treatment plants (WWTPs) to remove contaminants of emerging concern (CECs) leads to their continuous release to the environment. Consequently, CECs are present at low concentrations in the treated wastewater (TWW), producing unpredicted and unwanted effects on living organisms as they are discharged into water receiving bodies. This work presents an ~~innovative~~ fast and reliable method for the determination of CECs in treated wastewater (TWW) based on the innovative application of a QuEChERS (quick, easy, cheap, effective, rugged and safe) ~~extraction~~ method for water extraction and determination by sensitive liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry (LC-QqLIT-MS/MS). The scope of the proposed QuEChERS-based method allows the monitoring of 107 CECs, including pharmaceuticals (58), antibiotics (16) and pesticides (33). The proposed method was successfully validated in urban TWW at two concentration levels (50 and 500 ng $L^{-1}$) and it is a feasible alternative to conventional and time-consuming solid-phase extraction (SPE) methodologies. 89% of the CECs presented mean recovery values in the 70-120 % range with relative standard deviations (RSDs) always <20 % (intra and inter-day precision), and limits of quantification (LOQs) in the range 5-500 ng $L^{-1}$ (89% of the compounds showed an LOQ ≤50 ng $L^{-1}$). The applicability of the method was demonstrated by the analysis of urban TWW samples (7 sampling events). In total, 35 CECs (23 pharmaceuticals, 2 antibiotics and 10 pesticides) were detected in the monitored samples with concentrations ranging from 5 to 677 ng $L^{-1}$.

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## Keywords

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## 1. Introduction

Contaminants of emerging concern (CECs) are chemical substances from anthropogenic origin present in the environment at trace and ultratrace levels ($\mu$g L$^{-1}$ - ng L$^{-1}$) [1]. CECs usually refer to a wide range of substances such as pesticides, pharmaceuticals, personal care products, flame retardants, hormones, antibiotic resistant bacteria and resistance genes (ARBs and ARGs), etc., being pharmaceuticals and pesticides the most frequently detected due to their widespread human use. CECs are continuously discharged into the environment mainly through wastewater treatment plant (WWTP) effluents since conventional wastewater treatments are not design to remove efficiently these compounds [2]. The presence of CECs in environmental compartments is a matter of current concern, mainly due to the undesirable ecological and toxicological effects that may cause on aquatic organisms as a consequence of their persistence in receiving water bodies [3]. Nevertheless, the monitoring for the adequate chemical status of environmental waters at the European context only focuses on a set of 45 priority substances (PS) according to the Directive 2013/39/EU [4]. Besides, 19 additional chemicals are included in the so-called Watch List according to the Decision recently published by the European Commission in 2020 for their possible future consideration as PS [5]. However, current research articles reflect the vast number of anthropogenic pollutants that can be detected in environmental waters [6,7] and for which the impact on aquatic ecosystems and humans is still unidentified. This means that current lists of PS and watch lists are under a constant update as a result as continuous wide-scope monitoring of CECs in water bodies.

The large number of CECs present in treated wastewaters (TWW) and their diverse physicochemical properties and concentrations point out the need of broad spectrum, comprehensive and sensitive analytical methodologies [6]. In general, many of them are target multi-residue methods with the aim of monitoring as many CECs as possible. It is well-known that liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) is the preferred technique for the analysis of CECs in environmental waters due to its high selectivity and sensitivity [8], as well as the relative medium-high polarity of this type of contaminants. Regarding sample preparation, the application of a wide variety of methodologies has been reported. However, solid-phase extraction (SPE) has been traditionally used for multi-residue analysis, due to advantages such as the preconcentration of the analytes [9]. Other reported extraction techniques include online SPE, solid-phase microextraction (SPME), multilayer SPE, the recently developed fabric-phase sorptive extraction (FSPE) and, in specific cases for the analysis of volatile compounds, the use of liquid-liquid extraction (LLE) [8,10]. Recently, the direct injection technique (DI) is gaining increasing attention as an alternative to reduce solvent consumption and analysis costs, but highly-sensitive MS instruments are required [11].

79    Nevertheless, the analysis of compounds such as antibiotics, which are pH-dependent, makes

80    difficult their inclusion in multiresidue analysis, since specific conditions are required for their

81    determination. For instance, several authors have reported the use of specific ion exchange or

82    graphitized carbon SPE cartridges alone or in tandem with HLB sorbent cartridges, or pH

83    adjustments prior to sample filtration/extraction, among others strategies [12]. Therefore,

84    robust, broad spectrum and versatile methodologies are still needed for sample extraction.

85    Alternatively, a strategy little explored so far for the determination of CECs in environmental

86    waters is the use of QuEChERS-based methods (acronymic name for quick, easy, cheap,

87    effective, rugged and safe). This method has its roots in the multiresidue analysis of polar and

88    non-polar pesticides in solid samples, such as fruits and vegetables [13]. Nowadays, it is the

89    most used extraction method for wide-scope analysis of pesticides in foodstuffs. ~~Basically, t~~The

90    QuEChERS method involves a solid-liquid extraction with an organic solvent, followed by the

91    induced liquid–liquid partitioning using an appropriate mixture of salts. Commonly, a

92    dispersive-solid phase extraction (d-SPE) is applied as clean-up. Due to its high versatility, it

93    has been recently and successfully applied to the extraction of CECs in solid environmental

94    commodities such as agricultural soils and crops irrigated with reclaimed water [14–17],

95    sediments, manure and sludge [18–20]. To our best knowledge, there are few works reporting

96    the use of QuEChERS-based protocols as sample extraction or as purification step for the

97    analysis of CECs in environmental waters. Thus, Kachhawaha et al. [21] analyzed 19

98    pharmaceuticals and personal care products in surface and sewage waters by LC-MS/MS using

99    an acetate salting-out buffer. Similarly, Abdel Ghani and Hanafi [22] reported a modified

100   QuEChERS method using also the acetate buffer for the analysis of 8 pesticides by GC-MS in

101   river and well water. Tsai et al. [23] studied the performance of different sorbents based on

102   silica and polymers using d-SPE for the extraction of 4 tetracycline antibiotics in surface water.

103   Łozowicka et al. [24] applied a QuEChERS procedure for the analysis of 28 pesticides,

104   including azoles and neonicotinoids, in agro-food industrial wastewater. Wang et al. [25]

105   optimized a d-SPE clean-up for the determination of 28 veterinary antibiotics in swine

106   wastewater from pig farms. None of the reported methods includes the analysis of a high

107   number of analytes nor the groups of CECs that are studied in the presented work.

108   The aim of this study was to optimize and validate a QuEChERS-based method for the

109   multiresidue determination of more than 100 CECs in TWW by LC-Qq~~Q~~-LIT-MS/MS,

110   including~~. The scope of the method included~~ compounds with different applications and

111   physic~~o~~-chemical properties such as~~:~~ 58 pharmaceuticals, ~~16~~ antibiotics and ~~33~~ pesticides. ~~The~~

112   ~~method was successfully validated in TWW and its applicability was demonstrated by its~~

113   ~~suitable performance on the monitoring of CECs in TWW samples.~~

114

115   **2. Materials and methods**

4

*2.1. Chemicals and apparatus*

A total of 107 target compounds (pharmaceuticals of various therapeutic classes, some of their metabolites, antibiotics and pesticides) were selected for this work. Analytical standards with purity ≥96% were purchased from Sigma-Aldrich (Steinheim, Germany) and Dr Ehrenstorfer (Augsburg, Germany). Formic acid (purity ≥98%), acetonitrile (ACN) and methanol (MeOH) LC-MS grade (99.9%) were purchased from Fluka (Buchs, Germany). Ultrapure water was produced using a Milli-Q water purification system from Millipore (Darmstadt, Germany). Stock solutions of each compound were prepared in ACN or MeOH at a minimum concentration of 1000 mg L$^{-1}$. Multi-compound working solutions were prepared in MeOH at 10 mg L$^{-1}$ (except for cefotaxime and cephalexin, which were prepared at a concentration 10 times higher due to their low sensitivity). A standard solution of $^{13}$C-caffeine (Sigma-Aldrich) in ACN was used as injection standard.

Salts used for QuEChERS extractions were: anhydrous MgSO$_4$ (purity ≥96%) and sodium phosphate dibasic dihydrate (Na$_2$HPO$_4$·2H$_2$O, purity ≥99%), supplied by Panreac (Castellar del Vallès, Barcelona, Spain); sodium chloride (NaCl, purity ≥99.9%) from JT Baker (Deventer, The Netherlands); trisodium citrate dihydrate (C$_6$H$_5$Na$_3$O$_7$.2H$_2$O, purity ≥98%), supplied by Fluka (Steinheim, Germany); sodium acetate trihydrate (C$_2$H$_3$NaO$_2$ · 3H$_2$O, purity ≥99.5%), acetic acid (purity ≥99%), citric acid monohydrate (purity ≥98%), disodium ethylenediaminetetraacetic acid (Na$_2$EDTA, purity ≥99%) and sodium citrate dibasic sesquihydrate (C$_6$H$_6$Na$_2$O$_7$·1.5H$_2$O, purity >99%), supplied by Sigma-Aldrich (Steinheim, Germany). The sorbents used for d-SPE were primary-secondary amine (PSA), C18, Supel QuE Z-Sep and Supel QuE Z-sep+, all purchased from Supelco (Bellefonte, PA, USA).

*2.2. Sample collection*

Real samples of TWW were collected from the effluent of the secondary treatment of El Toyo WWTP, Almería (36º51'30" N, 2º19'48" W). This plant treats wastewater produced by 52000 population equivalents. Due to its location, El Toyo WWTP receives water mainly from urban areas and from a hospital located nearby. The treatment line consists of a pretreatment (roughing filtration, desanding and degreasing), a primary treatment (primary decantation) and a biological treatment consisting of an extended aeration followed by a secondary decantation. TWW samples were taken at the end of the pipe of the treatment plant, after finishing the secondary treatment. In every sampling event, 1 L of sample was stored in pre-rinsed amber glass bottles and kept at -20°C until analysis (mainly 24 h after the sampling).

In order to evaluate the applicability of the developed method, 7 real samples were collected from the same WWTP in different days in March (1 sample), April (3 samples) and May (3 samples) of 2019. Raw samples (non-filtered) were then analyzed with the validated method.

153

### 2.3. Sample extraction and clean-up

A modified version of the QuEChERS procedure [17] was optimized and validated for the extraction of the target CECs in TWW (referred as Method 5 in Results and discussion section). 10 mL of TWW sample were put into a 50-mL polypropylene tube and 10 mL of ACN: MeOH (85:15, *v/v*) were added. The mixture was vortexed for 3 min and then, 4 g of $MgSO_4$, 1 g of NaCl, 1 g of citric acid monohydrate and 0.5 g of trisodium citrate dihydrate were added and vortexed for 3 min. ~~Afterwards~~Then, the sample was centrifuged at 3500 rpm for 10 min (P-select Mixtasel, JP Selecta, Barcelona, Spain). For the d-SPE clean-up, 5 mL of the supernatant were put into a 15-mL polypropylene tube containing 750 mg of $MgSO_4$ and 125 mg of Z-sep. The tube was vortexed for 45 s and centrifuged at 3500 rpm for 10 min (P-select Mixtasel-BL, JP Selecta). A 150-µL aliquot of the extract was transferred to a 350-uL glass insert in a screw-cap vial, evaporated under $N_2$ stream until dryness and reconstituted with 150 µL of $H_2O$:ACN (90:10, *v/v*), including the injection quality control standard ($^{13}C$-caffeine) at 1 µg/L, before LC injection. The concentration factor for the extraction method was one.

Other four methods (referred as Method 1, 2, 3 and 4 in Results and discussion section) were also tested as it can be seen in Figure 1. Briefly, the studied methods included the QuEChERS citrate EN 15662 [26], the QuEChERS AOAC [27]~~,~~; a QuEChERS using the McIlvaine buffer [28] and a modified QuEChERS citrate EN 15662 using EDTA [29].

### 2.4. LC-QqLIT-MS/MS analysis

The analysis of the selected CECs was carried out using an Agilent 1200 series HPLC system (Agilent Technologies, Foster City, CA, USA) coupled to a hybrid triple quadrupole-linear ion trap-mass spectrometer (5500 QTRAP® LC/MS/MS system, Sciex Instruments, Foster City, CA, USA). Chromatographic separation was carried out using a Kinetex C18 core-shell analytical column (150 x 4.6 mm, 2.6 µm particle size, Phenomenex, Torrance, CA, USA). The mobile phases consisted of (A) 0.1% of formic acid in Milli-Q water and (B) MeOH. The initial proportion of solvent B was 10%, which was kept constant for 0.5 min and then increased to 50% in 2.5 min, to 90% in 4 min and to 100% in 2.5 min. This composition was kept constant for 3.5 min and decreased to 10% in 0.1 min. The total analysis run time was 21.1 min. The injection volume was 10 µL and the flow rate was 0.~~5~~4 mL min$^{-1}$. The Turbo IonSpray interface operated in positive polarity in all cases (ESI+). The ionization source parameters were as follows: curtain gas, 25 (arbitrary units); CAD gas, high; ion spray voltage, 5000 V; temperature, 500 ºC; gas 1, 50 psi; gas 2, 50 psi. $N_2$ was used as the collision and nebulization gas. The mass spectrometer operated in multiple reaction monitoring (MRM) using the Scheduled MRM™ algorithm. The MRM method was set with a 40-s time window and a target scan time of 0.5 s~~, which performs the monitoring of each transition in a time window of ± 0.4~~

190  min of the retention time of each analyte. To confirm the presence of the analytes, two SRM

191  transitions must show the specified retention time (RT; defined by the RT of the calibration

192  standards with a tolerance of ±1 min) and the adequate SRM2/SRM1 ratio (tolerance ±30%).

193  The MS parameters applied for each compound were optimized and are presented in Table A.1.

194  Figure A.1 shows the total ion chromatogram (TIC) of the target compounds analyzed with the

195  optimized method. Analyst 1.6.2 software was used for data acquisition and MultiQuant 3.0.2

196  for data quantification.

197

198  *2.5. Analysis by LC-QTOF-MS*

199  The description of the conditions for LC-QTOF-MS analyses can be found in Supplementary

200  Information (Section A.1).

201

202  *2.6. Method validation*

203  The validation study was carried out using urban TWW. Due to the impossibility of obtaining

204  blank samples, they were previously analyzed and the signal of the present CECs was subtracted

205  for the calculations. Parameters evaluated in the validation study included: linearity, trueness (in

206  terms of recovery), intra-day and inter-day precision (expressed as relative standard deviation,

207  RSD), limits of quantification (LOQs) and matrix effect (ME). Method performance

208  acceptability criteria proposed by the SANTE Guidelines [30] were also adopted in our study.

209    Linearity was studied using matrix-matched calibration standards (TWW) prepared at five

210  concentration levels, ranging from 5 to 5000 ng L$^{-1}$ (10 times more for cephalexin and

211  cefotaxime). Satisfactory linearity was assumed when determination coefficients (R$^2$) were >

212  0.9900. ME was studied comparing the slope of the calibration curves prepared in pure solvent

213  (H$_2$O:ACN, 90:10, *v/v*) with the slope of the matrix-matched calibration curves, according to the

214  following equation: ME (%) = (Slope matrix extract curve/ Slope solvent curve - 1) x 100 [16].

215  Negative values of ME indicated signal suppression effect and positive ones, signal

216  enhancement. LOQs were calculated experimentally by adding decreasing concentrations of the

217  target CECs to matrix extracts, in a range from 0.1 to 50 ng L$^{-1}$. LOQs were considered when

218  the signal-to-noise (S/N) ratio of the quantification transition was ≥10. In case the contaminants

219  were present in the samples, LOQs were established as the minimum concentration that

220  produced a significant peak area in comparison with the non-enriched spiked sample extract

221  [11]. For trueness, recoveries at two concentration levels (50 and 500 ng L$^{-1}$, *n=5*) were

222  evaluated. Acceptable mean recoveries were considered in the range 70-120 %, with an

223  associated RSD ≤20%. In certain cases, recoveries in the range 60-130% could also be accepted

224  provided the RSD values are <20%. Method intra-day precision was calculated from the

225  recovery studies (*n=5*). For inter-day precision, RSD values were calculated by the extraction of

226  spiked samples at the previously indicated concentration levels for three consecutive days.

227 Quantification of CECs included in the analytical method was carried out by preparing matrix-
228 matched calibration curves in order to minimize ME.
229

230 **3. Results and discussion**

231

232 *3.1. Development of the LC-QqLIT-MS/MS method*
233 The target analyte list includes compounds showing a variety of physicochemical properties and
234 polarities. A C18 core-shell analytical column was used for the analyte separation, which allows
235 achieving a chromatographic resolution similar to that of a typical UHPLC system without a
236 significant increase in the working pressure. Our group applied successfully this column in a
237 previous study [31] with a gradient elution using 0.1% of formic acid in Milli-Q water and
238 MeOH. The use of MeOH as organic eluent permitted to reduce costs in routine analysis and
239 even though pressure in the LC system increases with the use of MeOH, the applied conditions
240 permitted to obtain an adequate chromatographic separation of the target analytes below the
241 maximum pressure (Figure A.1., Supplementary Material).
242 Regarding the optimization of the MS/MS conditions (Table A.1.), the majority of the target
243 compounds were already characterized in our laboratory [11]. For the additional compounds,
244 they were optimized to maximum sensitivity by direct infusion of individual solutions at 10 µg
245 $L^{-1}$ in MeOH. All the target analytes were monitored in ESI positive, and thus, no polarity
246 switching was needed. Other MS parameters, such as time window and target scan time, were
247 set according to previous experience in our lab for multiresidue methods with the aim of
248 obtaining adequate peak shape and sensitivity. MS/MS parameters and RT are listed in Table
249 A.1. (Supplementary Material). The final developed method showed a running time of 21 min,
250 which is a feasible time to increase sample throughput in view of its application in routine
251 monitorization of TWW.

252

253 *3.2.~~1~~ Optimization of the sample extraction procedure*
254 Five different QuEChERS-based protocols were tested in order to optimize the best extraction
255 conditions for the determination of the target CECs in TWW. Since one of the key parameters
256 that affect method efficiency is the pH of the extraction process, the protocols chosen differed in
257 the acidity of the extraction system. To vary this parameter, diverse combinations of buffer salts
258 were evaluated. The methodologies investigated are described in the Experimental section
259 (subsection 2.3).
260 The strongest acidic conditions were applied in Method 5 (pH 2), followed by Method 3 (pH
261 3.6), Method 2 (pH 4.8) and, finally, Methods 1 and 4 (pH 5-5.5). Besides, ACN is used as
262 extraction solvent in all the tested methods except in Method 5, which uses a 15% of MeOH. To
263 compare the efficiency of the extraction step in each condition tested, recoveries of spiked

TWW (500 ng L$^{-1}$, *n=5)* were evaluated using a common d-SPE clean-up step. It consisted of the addition of MgSO$_4$ (750 mg) and C18 (125 mg) and subsequent stirring, centrifugation and evaporation as described in Section 2.3. Figure 2 summarized the overall recovery results obtained for each protocol. Method 5 showed the highest number of compounds recovered in the range 60-130% (98 CECs, 92% of the analytes), closely followed by Methods 1, 4 and 3 (88, 87 and 81% of the compounds, respectively). In contrast, Method 2 presented the worst results (63 CECs, 59% of the analytes). The more acidic conditions of Method 5 are obtained by the replacement of the dibasic sodium citrate sesquihydrate of the conventional QuEChERS-citrate protocol by citric acid monohydrate, which in combination with trisodium citrate dehydrate, provides a more acidic pH buffer compared to the rest of the tested QuEChERS versions [17,26–29]. Overall, no significant pH effect on recoveries was found for the majority of the CECs included in the scope of the analysis, except for antibiotics, which generally present a pH-dependent performance and require specific extraction conditions. Table 1 shows the average recoveries obtained for the antibiotics included in this study. The recoveries provided by Method 5 showed improved results for certain antibiotics belonging to different therapeutical classes, such as tetracyclines (doxycycline), macrolides (erythromycin), lincosamides (lincomycin) and some sulfonamides (sulfadiazine and sulfamethazine). This points out the fact that more acidic extraction conditions than those generally applied in multiresidue QuEChERS official methods [26,27] can increase the recoveries of selected antibiotics. Moreover, this improvement is achieved without worsening method performance of less pH-dependent compounds. Thus, pH adjustment should be considered for the performance of antibiotic analyses compatible with multi-residue methods. However, it was not possible to establish a correlation between pH and the different pK$_a$ of the investigated antibiotics. There are likely other additional factors affecting recovery such as the co-extracted material from the matrix, which can be different depending on the applied pH and influence the final recovery of the compound. Apparently, the use of MeOH to increase the polarity of the extraction solvent (ACN:MeOH, 85:15, *v/v*) is beneficial for the extraction of the more polar CECs, such as antibiotics and other pharmaceuticals. Similarly, MeOH has been applied as QuEChERS extraction solvent for the analysis of highly polar pesticides in foodstuffs [32]. Concerning the use of EDTA-Na$_2$ as a chelating agent to prevent the formation of chelation complexes of tetracycline and fluoroquinolone antibiotics with multivalent cations present in the matrix [33], it has been successfully applied for the extraction of veterinary antibiotics in swine wastewater, using only d-SPE as sample purification step [25]. In the present study, this effect has been evaluated by comparing Method 4 and Method 1, which is equal but including EDTA. As expected, several compounds improved the recovery, namely clindamycin, doxycycline, flumequine, lincomycin, nalidixic acid, and the four sulfonamides analyzed. However, the results obtained with Method 5 were overall the best ones. This method shows the more acidic

301  extraction pH (pH=2), indicating that the pH effect seems to be more important than the
302  chelation effect to increase antibiotic recovery.

303  Few methodologies based on the QuEChERS method for the determination of CECs in
304  environmental waters are reported in literature. Among them, two studies used more acidic
305  extraction conditions than those indicated in the official protocols. For instance, Kachhawaha et
306  al. [21] reported a final extract with a pH around 3 using acetate buffer [27] for the analysis of
307  19 pharmaceuticals and personal care products in surface and sewage waters. However, pH was
308  increased up to 6.5 after the extraction to achieve adequate recoveries. Besides, Tsai et al. [23]
309  optimized a d-SPME strategy for the specific determination of tetracyclines in water samples,
310  using ACN as extraction solvent acidified with perchloric acid, which lead to an extraction pH =
311  2.7.

312  Considering the obtained results for the five tested methods, Method 5 was finally selected for
313  method validation: recoveries were favorable for a wide range of antibiotics and it showed the
314  highest number of extracted CECs.

315

316  *3.23. Optimization of the d-SPE clean-up*

317  Depending on the type of matrix and analytes, the performance of the d-SPE clean-up stage
318  usually applied in QuEChERS-based methods is optional. In this study, a clean-up stage was
319  included considering that TWW is a complex, non-homogeneous and variable matrix with the
320  aim of achieving an efficient qualitative and quantitative method performance. This is
321  particularly important for TWW from secondary treatments that may still contain high amount
322  of organic matrix. Different d-SPE sorbents were tested to remove co-extracted matrix
323  components: C18 removes non-polar substances; PSA eliminates fats, sugars, polar organic
324  acids and pigments; Z-sep (zirconium oxide) removes compounds with electron-donating
325  functional groups; and Z-sep+ (mixture of zirconium oxide (Z-sep) and C18) increases twofold
326  the interaction with matrix components [20]. In order to evaluate their efficiency in the
327  elimination of interferences as well as their effect in the analytes extraction, recoveries were
328  studied using Method 5 at a spiked concentration level of 500 ng L$^{-1}$ (*n=3*). Four different
329  adsorbent combinations were evaluated, which are detailed below (in all cases, 750 mg of
330  MgSO$_4$ were also added): i) 125 mg of C18; ii) 125 mg of C18 + 125 mg of PSA; iii) 125 mg of
331  Z-sep; and iv) 125 mg of Z-sep+. Figure 3 shows the recovery results of the different clean-ups
332  tested. As it can be observed, the highest number of CECs recovered in the range 60-130% was
333  achieved by using Z-sep (94% of the analytes); closely followed by the mixture C18+PSA
334  (93%), C18 (89%) and, finally, Z-sep+ (90%). The number of compounds with recoveries
335  below 60% was higher when using Z-sep+. Additionally, full-scan LC-MS analyses were
336  performed in order to determine the effect of the different clean-ups on the elimination of
337  interferences. Figure 4 shows the total ion chromatograms (TIC) obtained under all the

338      conditions tested. As can be seen, a large number of predominantly polar interferences are
339      eluted at the beginning of the chromatogram, which decreases before the elution of the first
340      target compound (ranitidine, 2.7 min, LC-QTOF-MS analysis conditions). Based on TIC
341      evidences, the cleanest extracts for the first eluting chromatographic peaks were obtained using
342      Z-sep and C18 + PSA, while Z-sep+ and C18 offered a less efficient elimination of polar
343      interferences. In the central region of the chromatogram, where most of the compounds elute,
344      the best results were obtained with C18 and Z-sep. Due to the better performance of Z-sep in
345      terms of trueness and its efficient removal of polar compounds, Z-sep was finally chosen as
346      optimal clean-up adsorbent together with $MgSO_4$ for method validation.

347

348      *3.~~3~~4. Method validation*
349      The proposed QuEChERS-based methodology was validated in terms of linearity, ME, accuracy
350      (expressed as trueness), inter and intra-day precision and LOQs for the simultaneous
351      determination of 107 CECs in TWW. Method validation results are presented in Table 2.

352

353      3.~~3~~4.1 Linearity, matrix effect, trueness and precision
354      Adequate linearity results were obtained using matrix-matched calibration curves with $R^2$
355      $\geq$0.9930 for the selected CECs. 93% of the analytes presented low ME (ME $\leq$20%, 62 CECs)
356      and medium (20% < ME < 50%, 39 CECs), whereas only 6 compounds showed a strong ME
357      (ME $\geq$50%). The most frequent ME observed was signal suppression (87 analytes) while
358      enhancement was produced by 20 CECs.
359      Accuracy (evaluated as trueness) and intra-day precision were assessed at two concentration
360      levels: 50 and 500 ng $L^{-1}$ (*n=5*), covering low and medium-high concentrations of the average
361      linearity range of the target compounds. In general, all CECs showed successful recoveries in
362      the range 70-120% with RSD values $\leq$20%, at least at one concentration level (Table 2). In
363      certain cases, recoveries in the range 60-70% and 120-130% were also accepted if the RSD
364      values were $\leq$20% [15,31]. Taking into account the large number of CECs under study and their
365      diverse physicochemical properties, recovery results were considered satisfactory. Only two
366      CECs presented recoveries higher than 120%: cetirizine (126%) and doxycycline (145%) at 500
367      ng $L^{-1}$ with adequate RSD values. Trazodone, cephalexin, erythromycin, josamycin and
368      terbutryn, showed recovery values in the range 56-69%, which were accepted because the RSD
369      values were $\leq$20%), except for terbutryn at 50 ng $L^{-1}$, with an RSD of 22%. Acetanilide, N-
370      desmethycitalopram, acetamiprid, boscalid and terbutryn showed RSD $\leq$25% (50 ng $L^{-1}$) while
371      flecainide and ratinidine, $\leq$24% (500 ng $L^{-1}$). Intra-day precision was evaluated at the same
372      spiking levels (*n=3*). The results indicated that 89% of the compounds (95 CECs) showed
373      adequate inter-day precision values at both levels, 90% at 50 ng $L^{-1}$ and 98% at 500 ng $L^{-1}$.
374      Some compounds such as the antibiotics clindamycin, doxycycline, erythromycin and

11

375 lincomycin, showed a higher inter-day RSD variability, especially at the lowest concentration
376 tested (50 ng L$^{-1}$).

377     Overall, recovery and precision results presented in this work are in line with those reported
378 by various authors using a QuEChERS approach. Kachhawaha et al. [21] managed recoveries in
379 the range 86-106% (referred to their lowest spiked level, 100 ng L$^{-1}$) for atenolol,
380 carbamazepine, erythromycin, fluoxetine, metoprolol, propranolol, trimethoprim and valsartan
381 in surface and sewage water. Tsai et al. [23] found a similar recovery value for doxycycline
382 (100.6%) at 10000 ng L$^{-1}$ in surface water. Wang et al. [25] reported comparable recoveries for
383 sulfadiazine, lincomycin, doxycycline, josamycin and trimethoprim (67.1-95.6%, lowest spiked
384 level, 1000 ng L$^{-1}$) in swine wastewater, although recoveries for sulfathiazole and roxithromycin
385 (<70% in both cases) were rather lower than those herein obtained (Table 2). Regarding
386 pesticides, Łozowicka et al. [24] presented equivalent recoveries in agroindustrial wastewater
387 for acetamiprid, propiconazole, tebuconazole and thiacloprid (78-92%, lowest spiked level,
388 10000 ng L$^{-1}$). It must be noticed that these studies evaluated recovery values at higher
389 concentrations than the studied in the present work (except the first study cited).

390     The recovery and precision results were also compared to other reported methods using
391 typical SPE for the determination of CECs in environmental waters. Wu et al. [34] analysed 70
392 CECs and reported comparable recovery results for the compounds in common; precision
393 results could not be compared since it only reports instrumental RSD percentage and not
394 method RSD including extraction. Gros et al. [35] described a method for the analysis of 81
395 CECs and reported a significant number of compounds out of the 70-120% range (22-150%) at
396 400 ng L$^{-1}$ (spiking level), but in general the results were very similar in terms of recovery and
397 RSD with some exceptions (erythromycin 137%, loratadine 130%, paroxetine 145%,
398 thiabendazole 22% or salbutamol 30%), where the present study provided better validation
399 results. The method by Papageorgiou et al. [36] reported comparable recoveries for 138 CECs
400 to the presented herein but at a much higher spiking level (5000 ng L$^{-1}$). Again, reported RSD
401 data referred to the instrumental performance, so further comparison of precision was not
402 possible. It is important to notice that any of the aforementioned SPE methods performed the
403 validation at low levels like the validated in the present work (50 ng L$^{-1}$). Recoveries of
404 pesticides applying the validated method were also in agreement with the SPE results of a
405 previous work reported by our group [31]. This evidences the capability of the proposed
406 QuEChERS-based method to extract efficiently a wide variety of CECs with different
407 physicochemical characteristics compared to traditional sample preparation approaches such as
408 SPE.

409

410 3.34.2 Limits of quantification (LOQs)

One of the main limitations in the determination of the experimental LOQs is the lack of real TWW blank samples for the majority of the compounds. This fact can lead to an overestimation of the LOQs for analytes occurring at high concentrations in the samples used as blank. In order to avoid the establishment of non-reliable LOQs, they were estimated as the lowest matrix-matched concentration which yielded a substantial increase on the chromatographic peak signal in comparison with the non-spiked sample extract for those CECs present in the blank sample, as previously reported [11]. From a conservative perspective, the use of this approach intended to provide realistic LOQs considering that they can hardly be experimentally tested on real blank samples, while keeping the sensitivity of the method at the lowest reliable levels. Obviously, the calculated LOQs are consequently an approximation, and thus, the quantitative results in real samples may sometimes be lower than the estimated values. Theses quantifications can only be accomplished as long as they meet the established identification/confirmation criteria. Experimental LOQs were determined by the analysis of matrix-matched standards in the range 5-500 ng $L^{-1}$, and 89% of the compounds showed an LOQ $\leq$50 ng $L^{-1}$ (Table 2), which demonstrates the high sensitivity of the method. Barely, 12 analytes (11%) showed LOQs between 100 and 500 ng $L^{-1}$. To our best knowledge, evidence of the low LOQs achieved and taking into account the different methodologies, commodities and instruments reported, this work provides the lowest LOQ levels when applying a QuEChERS-based method to the analysis of CECs in environmental waters [21,23–25]. When compared with traditional extraction techniques implying sample enrichment such as SPE, the LOQs obtained in this study were equivalent to those reported (for the pharmaceuticals and antibiotics in common) in the study by Wu et al. [34], with some LOQs lower for analytes such as lincomycin, salbutamol, fluoxetine, and metoprolol. Another study analyzing also a high number of CECs in TWW reported similar LOQs for many of the compounds in common and lower LOQs for certain antibiotics and pharmaceuticals (e.g. nadolol, cephalexin and erythromycin) [35]. An additional study [36] reported overall lower LOQs for some of the target CECs of the present work. In these reports, LOQs were calculated apparently by extrapolation at a S/N ratio of 3 and 10. It must be noticed that the procedure used in this work to estimate LOQs is quite conservative: only real concentrations were injected and extrapolation calculations based on blank injections were not applied so this can lead to higher but more realistic LOQs. In the case of pesticides, the LOQs reported in an SPE-based method specifically optimized for this type of CECs showed in general lower LOQs [31]. In this case, the SPE conditions were exclusive for pesticide analysis, not including any other CECs like in the present study. Obviously, multiresidue methods require applying compromise solutions, with less favorable extraction conditions for certain compounds or sub-classes.

In any case, the developed methodology provides sufficient sensitivity for the monitoring of the CECs considered as PS under the Directive 2013/39/EU [4] (atrazine, chlorfenvinphos,

448 chlorpyrifos, diuron, isoproturon, quinoxyfen, simazine and terbutryn). Their individual LOQs
449 are below the maximum allowable concentration for the Environmental Quality Standards
450 (MAC-EQS) in surface waters in all cases [4]. Similarly, the sensitivity of the method enables
451 the monitoring of the compounds included in the recently published European Watch List [5]
452 (imazalil, prochloraz, tebuconazole, trimethoprim and venlafaxine), for which the provided
453 LOQs are below the maximum acceptable method detection limits (MDL). Only for
454 clotrimazole, the estimated LOQ (100 ng L$^{-1}$) was higher than the maximum acceptable MDL
455 (20 ng L$^{-1}$). From the economic point of view, the present method shows a lower cost per
456 sample than traditional SPE. Considering the prices in our laboratory, the cost per sample of an
457 SPE analysis (solvent consumption + SPE cartridge ≈ 5 €/sample) is 2.5 times higher than the
458 cost per sample using the proposed QuEChERS method (solvent consumption + salts + d-SPE
459 sorbents + tubes ≈ 2 €/sample). This difference is mainly attributed to the cost of the SPE
460 cartridge.

461

462 *3.4̶5. Application to real samples*

463 The applicability of the validated method was demonstrated by the analysis of seven TWW
464 samples from El Toyo WWTP, collected from March to May 2019. Concentration values (ng L$^{-1}$)
465 for each compound can be found in Table 3. A total of 35 CECs were determined,
466 representing the 34% of the CECs included in the scope of analysis. Up to 23 pharmaceuticals,
467 2 antibiotics and 10 pesticides were detected at concentrations ranging from 5 ng L$^{-1}$
468 (lincomycin, antibiotic) to 677 ng L$^{-1}$ (cetirizine, antihistaminic). 21 CECs were present in all
469 the TWW samples, which demonstrates the necessity of broad-spectrum methodologies. As can
470 be observed in Table 3, the total load of the analyzed samples ranged from 1817 to 4909 ng L$^{-1}$.
471 CECs such as cetirizine (677 ng L$^{-1}$), flecainide (676 ng L$^{-1}$, boscalid (619 ng L$^{-1}$), venlafaxine
472 (382 ng L$^{-1}$), valsartan (365 ng L$^{-1}$), citalopram (235 ng L$^{-1}$), amisulpride (232 ng L$^{-1}$), diuron
473 (223 ng L$^{-1}$), N-desmethylcitalopram (142 ng L$^{-1}$) and carbamazepine (116 ng L$^{-1}$) showed the
474 highest concentrations. It is important to highlight the presence of chlorpyrifos and diuron that
475 are categorized as PS [4], as it can be seen in Figure 5. Among them, chlorpyrifos in sampling 2
476 was quantified at a concentration exceeding the MAC-EQS defined for surface waters, which is
477 100 ng L$^{-1}$ [4]. Regarding the compounds included in the Watch List [5], tebuconazole
478 (pesticide) and venlafaxine (antidepressant) were quantified (Figure 5) at concentrations in the
479 range 24-84 ng L$^{-1}$ and 172-382 ng L$^{-1}$, respectively.

480

481 **4. Conclusions**

482 In this work, a new approach using the ~~innovative multiresidue~~ QuEChERS~~-based~~ method~~ology~~
483 has been successfully optimized and validated for the multiresidue ~~simultaneous~~ determination
484 of 107 CECs (58 pharmaceuticals, 16 antibiotics and 33 pesticides) in TWW by LC-QqLIT-

485 MS/MS. During the optimization stage, pH was as a key parameter, especially for the

486 determination of pH-dependent compounds such as antibiotics. The lack of blank WW samples

487 lead to the application of conservative strategies for the estimation of LOQs, which results in

488 the definition of LOQs higher than the real ones if a real blank sample were available. However,

489 the concentrations lower than the estimated LOQs can be reported in real TWW samples

490 provided the identification/confirmation criteria are fulfilled. It was demonstrated that the

491 validated QuEChERS-based method is a fast, feasible and reliable procedure to determine CECs

492 in TWW samples, without requiring different sample pre-treatments despite the high number

493 and nature of the studied compounds. Method sensitivity and robustness enabled the

494 determination of PS and CECs included in the European Watch List for the monitoring of

495 surface waters. The proposed methodology implies the need of less sample volume: from 100-

496 100 mL in typical SPE methods to 10 mL in the proposed method. The reduction of costs in

497 terms of time (higher sample throughput) is significant as well as in terms of reagents and cost

498 per sample. The optimized method could be applied to other environmental waters such as

499 surface waters or drinking water. Further developments of the proposed method would include

500 more investigation  in terms of sample concentration for waters with occurring CECs at lower

501 concentrations than in TWW (to increase concentration factor when needed) and miniaturization

502 strategies (to reduce costs in the d-SPE stage).

503

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510

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661

**Table 1.** Recovery (*n=5*) and RSD values (%) obtained at a spiking level of 500 ng L$^{-1}$ for the target antibiotics with each protocol tested (d-SPE clean-up with MgSO$_4$+C18 in all cases).

| Compound | Recovery % (RSD, %) | | | | |
|---|---|---|---|---|---|
| | Method 1[a] (pH 5-5.5) | Method 2[b] (pH 4.8) | Method 3[c] (pH 3.6) | Method 4[d] (pH 5-5.5) | Method 5[e] (pH 2) |
| Cefotaxime | 86 (2) | 56 (19) | 25 (17) | 122 (13) | 77 (12) |
| Cephalexin | 94 (9) | NA[f] (-) | 12 (6) | 78 (13) | NA (-) |
| Clindamycin | 53 (4) | 75 (7) | 44 (9) | 94 (6) | 96 (10) |
| Doxycycline | 18 (50) | NR[g] (-) | NR (-) | 36 (23) | 140 (1) |
| Erythromycin | NR (-) | 18 (40) | 53 (40) | NR (-) | 71 (17) |
| Flumequine | 82 (3) | 44 (58) | NR (-) | 97 (5) | 126 (6) |
| Josamycin | 70 (6) | NR (-) | 90 (1) | 74 (20) | 58 (6) |
| Lincomycin | 28 (10) | 50 (6) | 21 (17) | 62 (11) | 92 (5) |
| Metronidazole | 91 (2) | 77 (7) | 111 (7) | 88 (7) | 84 (1) |
| Nalidixic acid | 71 (4) | 39 (56) | NR (-) | 94 (8) | 140 (4) |
| Roxithromycin | 74 (3) | 55 (29) | 95 (3) | 42 (30) | 77 (2) |
| Sulfadiazine | 15 (11) | NR (-) | 125 (25) | 62 (30) | 97 (10) |
| Sulfamethazine | 22 (11) | 63 (23) | 118 (20) | 88 (36) | 92 (6) |
| Sulfamethizole | 19 (8) | 63 (26) | 94 (20) | 65 (33) | 98 (14) |
| Sulfathiazole | 20 (19) | 64 (30) | 83 (16) | 64 (29) | 84 (3) |
| Trimethoprim | 103 (9) | 56 (10) | 97 (8) | 89 (13) | 98 (4) |

[a]QuEChERS citrate; [b]QuEChERS AOAC; [c]QuEChERS McIlvaine; [d]QuEChERS citrate + EDTA; [e] acidified QuEChERS citrate; [f]NA: Data ~~N~~not available; [g]NR: not recovered.

**Table 2.** Validation results obtained for the target CECs in TWW with the optimized QuEChERS method at two concentration levels (50 and 500 ng L$^{-1}$).

| | Linearity (R$^2$) | Range$^a$ (ng L$^{-1}$) | ME$^b$ (%) | LOQ$^c$ (ng L$^{-1}$) | 50 ng L$^{-1}$ (n=5) Recovery (%) | RSD$^d$ (%) | 500 ng L$^{-1}$ (n=5) Recovery (%) | RSD$^d$ (%) | Inter-day precision (RSD, %)$^e$ 50 ng L$^{-1}$ | 500 ng L$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **Pharmaceuticals** | | | | | | | | | | |
| Acetanilide | 0.9997 | 50-1000 | 17 | 50 | 73 | 21 | 96 | 7 | 19 | 1 |
| Acridone | 0.9997 | 50-5000 | 17 | 50 | 102 | 4 | 105 | 6 | 5 | 2 |
| Alfuzosin | 0.9999 | 10-5000 | -15 | 10 | 108 | 13 | 111 | 7 | 14 | 6 |
| Amisulpride | 0.9996 | 5-1000 | -20 | 5 | 81 | 11 | 93 | 8 | 13 | 4 |
| Amitriptyline | 0.9983 | 50-5000 | -12 | 50 | 107 | 12 | 103 | 7 | 11 | 9 |
| Atenolol | 0.9996 | 10-1000 | 11 | 10 | 108 | 6 | 91 | 6 | 13 | 6 |
| Betamethasone | 0.999 | 10-1000 | -16 | 10 | 88 | 15 | 95 | 6 | 11 | 4 |
| Carbamazepine | 0.9991 | 5-1000 | -23 | 5 | 104 | 18 | 83 | 9 | 10 | 7 |
| Cetirizine | 0.9998 | 5-1000 | 8 | 5 | 105 | 11 | 126 | 7 | 11 | 12 |
| Citalopram | 0.9978 | 10-500 | -23 | 10 | 82 | 18 | 106 | 6 | 13 | 4 |
| Clomipramine | 0.9999 | 50-5000 | -18 | 50 | 86 | 12 | 107 | 3 | 10 | 7 |
| Clotrimazole | 0.9998 | 100-5000 | -4 | 100 | < LOQ$^f$ | - | 98 | 1 | < LOQ | 1 |
| Cyclophosphamide | 0.9973 | 50-1000 | -28 | 50 | 86 | 7 | 107 | 3 | 11 | 6 |
| Diazepam | 0.9982 | 10-1000 | -30 | 10 | 85 | 10 | 100 | 8 | 15 | 6 |
| Diphenhydramine | 0.9984 | 10-1000 | -17 | 10 | 101 | 3 | 88 | 6 | 5 | 6 |
| Domperidone | 0.9989 | 100-5000 | 14 | 100 | < LOQ | - | 105 | 8 | < LOQ | 11 |
| Donepezil | 0.9939 | 50-1000 | -21 | 50 | 85 | 11 | 112 | 6 | 3 | 7 |
| EDDP$^g$ | 0.9997 | 50-5000 | -10 | 50 | 103 | 8 | 91 | 5 | 6 | 5 |
| Famotidine | 0.9981 | 10-5000 | -25 | 10 | 83 | 12 | 79 | 17 | 13 | 3 |
| Fenofibrate | 0.9986 | 10-5000 | -39 | 10 | 95 | 3 | 102 | 5 | 3 | 3 |
| Fenofibric acid | 0.9994 | 10-5000 | -24 | 10 | 99 | 20 | 96 | 7 | 17 | 10 |
| Flecainide | 0.9939 | 5-1000 | -15 | 5 | 105 | 19 | 104 | 24 | 5 | 5 |
| Fluoxetine | 0.9998 | 5-5000 | -28 | 5 | 107 | 10 | 93 | 6 | 6 | 5 |
| Ifosfamide | 0.9997 | 50-1000 | -47 | 50 | 85 | 10 | 89 | 6 | 9 | 10 |
| Iminostilbene | 0.9995 | 50-5000 | -17 | 50 | 85 | 6 | 98 | 9 | 18 | 3 |
| Indomethacin | 0.9991 | 5-1000 | -16 | 5 | 101 | 7 | 89 | 4 | 9 | 6 |
| Labetalol | 0.9998 | 50-1000 | -5 | 50 | 95 | 9 | 103 | 4 | 8 | 4 |
| Lidocaine | 0.9999 | 5-1000 | -8 | 5 | 95 | 16 | 105 | 6 | 17 | 5 |
| Loratadine | 0.9999 | 10-1000 | -2 | 10 | 104 | 7 | 107 | 5 | 12 | 9 |
| Mefenamic acid | 0.9998 | 5-1000 | -14 | 5 | 112 | 9 | 114 | 2 | 14 | 15 |
| Memantine | 0.9997 | 5-1000 | -31 | 5 | 108 | 6 | 89 | 5 | 12 | 7 |
| Mepivacaine | 0.9999 | 5-1000 | -21 | 5 | 96 | 3 | 101 | 4 | 6 | 3 |
| Methadone | 0.9984 | 10-1000 | -4 | 10 | 90 | 7 | 102 | 3 | 2 | 9 |
| Metoclopramide | 0.9999 | 50-5000 | -17 | 50 | 100 | 9 | 98 | 3 | 11 | 1 |
| Metoprolol | 0.9997 | 10-1000 | -28 | 10 | 101 | 1 | 93 | 6 | 1 | 4 |
| Mevastatin | 0.9996 | 50-1000 | -59 | 50 | 95 | 17 | 95 | 6 | 14 | 12 |
| N-Desmethylcitalopram | 0.9999 | 10-1000 | -44 | 10 | 102 | 22 | 100 | 4 | 5 | 3 |
| Nadolol | 0.9994 | 100-5000 | -32 | 100 | < LOQ | - | 82 | 13 | < LOQ | 11 |
| Niflumic acid | 0.9997 | 5-1000 | 9 | 5 | 105 | 5 | 100 | 2 | 12 | 3 |
| Nitrendipine | 0.9997 | 500-5000 | -27 | 500 | < LOQ | - | 97 | 5 | < LOQ | 3 |

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| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Paroxetine | 0.9988 | 100-5000 | -24 | 100 | < LOQ | - | 98 | 10 | < LOQ | 2 |
| Pentoxifylline | 0.9998 | 5-1000 | -26 | 5 | 88 | 11 | 96 | 8 | 8 | 2 |
| Primidone | 0.9997 | 100-5000 | -43 | 100 | < LOQ | - | 104 | 8 | < LOQ | 7 |
| Propranolol | 0.9999 | 50-5000 | -22 | 50 | 110 | 7 | 104 | 3 | 19 | 6 |
| Propafenone | 0.9995 | 5-5000 | -18 | 5 | 87 | 8 | 90 | 4 | 5 | 6 |
| Propyphenazone | 0.9999 | 50-5000 | -2 | 50 | 97 | 9 | 93 | 9 | 2 | 3 |
| Ranitidine | 0.9999 | 50-5000 | -44 | 50 | 111 | 8 | 71 | 22 | 3 | 12 |
| Salbutamol | 0.9997 | 10-5000 | -11 | 10 | 86 | 5 | 95 | 7 | 3 | 1 |
| Simvastatin | 0.9997 | 50-1000 | -57 | 50 | 87 | 7 | 89 | 5 | 5 | 7 |
| Sotalol | 0.9977 | 10-1000 | 2 | 10 | 97 | 9 | 102 | 10 | 12 | 6 |
| Tamoxifen | 0.9999 | 50-1000 | -8 | 50 | 94 | 7 | 98 | 4 | 21 | 10 |
| Terbutaline | 0.9995 | 50-1000 | 13 | 50 | 99 | 5 | 93 | 6 | 10 | 10 |
| Tramadol-N-oxide | 0.9999 | 5-1000 | -13 | 5 | 102 | 6 | 96 | 7 | 5 | 5 |
| Trazodone | 0.9999 | 10-1000 | -21 | 10 | 66 | 18 | 83 | 9 | 13 | 8 |
| Triamterene | 0.9981 | 100-1000 | -39 | 100 | < LOQ | - | 94 | 7 | < LOQ | 7 |
| Valsartan | 0.9968 | 10-5000 | -25 | 10 | 81 | 7 | 74 | 8 | 12 | 17 |
| Venlafaxine | 0.9999 | 5-1000 | -14 | 5 | 111 | 5 | 74 | 7 | 22 | 13 |
| Verapamil | 0.9988 | 10-5000 | -3 | 10 | 98 | 6 | 87 | 4 | 6 | 7 |
| **Antibiotics** | | | | | | | | | | |
| Cefotaxime[h] | 0.9977 | 500-50000 | 4 | 500 | 86 | 14 | 103 | 7 | 20 | 12 |
| Cephalexin[h] | 0.9997 | 500-50000 | -57 | 500 | 112 | 8 | 60 | 19 | 15 | 5 |
| Clindamycin | 0.9987 | 5-1000 | -5 | 5 | 97 | 5 | 94 | 8 | 26 | 4 |
| Doxycycline | 0.9999 | 50-5000 | -10 | 50 | 105 | 13 | 145 | 6 | 25 | 43 |
| Erythromycin | 0.9981 | 500-5000 | -58 | 500 | < LOQ | - | 61 | 20 | < LOQ | 28 |
| Flumequine | 0.9999 | 50-1000 | -15 | 50 | 90 | 12 | 82 | 3 | 18 | 14 |
| Josamycin | 0.9999 | 10-5000 | -28 | 10 | 56 | 7 | 74 | 11 | 24 | 5 |
| Lincomycin | 0.9997 | 5-1000 | 21 | 5 | 79 | 9 | 78 | 19 | 30 | 5 |
| Metronidazole | 0.9997 | 50-5000 | -36 | 50 | 97 | 10 | 91 | 10 | 22 | 6 |
| Nalidixic acid | 0.9998 | 10-1000 | 18 | 10 | 96 | 11 | 97 | 1 | 16 | 2 |
| Roxithromycin | 0.9990 | 5-5000 | -75 | 5 | 77 | 15 | 120 | 13 | 10 | 17 |
| Sulfadiazine | 0.9997 | 10-1000 | -20 | 10 | 101 | 8 | 87 | 13 | 5 | 10 |
| Sulfamethazine | 0.9996 | 50-500 | -6 | 50 | 85 | 11 | 91 | 10 | 22 | 6 |
| Sulfamethizole | 0.9996 | 5-1000 | -37 | 5 | 92 | 8 | 86 | 6 | 9 | 11 |
| Sulfathiazole | 0.9999 | 50-1000 | -22 | 50 | 96 | 6 | 83 | 9 | 11 | 16 |
| Trimethoprim | 0.9999 | 50-1000 | -28 | 50 | 90 | 12 | 78 | 11 | 18 | 14 |
| **Pesticides** | | | | | | | | | | |
| Acetamiprid | 0.9994 | 5-1000 | -52 | 5 | 80 | 21 | 109 | 7 | 17 | 6 |
| Atrazine | 0.9989 | 5-1000 | 8 | 5 | 89 | 12 | 98 | 5 | 6 | 2 |
| Azoxystrobin | 0.9999 | 5-1000 | -17 | 5 | 97 | 7 | 96 | 5 | 1 | 2 |
| Boscalid | 0.9982 | 10-5000 | -30 | 10 | 91 | 25 | 106 | 3 | 14 | 5 |
| Buprofezin | 0.9984 | 5-1000 | -8 | 5 | 87 | 9 | 99 | 3 | 6 | 3 |
| Carbendazim | 0.9988 | 50-1000 | 14 | 50 | 117 | 10 | 91 | 7 | 14 | 7 |
| Chlorfenvinphos | 0.9996 | 50-1000 | -19 | 50 | 80 | 12 | 99 | 5 | 15 | 2 |
| Chlorpyrifos | 0.9991 | 5-1000 | -47 | 5 | 97 | 8 | 105 | 4 | 9 | 2 |
| Cyantraniliprole | 0.9996 | 5-1000 | -22 | 5 | 95 | 9 | 85 | 14 | 1 | 13 |
| Cyprodinil | 0.9999 | 100-5000 | 18 | 100 | < LOQ | - | 97 | 3 | < LOQ | 2 |
| Dimethoate | 0.9993 | 50-5000 | -39 | 50 | 86 | 7 | 103 | 2 | 15 | 2 |
| Dimethomorph | 0.9996 | 50-5000 | -24 | 50 | 89 | 7 | 90 | 4 | 7 | 6 |
| Diuron | 0.9995 | 5-1000 | -13 | 5 | 81 | 2 | 100 | 3 | 11 | 6 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fenhexamid | 0.9988 | 10-1000 | -15 | 10 | 85 | 14 | 88 | 3 | 15 | 8 |
| Imazalil | 0.9987 | 100-5000 | -11 | 100 | < LOQ | - | 101 | 2 | < LOQ | 7 |
| Isoproturon | 0.9996 | 50-1000 | -3 | 50 | 96 | 16 | 109 | 4 | 9 | 7 |
| Metalaxyl | 0.9979 | 10-1000 | -23 | 10 | 96 | 4 | 98 | 4 | 2 | 6 |
| Methiocarb | 0.9995 | 50-5000 | -19 | 50 | 98 | 5 | 90 | 4 | 12 | 14 |
| Myclobutanil | 0.9994 | 50-1000 | -12 | 50 | 103 | 4 | 103 | 3 | 4 | 1 |
| Oxadiazon | 0.9999 | 10-5000 | -37 | 10 | 103 | 4 | 103 | 2 | 3 | 1 |
| Pirimicarb | 0.9998 | 10-1000 | 0 | 10 | 102 | 5 | 96 | 6 | 12 | 2 |
| Prochloraz | 0.9992 | 5-1000 | 4 | 5 | 91 | 3 | 101 | 3 | 6 | 6 |
| Propamocarb | 0.9996 | 10-5000 | -6 | 10 | 91 | 5 | 99 | 2 | 11 | 6 |
| Propiconazole | 0.9997 | 10-5000 | 4 | 10 | 101 | 9 | 101 | 3 | 10 | 2 |
| Pyrimethanil | 0.9985 | 5-1000 | -1 | 5 | 105 | 6 | 102 | 4 | 5 | 1 |
| Quinmerac | 0.9993 | 50-1000 | -12 | 50 | 94 | 5 | 93 | 7 | 3 | 5 |
| Quinoxyfen | 0.9999 | 5-5000 | -1 | 5 | 112 | 10 | 104 | 1 | 16 | 8 |
| Simazine | 0.9995 | 50-1000 | 10 | 50 | 106 | 4 | 92 | 6 | 12 | 6 |
| Spirotetramat | 0.9999 | 5-1000 | -24 | 5 | 110 | 7 | 105 | 3 | 9 | 4 |
| Tebuconazole | 0.9995 | 10-1000 | -8 | 10 | 85 | 17 | 96 | 4 | 6 | 2 |
| Terbutryn | 0.9989 | 5-1000 | 7 | 5 | 69 | 22 | 90 | 5 | 20 | 6 |
| Thiacloprid | 0.9999 | 50-1000 | -43 | 50 | 100 | 6 | 91 | 6 | 14 | 6 |
| Thiabendazole | 0.9956 | 10-1000 | 7 | 10 | 79 | 8 | 102 | 6 | 23 | 2 |

[a]LR: linear range; [b]ME: matrix effect; [c]LOQ: limit of quantification; [d]Intra-day precision; [e]$n=3$; [f]Detected at a concentration <LOQ; [g]EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; [h]Compound evaluated at a concentration 10 times higher than the expressed in the Table heading (500 and 5000 ng/L) because of its low sensitivity.

**Table 3.** Results of the real TWW samples analysed using the validated QuEChERS-based developed method.

| Compound | Concentration (ng L$^{-1}$) | | | | | | | Mean ± SD[b] | FD[c] (%) |
|---|---|---|---|---|---|---|---|---|---|
| | S1[a] | S2 | S3 | S4 | S5 | S6 | S7 | | |
| **Pharmaceuticals** | | | | | | | | | |
| Acridone | 53 | 119 | 68 | 91 | < LOQ[d] | 51 | < LOQ | 251 ± 429 | 71 |
| Amisulpride | 82 | 222 | 37 | 20 | 39 | 64 | 232 | 99 ± 89 | 100 |
| Atenolol | < LOQ | 54 | 48 | 78 | 35 | 56 | 80 | 59 ± 18 | 86 |
| Carbamazepine | 32 | 77 | 76 | 109 | 57 | 100 | 116 | 81 ± 30 | 100 |
| Cetirizine | 191 | 424 | 408 | 575 | 292 | 578 | 677 | 449 ± 172 | 100 |
| Citalopram | 82 | 178 | 163 | 187 | 130 | 192 | 235 | 167 ± 49 | 100 |
| Diphenhydramine | 24 | 43 | 40 | 45 | 34 | 36 | 35 | 37 ± 7 | 100 |
| EDDP[e] | < LOQ | 75 | 83 | 94 | 61 | 80 | < LOQ | 79 ± 12 | 71 |
| Fenofibric acid | 22 | 49 | 28 | 19 | < LOQ | < LOQ | 17 | 27 ± 13 | 71 |
| Flecainide | 36 | 269 | 141 | 305 | 319 | 425 | 676 | 310 ± 205 | 100 |
| Indomethacin | <LOQ | 32 | 26 | 34 | 22 | 26 | 22 | 27 ± 5 | 86 |
| Lidocaine | 57 | 114 | 110 | 129 | 84 | 112 | 123 | 104 ± 25 | 100 |
| Memantine | 32 | 109 | 54 | 89 | 67 | 90 | 120 | 80 ± 31 | 100 |
| Mepivacaine | 12 | 26 | 19 | 23 | 10 | 30 | 21 | 20 ± 7 | 100 |
| Methadone | 32 | 61 | 60 | 69 | 53 | 51 | 52 | 54 ± 12 | 100 |
| N-Desmethylcitalopram | 87 | 142 | 112 | 135 | 105 | 117 | 85 | 112 ± 22 | 100 |
| Propanolol | <LOQ | <LOQ | <LOQ | 54 | <LOQ | <LOQ | <LOQ | 54 | 14 |
| Propafenone | 10 | 14 | 12 | 12 | 10 | 13 | 7 | 11 ± 2 | 100 |
| Ranitidine | 72 | 133 | 99 | 128 | <LOQ | 95 | 105 | 105 ± 23 | 86 |
| Sotalol | 42 | 83 | 67 | 83 | <LOQ | 48 | 73 | 66 ± 17 | 86 |
| Trazodone | 27 | 47 | 44 | 50 | 38 | 45 | 58 | 44 ± 10 | 100 |
| Valsartan | 158 | 302 | 365 | 340 | 260 | 287 | 222 | 276 ± 71 | 100 |
| Venlafaxine | 172 | 382 | 324 | 375 | 244 | 308 | 269 | 296 ± 75 | 100 |
| **Antibiotics** | | | | | | | | | |
| Clindamycin | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 30 | 30 | 14 |
| Lincomycin | <LOQ | <LOQ | <LOQ | <LOQ | 129 | <LOQ | 5 | 67 ± 88 | 29 |
| **Pesticides** | | | | | | | | | |
| Acetamiprid | <LOQ | 80 | 62 | 72 | 59 | 143 | 10 | 71 ± 43 | 86 |
| Azoxystrobin | 38 | 70 | 41 | 53 | 42 | 36 | 13 | 42 ± 17 | 100 |
| Boscalid | 261 | 619 | 212 | 274 | 245 | 194 | 148 | 279 ± 156 | 100 |
| Carbendazim | <LOQ | 106 | <LOQ | <LOQ | 63 | 120 | 64 | 88 ± 29 | 57 |
| Chlorpyriphos | 97 | 111 | 58 | 50 | 53 | 53 | 57 | 68 ± 25 | 100 |
| Diuron | 95 | 223 | 59 | 105 | 109 | 76 | 174 | 120 ± 58 | 100 |
| Fenhexamid | <LOQ | 80 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 80 | 14 |
| Propiconazole | 15 | 30 | <LOQ | <LOQ | 10 | <LOQ | <LOQ | 18 ± 10 | 43 |
| Pyrimethanil | 45 | 68 | 50 | 53 | 43 | 35 | 34 | 47 ± 12 | 100 |
| Tebuconazole | 43 | 84 | 26 | 25 | 32 | 28 | 24 | 37 ± 22 | 100 |
| **Total load (ng/L)** | **1817** | **4426** | **2892** | **3676** | **2645** | **3489** | **4909** | | |

[a]S, sampling; [b]SD, standard deviation; [c]FD, frequency of detection (only analytes with reported concentrations were considered); [d]<LOQ, concentration below the limit of quantification; [e]EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.
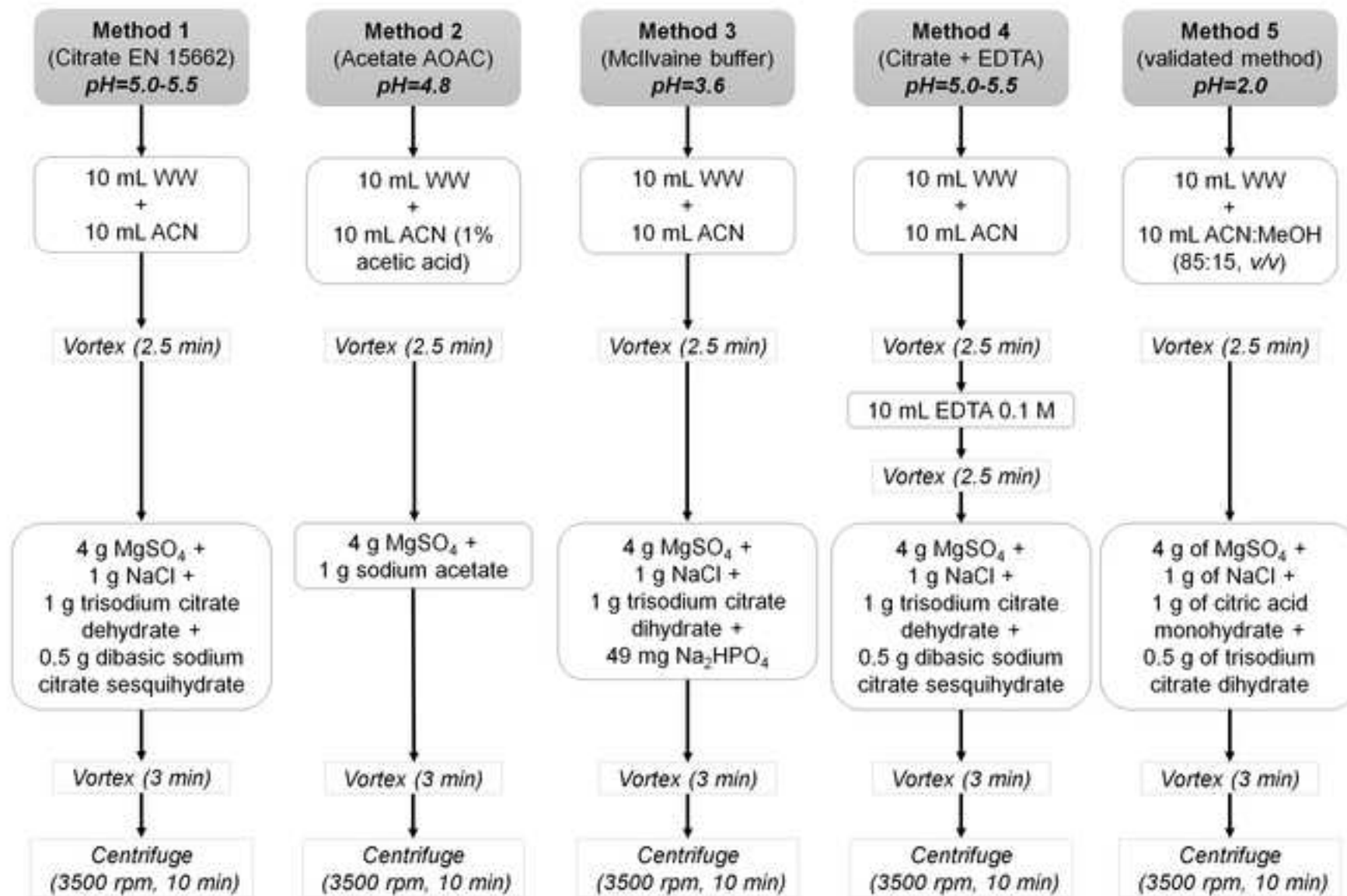
**Figure captions**

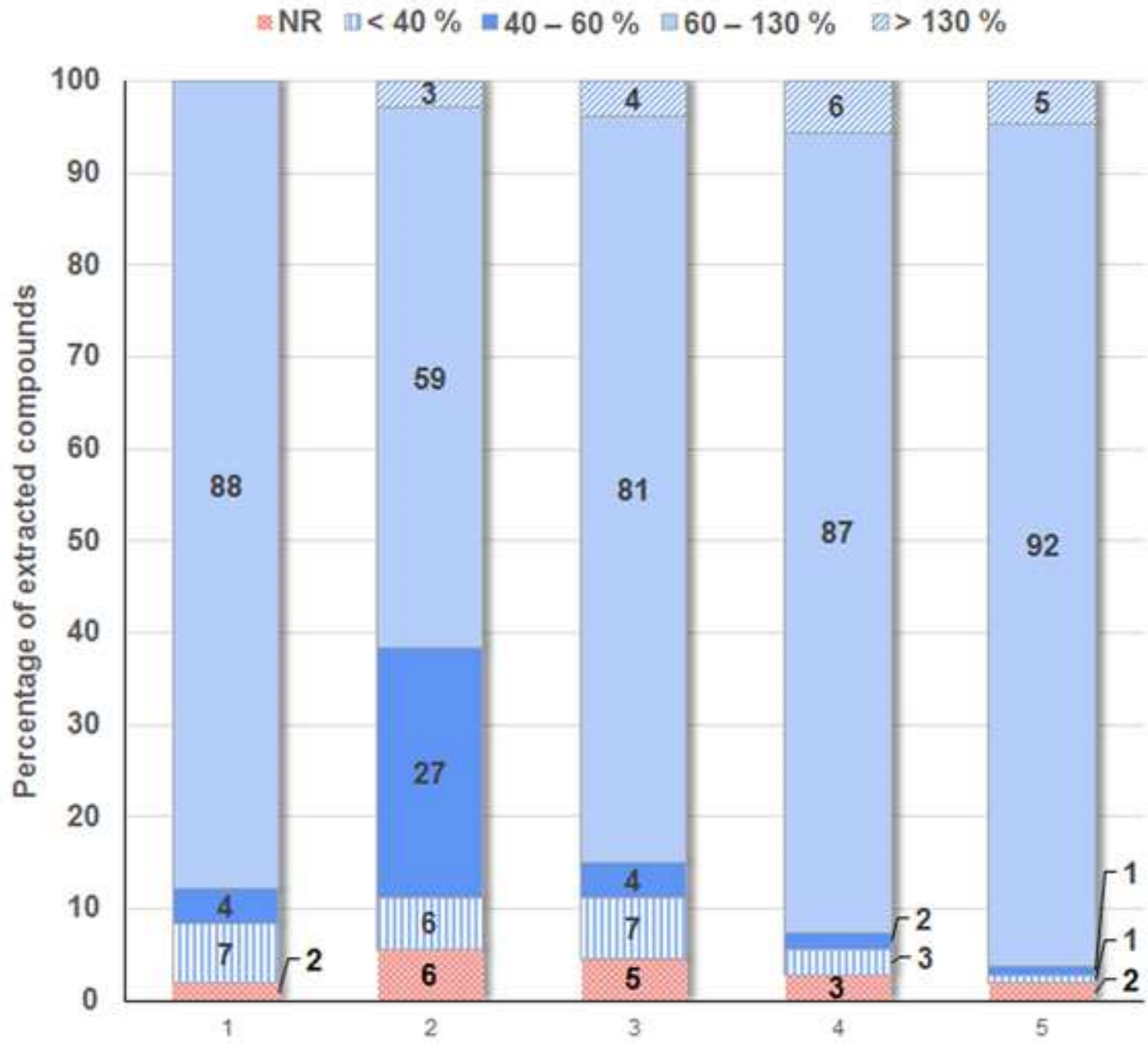**Figure 1.** Schemes of the different tested QuEChERS extraction methods.

**Figure 2.** Summary of recovery ranges and percentage of CECs (total = 107) obtained in spiked treated wastewater samples (500 ng L$^{-1}$, $n$=3) applying the evaluated extraction methods (NR/NA: not recovered/not available).

**Figure 3.** Summary of the results obtained for the different d-SPE clean-up obtained in terms of recovery and percentage of CECs (total = 107, spiked treated wastewater samples at 500 ng L$^{-1}$, $n$=3, NR/NA: not recovered/not available).

**Figure 4.** Comparison of the total ion chromatograms (TIC) obtained in the full scan mode by LC-QTOF-MS of the different d-SPE evaluated.

**Figure 5.** Extracted ion chromatograms (XIC) of a treated wastewater sample containing two priority substances (chlorpyrifos and diuron) and two compounds of the EU Watch List (tebuconazol and venlafaxine).

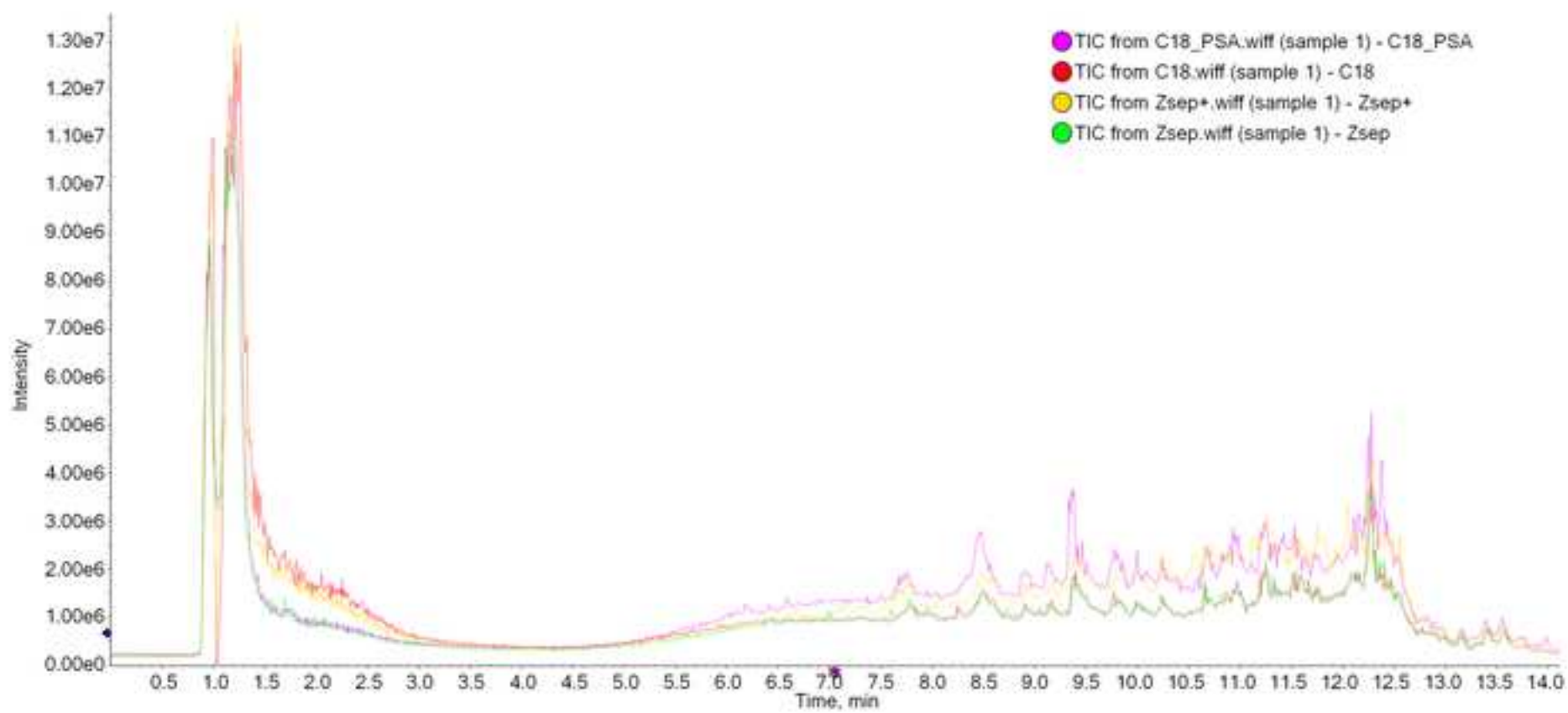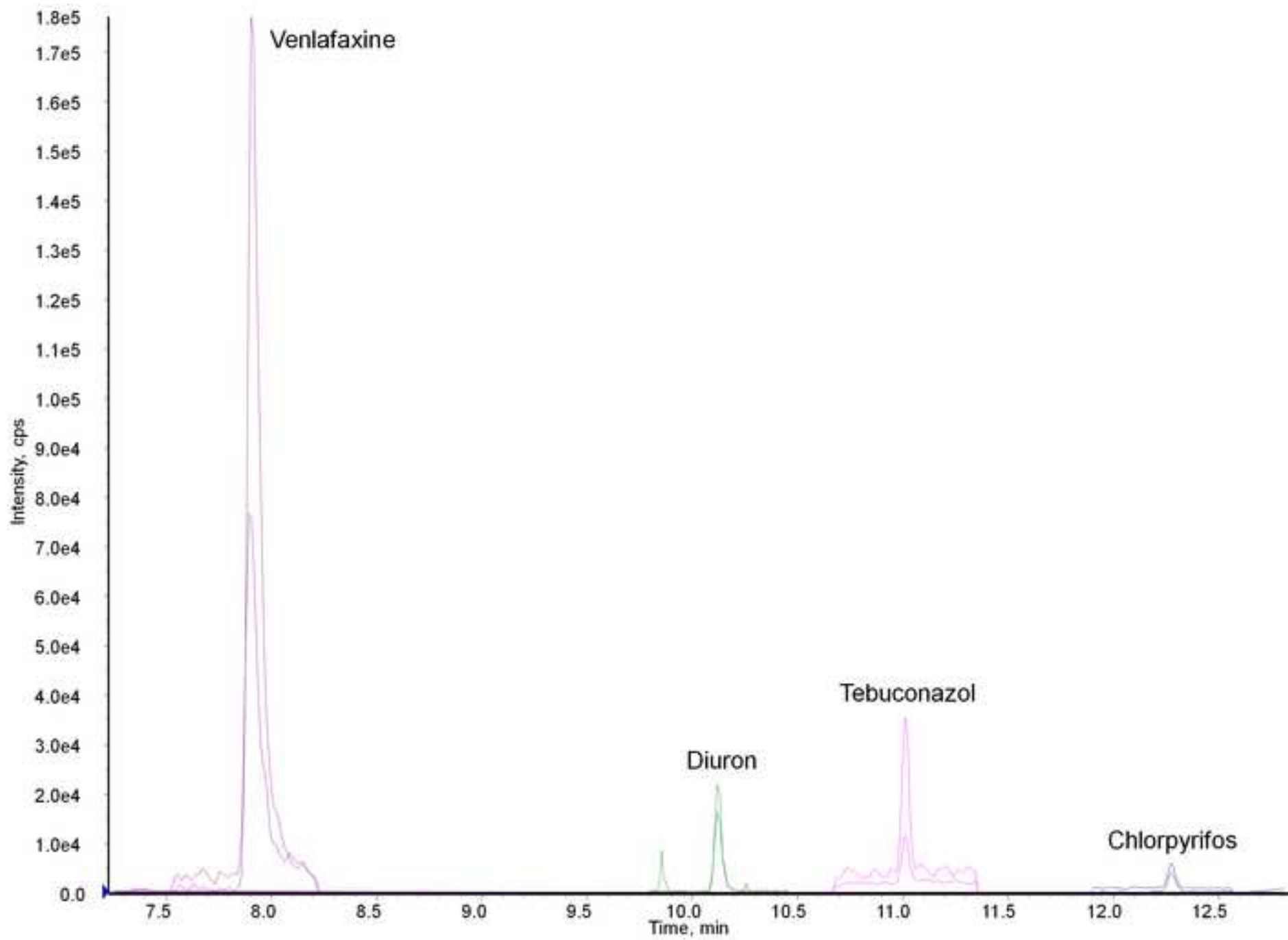| **Method 1** (Citrate EN 15662) *pH=5.0-5.5* | **Method 2** (Acetate AOAC) *pH=4.8* | **Method 3** (McIlvaine buffer) *pH=3.6* | **Method 4** (Citrate + EDTA) *pH=5.0-5.5* | **Method 5** (validated method) *pH=2.0* |
|---|---|---|---|---|
| 10 mL WW + 10 mL ACN | 10 mL WW + 10 mL ACN (1% acetic acid) | 10 mL WW + 10 mL ACN | 10 mL WW + 10 mL ACN | 10 mL WW + 10 mL ACN:MeOH (85:15, *v/v*) |
| Vortex (2.5 min) | Vortex (2.5 min) | Vortex (2.5 min) | Vortex (2.5 min) | Vortex (2.5 min) |
| | | | 10 mL EDTA 0.1 M | |
| | | | Vortex (2.5 min) | |
| 4 g $MgSO_4$ + 1 g NaCl + 1 g trisodium citrate dehydrate + 0.5 g dibasic sodium citrate sesquihydrate | 4 g $MgSO_4$ + 1 g sodium acetate | 4 g $MgSO_4$ + 1 g NaCl + 1 g trisodium citrate dihydrate + 49 mg $Na_2HPO_4$ | 4 g $MgSO_4$ + 1 g NaCl + 1 g trisodium citrate dehydrate + 0.5 g dibasic sodium citrate sesquihydrate | 4 g of $MgSO_4$ + 1 g of NaCl + 1 g of citric acid monohydrate + 0.5 g of trisodium citrate dihydrate |
| Vortex (3 min) | Vortex (3 min) | Vortex (3 min) | Vortex (3 min) | Vortex (3 min) |
| Centrifuge (3500 rpm, 10 min) | Centrifuge (3500 rpm, 10 min) | Centrifuge (3500 rpm, 10 min) | Centrifuge (3500 rpm, 10 min) | Centrifuge (3500 rpm, 10 min) |

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☒ 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.

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