Development and validation of an analytical method based on liquid chromatography-tandem mass spectrometry detection for the simultaneous determination of 13 relevant wastewater-derived contaminants in lettuce

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17 **Abstract**

18 The current work presents an analytical methodology developed and validated to study the potential
19 uptake of thirteen selected drugs (ten pharmaceuticals, one illicit drug and two transformation
20 products) into lettuce plants from contaminated water and soil. Some of the selected drugs (i.e.,
21 cocaine, methadone, cis-diltiazem, valsartan, and valsartan acid), commonly present in treated
22 wastewater, are investigated for the first time in plant tissues. The method is based on ultrasonic
23 solvent extraction (USE) with acetonitrile:methanol (1:1, v/v), and subsequent automated extract
24 clean-up and analysis by means of on-line solid-phase extraction - liquid chromatography - tandem
25 mass spectrometry (on-line SPE-LC-MS/MS). Optimum extraction conditions were selected after
26 evaluating analyte recoveries with four different extraction techniques (USE; solid-liquid extraction
27 (SLE), pressurized liquid extraction (PLE), and a “quick, easy, cheap, effective, rugged, and safe”
28 (QuEChERS)-based method) and six different solvent mixtures. Furthermore, two different clean-
29 up SPE sorbents were also evaluated. The developed methodology presents high sensitivity (with
30 limits of detection between 0.1 and 12.6 ng g⁻¹ d.w. and limits of quantification between 0.5 and
31 42.0 ng g⁻¹ d.w.); satisfactory accuracy (with analyte relative recoveries above 80% for all analytes
32 but for acridone and oxcarbazepine); and good repeatability (with relative standard deviations
33 below 9% for all analytes). As part of the validation procedure, the analytical method was applied to
34 the analysis of lettuce plants irrigated with water fortified with the selected compounds for the
35 entire growing period. Results obtained evidence the transfer of all investigated drugs into lettuce
36 leaves.

37

38 **Keywords:** Pharmaceuticals, illicit drugs, plant uptake, ultrasound extraction, on-line solid phase
39 extraction, liquid chromatography-mass spectrometry.

1. Introduction

Nowadays, water scarcity and the increasing water demand for agriculture have forced water authorities to seek alternative water resources. Consequently, countries in arid and semi-arid regions where water is a limited commodity (e.g., countries bordering the Mediterranean Sea) are increasingly using treated municipal wastewater to cover water demand (1, 2). Despite the fact that the safety and the suitability of reclaimed wastewater for agricultural irrigation has already been demonstrated (3, 4), it can also be a source of anthropogenic chemicals, such as pharmaceutically active compounds (PhACs) and personal care products into the environment (5), because they are not completely removed during wastewater treatment processes. Thus, the consumption of fresh crops irrigated with reclaimed water could represent a direct route of human exposure to wastewater derived contaminants (6).

In the recent years, numerous analytical methods have been developed to determine the occurrence of PhACs, drugs of abuse, pesticides and hormones in environmental aqueous matrices (drinking water, groundwater, surface water, and wastewater) (7-11). Most of these methods are based on solid phase extraction (SPE) of the analytes and liquid chromatography - mass spectrometry (LC-MS) detection. Despite the fact that SPE is a quite versatile technique useful to extract and preconcentrate a wide spectrum of organic compounds from environmental samples, it can also be connected in series to the LC-MS system. This allows the development of fully automated methodologies that have been proved to be more sensitive and reproducible than off-line analytical approaches, because the whole sample is transferred into the LC-MS system and it reduces sample handling and analysis time (12-14). On the contrary, few analytical methodologies exist in the peer-reviewed literature for the determination of such organic contaminants in environmental solid matrices, such as soils and plants, being even scarcer for the later (15-19).

Different sample preparation approaches have been used to extract PhACs from plant tissues. Boxal et al. extracted ten drugs of common veterinary use from lettuce and carrots with different solvent mixtures, by solid-liquid extraction or soxhlet extraction (20). PhACs commonly found in treated wastewater, including carbamazepine, diclofenac and ibuprofen, and various pesticides have been successfully extracted by means of pressurized liquid extraction (PLE) from lettuce using as extraction solvents acetone:hexane and ethyl acetate:hexane (21). Ultrasound extraction (USE) for instance with acetonitrile:water, or methyl-tert-butyl ether (MTBE) followed by acetonitrile has been used to extract various wastewater derived contaminants (PhACs, personal care products, steroid hormones) from different plant tissues (radish and ryegrass (22), and lettuce and spinach (23), and lettuce and tomato (24)). A solid-liquid extraction (SLE) method using methanol and methanol:water was recently developed by Riemenschneider et al. to extract several organic

74 contaminants from lettuce, cabbage and tomato (25). The extraction of PhACs with “quick, easy,
75 cheap, effective, rugged, and safe” (QuEChERS) -based approaches was also explore in several
76 occasions (25, 26), and it was reported to provide good analyte recoveries in celery and lettuce
77 matrices (26). SPE is usually the technique of choice to clean-up the plant extracts obtained (20, 22,
78 23, 25).

79 Measurement of polar organic compound concentrations in plant tissues is of great interest to
80 investigate their fate in edible crops, and represents a big challenge due to the low concentrations at
81 which these compounds may be present (usually in the ng g^{-1} d.w. range) and the complexity of
82 these matrices, as they contain pigments, fats, cellulose, and waxes in different proportions, which
83 may interfere with analyte extraction and instrumental analysis (23, 27). Co-extracted chlorophyll
84 and waxy or fatty materials from plant tissues may interfere with the accuracy and precision of the
85 results if not removed during sample treatment (28). Analytical methods reported to date in the
86 peer-reviewed literature to analyse PhACs in plant tissues require considerable time and effort in
87 the sample preparation process or specialized extraction equipment (15).

88 In this context, the main objective of the present study was to develop and validate an analytical
89 methodology based on liquid chromatography tandem mass spectrometry (LC-MS/MS) detection
90 for the simultaneous determination of thirteen drugs in lettuce leaves. These compounds were
91 chosen for their widespread use as well as their frequent detection in treated wastewater (10, 11).
92 For this, the performance of four extraction techniques, i.e., SLE, PLE, USE, and QuEChERS, to
93 extract the selected drugs from this matrix was compared. Lettuce was selected as the matrix of
94 study for several reasons: (i) it is the most cultivated leaf vegetable worldwide, (ii) it is a quick-
95 growing crop, (iii) it is easy to grow in greenhouse conditions, (iv) it is usually consumed without
96 being cooked or heated, which preserves the retained contaminants, (v) it has an extensive complex
97 root structure that may facilitate the uptake of organic contaminants from soil (29), and (vi) its
98 vegetative part consists merely of green leaves, which simplifies sample preparation. The list of
99 drugs selected included ten pharmaceuticals belonging to six different therapeutic classes, i.e.,
100 nonsteroidal anti-inflammatory drugs (NSAID) (diclofenac, DCF and ibuprofen, IBU), antibiotics
101 (trimethoprim, TMP), anticonvulsants (carbamazepine, CBZ, oxcarbazepine, OxCBZ, and
102 lamotrigine, LMG), antihypertensive drugs (cis-diltiazem, CDM, and valsartan, VST),
103 benzodiazepines (midazolam, MDZ), and opioid-agonists (methadone, MTD); one illicit drug
104 (cocaine, COC), and two transformation products (TPs) (acridone, ACR, and valsartan acid, VSA).
105 After validation, the optimized analytical approach was applied to the analysis of the selected drugs
106 in lettuces grown in soil pots and irrigated with a mixture of these drugs for the entire growing
107 period. To the authors’ knowledge, cocaine, methadone, cis-diltiazem, valsartan and valsartan acid

108 have not been previously investigated before in plant tissues, and this work represents the first
109 occurrence data on the potential accumulation of these compounds in lettuce plants.

110

111 **2. Materials and methods**

112 **2.1. Chemicals reagents**

113 High purity standards used for method development and analytical purposes were purchased from
114 Sigma-Aldrich (St. Luis, MO, USA), and Cerilliant (Round Rock, TX, U.S.). COC hydrochloride
115 salt and MTD hydrochloride salt used in the mixtures used for irrigation were provided as a
116 concession for research purposes (2009C00124) by the Division of Narcotic Drugs and
117 Psychotropic Substances of the Spanish Agency of Pharmaceuticals and Medical products. Medical
118 formulations were used for irrigation in the case of MDZ (EFG injectable solution, 5 mg mL⁻¹ of
119 active principle, Normon, Madrid, Spain), VST (Valsartan 40 mg, Kern Pharma S.L., Barcelona,
120 Spain), VSA (≥95%, synthtized and purified following the procedure described by Nödler et al.
121 (30)), and OxCBZ (Trileptal[®] 300 mg, Novartis, Switzerland). The chemical structure of the target
122 drugs, as well as their purity, suppliers, and selected physical-chemical properties are shown in
123 Table 1.

124 Isotopically labelled compounds used as surrogate standards (SS) (DCF-d₄, IBU-d₃, TMP-d₃, CMZ-
125 d₁₀, VST-d₃, VSA-d₄, LMG-¹³C₃, MDZ-¹³C₆, COC-d₃, MTD-d₃) were purchased from Cerilliant,
126 Alsachim (Illkirch Graffenstaden, France) or Toronto Research Chemicals (Toronto, ON, Canada).

127 Individual stock standard solutions with a concentration of 1,000 µg mL⁻¹ were prepared in
128 methanol (MeOH) and stored at -20 °C. A fresh irrigation solution was prepared before each use by
129 dilution of a water-based stock standard mixture of the target analytes (2 µg mL⁻¹) with tap water to
130 a final concentration of 200 ng mL⁻¹. Working mixtures containing the target analytes and/or the
131 isotopically labelled compounds were prepared in MeOH at appropriate concentrations to be used in
132 the validation studies and for calibration purposes. Bond Elut QuEChERS Method Extraction Kit
133 and Bond Elut QuEChERS Dispersive SPE Kit for general fruits and vegetables were purchased
134 from Agilent Technologies (Santa Clara, CA, USA). High performance liquid chromatography
135 (HPLC)-grade solvents were purchased from Merck (Darmstadt, Germany): acetonitrile (ACN)
136 (≥99.9%), MeOH (≥99.9%), ethyl acetate (EtAc) (≥99.5%), *n*-hexane (HEX) (≥99.0%), acetone
137 (Acet) (≥99.7%), and water. Chromasolv[®] grade MTBE (≥99.9%), formic acid (≥96%, ACS
138 reagent) and sodium thiosulfate pentahydrate salt (≥99.5%) were supplied by Sigma-Aldrich.
139 Polytetrafluoroethylene (PTFE) filters (13 mm, 0.2 µm) were purchased from Millipore
140 (Carrigtwohill, Cork, Ireland).

142 **Table 1.** Target analytes: molecular structure, physical-chemical properties, purity and supplier.

Analyte	Abbrev.	Structure	Use	CAS number	Formula	MW (Da)	Log K _{ow}	pK _a	Water Solubility (mg L ⁻¹)	Purity* (%)	Supplier*
Acridone (10H-acridin-9-one)	ACR		Transformation product	578-95-0	C ₁₃ H ₉ NO	195.22	2.84	-1.31 16.61	13.53	>98%	Sigma
Carbamazepine (5 <i>H</i> -dibenzo[<i>b,f</i>]azepine-5-carboxamide)	CBZ		Anticonvulsant, treatment of epilepsy and neuropathic pain	000298-46-4	C ₁₅ H ₁₂ N ₂ O	236.27	2.45	2.3	112	99	Sigma
(+)-Cis-Diltiazem hydrochloride (2 <i>S</i> ,3 <i>S</i>)-(+)- <i>cis</i> -3-Acetoxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5 <i>H</i>)-one hydrochloride)	CDM		Antihypertensive drug, treatment of hypertension and prevention of angina pectoris	33286-22-5	C ₂₂ H ₂₆ N ₂ O ₄ S·HCl	450.98	2.70	12.86 8.18	465	≥99	Sigma
Cocaine hydrochloride** (3β-Hydroxy-1 <i>αH</i> ,5 <i>αH</i> -tropane-2β-carboxylic acid methyl ester benzoate hydrochloride)	COC		Illicit drug, stimulant recreational drug	53-21-4	C ₁₇ H ₂₁ NO ₄ ·HCl	339.81	-2.91	8.85	7.14x10 ⁵	>99%/ >98.5%	Cerilliant/ AEMPS
Diclofenac (2-(2,6-dichloranilino)phenylacetic acid)	DCF		NSAID	15307-86-5	C ₁₄ H ₁₁ N ₁ O ₂ Cl ₂	296.15	4.51	4.15	2.37	≥98.5	Sigma
Ibuprofen (2-(4-(2-methylpropyl)phenyl)propanoic acid)	IBU		NSAID	15687-27-1	C ₁₃ H ₁₈ O ₂	206.29	3.97	4.91	21	≥98	Sigma
Lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine)	LMG		Anticonvulsant, treatment of epilepsy and bipolar disorder	84057-84-1	C ₉ H ₇ Cl ₂ N ₅	255.00	0.99	14.98, 5.87	3127	≥98	Sigma

Midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4 <i>H</i> -imidazo[1,5- <i>a</i>][1,4]benzodiazepine)	MDZ		Benzodiazepine, anesthetic	59467-70-8	C ₁₈ H ₁₃ ClFN ₃	325.78	4.33	6.57	0.1343	>99%/n.a.	Cerilliant/ Normon
(±)-Methadone hydrochloride** (6-Dimethylamino-4,4-diphenylheptan-3-one hydrochloride)	MTD		Opioid, pain treatment; opioid dependence treatment	1095-90-5	C ₂₁ H ₂₇ NO·HCl	345.91	1.40	18.78 9.12	1.2E+005	>99%	Cerilliant/ AEMPS
Ox-Carbazepine (10,11-dihydro-10-oxo-5 <i>H</i> -dibenz(b,f)azepine-5-carboxamide)	OxCBZ		Anticonvulsant, treatment of epilepsy	28721-07-5	C ₁₅ H ₁₂ N ₂ O ₂	252.27	1.1	-0.65°	308.1	n.a.	Novartis
Trimethoprim (5-(3,4,5-Trimethoxybenzyl)pyrimidine-2,4-diamine)	TMP		Antibiotic, treatment of bladder infections	738-70-5	C ₁₄ H ₁₈ N ₄ O ₃	290.32	0.91	7.12	400	98	Sigma
Valsartan acid (2'-(1 <i>H</i> -Tetrazol-5-yl)-[1,1'-biphenyl]-4-carboxylic acid)	VSA		Transformation product	164265-78-5	C ₁₄ H ₁₀ N ₄ O ₂	266.08	4.0	3.6	1.4	≥95	Synthesis [1]
Valsartan (((<i>S</i>)-3-methyl-2-(<i>N</i> -{[2'-(2 <i>H</i> -1,2,3,4-tetrazol-5-yl) biphenyl-4-yl]methyl}pentanamido) butanoic acid)	VST		Antihypertensive drug, treatment of high blood pressure, congestive heart failure and heart attack	137862-53-4	C ₂₄ H ₂₉ N ₅ O ₃	435.52	3.65	4.37	1.4	n.a.	Kern Pharma

Experimental database match

US EPA; Estimation Program Interface (EPI) Suite. Ver.3.12. <http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm>

*If two data are available, it reads as analytical standard/irrigation standard

** Log Kow values for these compounds corresponds to the free base, while solubility data corresponds to the salt

n.a. not available

148 Oasis HLB on-line solid phase extraction (SPE) cartridges (10 x 1 mm, 30-µm particle size, 2.5 mg)
149 were purchased from Waters Corporation (Milford, MA, USA), while Hysphere Resin GP on-line
150 SPE cartridges (10 x 2 mm, 5–15-µm particle size, 13 mg) were purchased from Spark Holland
151 (Emmen, The Netherlands).

152

153 **2.2. Sample preparation**

154 Lettuce (*Lactuca sativa* L.) cv. Maravilla de Verano-Canasta was selected for the study as it can be
155 cultivated in both outdoor and greenhouse setups at any time of the year and it is very resistant to
156 fungal diseases. A mature lettuce head was purchased from a local organic supermarket to perform
157 method optimization and validation studies. Grown plants irrigated with a mixture of the selected
158 drugs (see section 2.6 for further details) were investigated to evaluate drug uptake. Before
159 extraction, lettuce leaves were gently hand-washed with tap water to remove soil residues and
160 subsequently rinsed with deionized water. Then, leaves were gently blot dried with paper towel, and
161 stored at – 20° C for at least 48 h. Frozen leaves were then freeze-dried for 3 days or longer if
162 needed (until no ice was left) to remove residual water using a freeze-dry system (LyoAlfa 6,
163 Telstar Technologies, Terrassa, Spain). Freeze-dried samples were ground to powder using a
164 laboratory grinder with a stainless steel grinding chamber (Snijders, Tilburg, Holland) and stored in
165 20 mL glass vials in the dark at – 20° C until extraction.

166

167

168 **2.3. Sample extraction and clean-up**

169 Four extraction techniques were assessed and compared in this study: (i) SLE, (ii) PLE, (iii) USE,
170 and (iv) QuEChERS. For each technique, six different extraction solvent mixtures were tested:
171 ACN:MeOH (1:1, v/v), MTBE:MeOH (1:1, v/v), HEX:Acet (1:1, v/v), MTBE:ACN (1:1, v/v),
172 EtAc:MeOH (1:1, v/v), and 0.5% formic acid in ACN:MeOH (1:1, v/v). Detailed extraction
173 protocols are described in section 2.3.1. The effects of a clean-up step on analyte recovery were also
174 evaluated. For this, leave lettuce extracts obtained with the different extraction techniques and
175 extraction solvent mixtures were filtered through 0.2-µm PTFE filters, liquid-liquid extracted with
176 hexane (hexane washed), or pre-concentrated by SPE on either Oasis HLB or HySphere Resin GP
177 followed by LC-MS/MS analysis (see section 2.3.2. for further details).

178 In the optimized approach, 0.5 g of a freeze-dried lettuce leave sample were placed into 50-mL
179 polypropylene centrifuge tubes and spiked with a known amount of the isotopically labelled
180 compounds (100 ng g⁻¹ d.w. equivalent to 0.5 ng/mL in the final extract). Spiked samples were left
181 in the fume hood for 1 hour to allow the MeOH of the spiking solution to evaporate, and then
182 placed in a fridge overnight. Analytes were extracted from lettuce leaves by means of two
183 consecutive USE cycles using 16 mL of a mixture ACN:MeOH (1:1, v/v) using a Fisherbrand
184 FB15064 ultrasonic water bath (Fisher Scientific, Pittsburgh, PA, USA). Before adding the first
185 extraction solvent, 2 mL of water were added to the lettuce sample. Each 20-min USE cycle was
186 performed after vortex-mixing the extraction solvent and the sample for 2 min. After each USE
187 cycle, sample was centrifuged at 4000 rpm for 30 min, and the supernatant was transferred to a 40-
188 mL glass vial. Extraction solvents were combined and evaporated until 2 mL under a gentle stream
189 of nitrogen at 24 °C using a TurboVap® LV (Biotage AB, Uppsala, Sweden). Then, the 2 mL
190 extract was diluted with HPLC water to 100 mL, and filtered through a 0.2 µm PTFE filter. A 10
191 mL aliquot of the filtrate was transferred to a vial for extract clean-up by on-line SPE.

192 For clean-up, 5 mL of the extract were preconcentrated onto Oasis HLB cartridges using an
193 automated on-line SPE sample processor Symbiosis™ Pico (Spark Holland). On-line SPE
194 cartridges were conditioned with 2 mL of ACN and 2 mL of MeOH, and equilibrated with 2 mL of
195 water, at a flow rate of 5 mL min⁻¹. Extract loading was performed at a slower flow rate (1 mL min⁻¹)
196 to enhance analyte retention onto the sorbent. After extract load, the cartridge was washed with 1
197 mL of water to complete the transfer of the sample and remove interferences such as inorganic salts,
198 and trapped analytes were eluted from the cartridge to the LC column with the LC mobile phase.
199 This clean-up step contributed to improve method sensitivity and reduce matrix interferences.

200

201 2.3.1. Sample extraction protocols

202 In order to select the best extraction approach, 0.5 g of freeze-dried analyte-free lettuce was
203 weighed and spiked with the target compounds (100 µL of 2000 ng mL⁻¹ solution in MeOH) and the
204 surrogate standard (SS) solution (100 µL of 1000 ng mL⁻¹ solution in MeOH) in order to obtain a
205 final concentration of 400 ng g⁻¹ d.w. and 200 ng g⁻¹ d.w. for the target compounds and the SS,
206 respectively. The spiked matrix was vortex-homogenized and left under the hood for one hour to
207 allow the MeOH to evaporate, and overnight in the fridge for stabilization. Then, the spiked
208 samples were extracted following the different extraction protocols indicated below with different
209 extraction solvent mixtures. All tests were performed in duplicate:

210 (i) *Solid-Liquid Extraction (SLE)*. For SLE, a modified method for molluscs was used (1). Twenty
211 mL of the solvent mixture (1:1, v/v) was added into the 50 mL polypropylene centrifuge tube
212 where the spiked sample was and vortexed for 2 min. The tube was shaken at 500 rpm at room
213 temperature (25° C) for 16 h using a flask shaker SF1 (Bibby Scientific Ltd, Staffordshire, UK).
214 Then, the tube was centrifuged at 400 rpm (4° C) for 40 min by a centrifuge 5810 R (Eppendorf
215 AG, Hamburg, D), and the supernatant collected for further clean-up.

216 The following solvent mixtures were tested: ACN:MeOH (1:1 v/v), MTBE:MeOH (1:1, v/v),
217 HEX:Acet (1:1, v/v), MTBE:ACN (1:1, v/v), EtAc:MeOH (1:1, v/v), and 0.5% formic acid in
218 ACN:MeOH (1:1, v/v).

219
220 (ii) *Pressurized Liquid Extraction (PLE)*. This technique has been previously proved to
221 satisfactorily extract pharmaceuticals from vegetable and soil matrices (38). PLE was performed
222 with an accelerated solvent extractor ASE 350 system (Thermo Scientific-Dionex, Sunnyvale,
223 CA). Packing of the 22-mL stainless steel ASE extraction cell is illustrated in Figure 1. Firstly, a
224 2-cm glass cellulose filter (10 µm, Dionex, Leeds, UK) was placed at the bottom of the cell
225 before adding the adsorbent layers responsible for the in-cell clean-up of the sample extract.
226 This was done to avoid fine sample particles leaving the cell and plugging of the system. The
227 order followed to pack the cell was 3g of alumina (Al₂O₃), 3 g of fluorisil, the spiked lettuce
228 sample, 0.5 g hydromatrix, and 3 g of alumina. The upper void space in the cell was completely
229 filled with hydromatrix, and finally a cellulose filter was place on top before closing the cell.

230
231 **Figure 1** - Packing of the ASE extraction cell.

232 Lettuce samples were initially extracted using each one of the solvent mixture (1:1, v/v)
233 tested for SLE extraction (see above) and two extraction cycles with the following
234 conditions: 5 min heat time and 5 min static time, 60% of flush cell volume, and 120 s of
235 purge time. Temperature and pressure were kept steady at 50° C and 1600 psi, respectively.

After the static period, the eluate, was purged into a collection vial (see Figure 2). The cell was left in the system and further extracted with a mixture of MeOH:Acet (1:1, v/v) at a temperature of 80 °C and a flush cell volume of 80%. PLE extracts were combined (about 50 mL in total) and evaporated to 20 mL under a gentle stream of nitrogen at 24°C in a TurboVap® LV station. Hydromatrix spiked with the compounds of interest was also extracted using the same conditions to evaluate the absolute recovery of the PLE extraction method. All PLE extracts were kept for further clean-up.

Figure 2 –PLE extracts: Hydromatrix on the left (3 PLE cycles), two PLE cycles with MeOH:ACN (1:1, v/v) in dark green, and one PLE cycle with MeOH/Acet (1:1, v/v) in light green.

(iii) *Ultrasonic Solvent Extraction (USE)*. Sixteen mL of one of the mixture solvents (e.g, ACN for the ACN:MeOH mixture) was added into the 50 mL polypropylene centrifuge tube where the spiked sample was and vortexed for 2 min. Then, it was sonicated in a Fisherbrand FB15064 ultrasonic water bath (Fisher Scientific, Pittsburgh, PA, USA) for 20 min, and centrifuged at 4000 rpm for 30 min. The supernatant was collected in a 40-mL glass vial, and the USE protocol was performed once more using the other solvent of the tested mixture (e.g., MeOH for the ACN:MeOH mixture). The extracts were combined (about 28 mL in total), and evaporated until 20 mL under a gentle stream of nitrogen at 24° C in a TurboVap® LV station. Analyte recoveries after two USE cycles using different solvent mixtures (ACN:MeOH and HEX:Acet) were also evaluated: two cycles of each solvent mixture, and one cycle of ACN:MeOH in combination with one cycle of HEX:Acet. All USE extracts were kept for further clean-up.

(iv) *QuEChERS Extraction (QUE)*. The QuEChERS method is an extraction and cleanup method introduced by Anastassiades et al. (39). This extraction method has been widely extended to extract pesticide residues from different matrices, as it allows removing sugars, lipids, organic

acids, sterols, proteins, pigments and excess of water that could interfere with chromatographic analysis. In our study, QuEChERS Extract Pouch (Agilent Bond Elut) containing 4 g MgSO_4 , 1 g of NaCl and 1 g of sodium citrate and 20 mL of the solvent mixture were added to the 50 mL centrifuge tube containing the spiked lettuce sample. The tube was hand-shaken vigorously for 5 min. Then it was placed for 15 min in an ultrasonic bath, vortexed for 5 min, and centrifuged at 4000 rpm for 30 min. Five mL of the supernatant (about 16 mL in total) was transferred into a 15 mL centrifuge tube containing Dispersive – SPE sorbents, (PSA and magnesium sulfate, Agilent Bond Elut), and 5 mL were kept for hexane-wash based clean-up. The tube was vortexed for 2 min and then centrifuged at 4000 rpm for 30 min. The obtained supernatant was transferred into a glass vial, dried under a gentle stream of nitrogen 24°C , and finally reconstituted with 1.0 mL of $\text{H}_2\text{O}:\text{MeOH}$ (80:20, v/v) and filtered through 0.2- μm PTFE filters before LC-MS analysis.

2.3.2. Extract clean-up protocols

Each final extract was split in different fractions. One extract fraction (5 mL, with a theoretical mass of 100 ng and 50 ng of each target analyte and SS, respectively) was evaporated to dryness under nitrogen and then reconstituted into 1 mL of $\text{H}_2\text{O}:\text{MeOH}$ (80:20, v/v). The reconstituted extract was filtered through 0.2 μm PTFE filters to remove solid particles, transferred to a 2 mL vial and directly injected into the LC-MS/MS system. Filtration was done for all extracts but those obtained after the first step of the QuEChERS extraction.

The other extract fraction (5 mL, with a theoretical mass of 100 ng of each target analyte and 50 ng of each target analyte and SS, respectively) was solvent exchanged to ACN under nitrogen evaporation, and washed with 3 mL of ACN-saturated hexane (34) by hand-shaking for 10 sec. (3x2 mL). The hexane phase containing chlorophyll, fatty and waxy materials was removed after liquid-liquid extraction and discarded. At least three hexane washes were usually performed to clean lettuce extracts of undesirable compounds, except in the case of ASE extracts that only required two cycles of hexane wash. Washed extracts (see SLE extracts in Figure 3) were evaporated to dryness under nitrogen (24°C) in a Turbo-Vap® LV station and reconstituted with 1 mL of $\text{H}_2\text{O}:\text{MeOH}$ (80:20, v/v). This evaporated extract was also filtered through 0.2- μm PTFE filters before analysis by HPLC-MS/MS.

Figure 3 – SLE extracts before (on the left) and after (on the right) ACN-saturated hexane wash

2.4. LC-MS/MS analysis

LC-MS/MS analyses were carried out in a system consisting of a HPLC SymbiosisTM Pico, operated in XLC mode, connected in series with a 4000QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer equipped with a Turbo Ion Spray source (Sciex, Redwood City, CA, U.S.A.), operated in positive ionization mode (ESI+). The analytical instrumentation was controlled with the Analyst 1.4.2 software (Sciex) that had the Symbiosis software (Spark Holland) integrated.

HPLC separation of the analytes was performed using a 125 x 2 mm Purospher STAR RP-18 endcapped column (5 μ m particle size) (Merck, Darmstadt, Germany) and a mobile phase consisting of ACN and water, both acidified with 0.1 % formic acid at a flow rate of 0.3 mL min⁻¹. Initial conditions (5% of ACN) were maintained for 2 min before increasing the organic solvent from 5% to 50% over next 19 min and then to 100% in the following 2 min. Pure organic conditions were held for 5 min before returning to initial conditions that were maintained for 10 min to allow column re-equilibration before the next injection. This gradient provided a chromatographic analysis time of 38 min.

MS/MS analyses were performed in the selected reaction monitoring (SRM) mode. Thus, the transitions from the precursor ion to two of its main fragment ions were registered for each target compound. Only one SRM transition was selected for each SS. Selected SRM transitions for each analyte and isotopically labelled compounds and the optimal instrumental conditions set for their analysis are summarized in Table 2.

318 2.5. Method validation

319 The performance of the optimized analytical approach was evaluated in terms of linearity, trueness
320 (analyte recovery), precision (repeatability), sensitivity, and matrix effects.

321 Quantification was performed by the IS method. For this, a fixed and known amount of SS (100 ng
322 g⁻¹ d.w.) was added to the samples, calibration standards, and blanks. The calibration curve was
323 then constructed using linear weighted least-squares regression (1/x as weighting factor) by plotting
324 the ratio of the analyte signal to the SS signal as a function of the analyte concentration. Calibration
325 standards were prepared by spiking the appropriate amount of standards in compound-free lettuce
326 extracts, and expanded within the calibration range 0.0025-2.5 ng mL⁻¹ (equivalent to 0.5-500 ng g⁻¹
327 d.w. in lettuce leaves).

328 Extraction recoveries were evaluated from replicate analysis (n=6) of compound-free lettuce leave
329 samples fortified with the thirteen target compounds before the extraction process at two
330 concentration levels (50 ng g⁻¹ and 400 ng g⁻¹ d.w.). Absolute recoveries (AR) were determined by
331 comparing the peak areas obtained in the spiked lettuces with those obtained in standard solutions at
332 equivalent concentrations (0.25 ng mL⁻¹ and 2 ng mL⁻¹, respectively). Analyte relative recoveries
333 (RR) were evaluated by comparing its AR with that obtained for the SS used for its quantification.
334 Method precision, expressed as method repeatability, was calculated as the relative standard
335 deviations of the RR obtained after analysis of the lettuce leave samples (n=6) prepared for the
336 recovery study.

337 Method detection limits (MDL) and method quantification limits (MQL) were estimated as the
338 concentration of analyte that provides a signal-to-noise (S/N) of 3 and 10, respectively. For those
339 analytes found at high concentrations in the samples or that were not found in the lettuce samples,
340 MDLs and MQLs were estimated from the S/N values observed in the samples fortified at the
341 lowest level (50 ng g⁻¹ d.w.) in the recovery study.

342 In order to evaluate the matrix effects (the potential ion suppression or ion enhancement produced
343 by the matrix components), compound-free lettuce samples (n=3) were extracted with the optimized
344 protocol and the extract was spiked with the target compounds at 1 ng mL⁻¹ (equivalent to 200 ng g⁻¹
345 d.w.) just before on-line SPE-LC-MS/MS analysis. The peak areas obtained in these samples were
346 compared to those obtained in on-line SPE-LC-MS/MS analysis of HPLC water spiked at an
347 equivalent concentration (1 ng mL⁻¹).

349 **Table 2.** Optimum conditions for the LC-ESI(+)-MS/MS analysis of the target analytes.

Analyte	t _R (min)	SRM transitions ^a	DP(V)-CE(eV)- CXP(V)	SRM1/ SRM2
TMP	9.2	291 > 230 291 > 123	72-35-10 86-31-20	1.2
<i>TMP-d</i> ₃	9.2	294 > 230	86-37-10	
LMG	11.2	256 > 211 256 > 109	86-37-12 86-70-6	1.1
<i>LMG-¹³C</i> ₃	11.2	259 > 214	86-37-12	
COC	12.5	304 > 182 304 > 77	70-30-10 70-90-15	3.3
<i>COC-d</i> ₃	12.5	307 > 77	70-90-15	
MDZ	15.1	326 > 291 326 > 249	102-38-16 102-52-18	4.3
<i>MDZ-¹³C</i> ₆	15.1	332 > 297	106-39-16	
VSA	15.3	267 > 206 267 > 178	66-25-12 66-43-14	2.7
<i>VSA-d</i> ₄	15.3	271 > 182	71-25-12	
OxCBZ*	15.4	253 > 236 253 > 180	61-21-14 61-41-14	1.3
CDM**	16.3	415 > 178 415 > 109	60-35-10 60-90-4	2.0
ACR*	16.4	196 > 167 196 > 139	101-48-14 101-72-10	2.9
CBZ	17.3	237 > 194 237 > 192	61-29-14 61-31-12	4.6
<i>CBZ-d</i> ₁₀	17.2	247 > 204	71-29-18	
MTD	18.3	310 > 265 310 > 105	45-20-15 45-40-15	2.7
<i>MTD-d</i> ₃	18.2	313 > 268	60-20-15	
VST	22.7	436 > 235 436 > 207	66-27-12 66-37-16	1.0
<i>VST-d</i> ₃	22.7	439 > 235	71-27-14	
IBU	23.9	207 > 161 207 > 119	63-14-9 63-32-5	2.0
<i>IBU-d</i> ₃	23.9	210 > 164	66-15-12	
DCF	24.0	296 > 214 298 > 216	40-45-16 46-45-12	0.9
<i>DCF-d</i> ₄	23.9	302 > 218	46-43-12	

^a Quantification SRM transition is highlighted in bold font

* Compound quantified using *CBZ-d*₁₀ as SS

** Compound quantified using *MDZ-¹³C*₆ as SS

383 Quality controls (QCs) were prepared by spiking analyte-free lettuce extracts at a concentration of
384 50 ng g⁻¹ d.w., and they were analysed every six samples throughout the analysis batch. Blanks
385 consisting of the lettuce used in the validation study fortified only with the SS mixture were also
386 analysed to confirm the absence of the target analytes in this matrix, and blanks consisting of HPLC
387 grade water were also injected after every QC to confirm the absence of carry-over or memory
388 effects.

389

390 **2.6. Experimental design for the uptake study**

391 In May 2016, nine plants (3 controls and 6 replicates) of lettuce (*Lactuca sativa* L.) cv. Maravilla de
392 Verano-Canasta at approximately four-leaf stage were purchased from a local plant nursery and
393 transplanted individually into 0.20 m deep polyethylene terephthalate (PET) pots filled with about
394 2,000 ± 20 g of organic substrate for horticulture each. Lettuce plants were left to grow on the
395 terrace of a three store building under natural sunlight. (IDAEA-CSIC, Barcelona, Spain). After five
396 days of acclimation, plants were daily irrigated with 100 mL of tap water containing the selected
397 drugs at a concentration of 200 ng mL⁻¹, by pouring the water directly onto the soil. Control plants
398 were irrigated with 100 mL of tap water. Sodium thiosulfate (1 mL of a 1.8% (m/v) solution) was
399 used to remove residual chlorine (≈ 0.6 mg L⁻¹) present in the tap water used for irrigation.
400 Application of fertilizers, pesticides and irrigation was performed as needed to ensure optimal crop
401 growth. No rainfall occurred during the experimental period. Mature lettuces were harvested after
402 60 days by cutting just below the cotyledonary node, and processed for analysis in triplicate as
403 described in sections 2.2 and 2.3.

404

405 **3. Results and discussion**

406 **3.1. Sample extraction and clean-up**

407 The most efficient extraction approach (described in section II as SI) was selected after evaluating
408 analyte AR in filtered extracts. For this, analyte peak areas obtained after LC-MS/MS analysis of
409 filtered extracts and standard solutions prepared with a mixture of H₂O:MeOH at equivalent
410 concentrations (100 ng mL⁻¹) were compared. Besides extraction efficiency, other factors such as
411 extraction time and cost were also considered in the final decision.

412 Analyte AR pointed the ACN:MeOH extraction mixture as one of the best options to extract the
413 target compounds from lettuce leaves, as it can be observed in Figure 4. Good recoveries were

414 achieved using this solvent mixture in USE, SLE, and QuEChERS. Overall, the poorest extraction
415 yields were obtained when using HEX:Acet as extraction solvents.

416 Although the analytes were successfully extracted with SLE using different extraction mixtures, i.e.,
417 ACN:MeOH (1:1, v/v) and MTBE:MeOH (1:1, v/v), this technique was discarded because
418 recoveries obtained for most analytes presented high variability (RSD >20 %) and it is a very time-
419 consuming technique (16 hours of extraction time).

420 QuEChERS has been the technique of choice to extract pharmaceuticals and personal care products
421 from plant tissues by several authors (26, 31, 32). However, it was discarded in our study because
422 we obtained recovery values <50 % for more than half of target compounds (see Figure 4).

423 As for PLE, it was discarded because the acidic compounds DCF, IBU, VST, and VSA were not
424 recovered at all by means of the tested PLE approaches. This could be attributed to the low
425 extraction temperature used (up to 80°C) to avoid thermal degradation of the analytes, or the use of
426 the incorrect extraction solvent, because these are factors that can severely affect the extraction
427 efficiency (33). Other authors used waters as extraction solvent to recover pharmaceuticals from
428 plant tissues (26). For instance, Chuang et al. (26) reported recoveries above 70% for CBZ and
429 TMP, using PLE with different mixtures of ACN:MeOH:H₂O. However, according to our results,
430 these compounds were also efficiently extracted using a different polar solvent mixture, i.e.,
431 MeOH:Acet, in the PLE methodology.

432 Therefore, taking all the aforementioned findings into consideration, the USE technology was
433 finally selected for further optimization. Since preliminary tests were performed using extraction
434 solvents individually in each USE cycle, the extraction recovery provided by solvent mixtures in
435 USE was also evaluated. For this, spiked lettuces were extracted with two USE cycles using
436 ACN:MeOH (1:1, v/v), two USE cycles using HEX:Acet (1:1, v/v), and one cycle of each of the
437 aforementioned mixtures. Results, shown in Figure 5, confirm the mixture of ACN:MeOH as the
438 best extraction solvent. Unfortunately, this extraction solvent mixture proved very difficult to
439 evaporate, as it left a viscous residue difficult to evaporate and re-suspend (experimentally observed
440 and also reported by Bragança et al. (31). For this reason, 2 mL of water were added to the
441 extraction solvent mixture. To solve the problem of suspended particles, all samples were filtered
442 through 0.2-µm PTFE filters.

443

Figure 4. Analyte recovery (%) in filtered extracts obtained with the different extraction techniques and solvents at a concentration of $100 \text{ ng g}^{-1} \text{ d.w.}$ (SLE: Solid-liquid extraction ($n=2$, lettuce leaves fortified with the target analytes), PLE (Blank) ($n=1$, hydromatrix fortified with the target analytes) and PLE ($n=2$, lettuce leaves fortified with the target analytes); USE: ultrasonic extraction ($n=2$, lettuce leaves fortified with the target analytes))

Figure 5. Analyte recovery (% , mean and relative standard deviation, n=2) in filtered extracts obtained with different extraction solvents in USE at a concentration of 100 ng g⁻¹ d.w.

Despite the fact that a good signal was obtained for most target analytes in LC-ESI-MS/MS analysis of filtered USE extracts, a clean-up approach was considered to avoid precipitation of matrix components in the ionization source and thus a frequent clean-up of the source, and ultimately, to increase method robustness. Following the recommendations provided in peer-reviewed analytical works (28, 34), the suitability of a clean-up step based on hexane washes was evaluated. Analyte recoveries in hexane washed extracts are provided in Figure 6. Results obtained indicated that this procedure was not effective for extract clean-up due to the loss of many analytes during the process. Furthermore, this step resulted in very high RSD values. This could be attributed to the fact that waxy or fatty components were not completely removed during the wash step (28), and/or labour-intensive manual sample handling.

An SPE-based clean-up of the extract was also evaluated, as this technique may increase method sensitivity due to extract pre-concentration and removal of matrix interferences that are not retained in the SPE sorbent, and can be coupled to the LC-MS instrument, which reduces sample handling and consequently, improves method precision. The extraction recoveries of the analytes on the two generic SPE sorbents, i.e., Oasis HLB and Hysphere Resin GP, was evaluated by analysing HPLC water fortified with the target analytes at a concentration of 0.1 ng mL⁻¹. Results obtained are shown in Figure 6. As it can be observed in the figure, both sorbents provided similar average extraction recoveries. However, Oasis HLB was chosen for the excellent RSD values (n=3) provided, < 10% except for DCF (15%).

Figure 6. Absolute recoveries (%) of target analytes in the SPE sorbents evaluated

3.2. LC-ESI-MS/MS analysis

Optimum MS conditions under positive and negative ionization were obtained for each target analyte after infusion of individual standard solutions ($0.5\text{--}1\ \mu\text{g mL}^{-1}$) into the MS. The carboxylic-acid bearing compounds DCF, IBU, VST and VSA were found to be amenable to both positive and negative ESI with the latter providing higher signal intensity. However, they were all analysed in the positive mode, so that all target compounds could be determined in a single analytical run, reducing analysis time and cost, and contributing with the latter to develop a more “green-chemistry” analytical approach.

Furthermore, the analysis of IBU in the positive ionization mode allowed to register two SRM transitions, which is an important criteria required by the EU legislation for reliable identification of organic residues in food (35). IBU molecule fragments poorly under the negative ionization mode, providing only one SRM transition for its analysis (Gros et al., 2006).

Different LC mobile phases with ACN, MeOH, or a mixture of both (1:1) as organic constituents and with and without pH modifiers in the aqueous phase (0.1% formic acid for pH 2.7, 10 mM of ammonium formate for pH 4, and 10 mM of ammonium acetate for pH 6) were tested in order to maximize the signal of the target analytes. The use of acidified methanol provided the highest MS signal for all target compounds but IBU, and good chromatographic peak shape in all cases.

496 However, methanol had to be replaced by acetonitrile to produce narrow and symmetric peaks when
497 switching to on-line SPE-LC-MS/MS mode. This could be attributed to the fact that acetonitrile has
498 a higher elution strength than methanol, required to properly elute the target compounds from the
499 SPE cartridge.

500 A representative total ion chromatogram (TIC) and extracted ion chromatograms (XIC) of the target
501 analytes obtained after on-line SPE-LC-ESI(+)-MS/MS analysis of a lettuce sample fortified at a
502 concentration of 50 ng g⁻¹ d.w. are shown in Figure 7.

503

Figure 7. Total ion chromatogram (a) and extracted ion chromatograms (b) obtained for the target analytes after on-line SPE-LC-ESI(+)-MS/MS analysis of a lettuce leave sample fortified at a concentration of 50 ng g⁻¹ d.w.

Figure 7. (Cont.) Total ion chromatogram (a) and extracted ion chromatograms (b) obtained for the target analytes after on-line SPE-LC-ESI(+)-MS/MS analysis of a lettuce leave sample fortified at a concentration of 50 ng g⁻¹ d.w.

3.3. Method validation

Method performance was evaluated in terms of linearity, analyte recovery, repeatability, sensitivity, and matrix effects as described in section 2.5. Validation results are provided in Table 3.

The MS signal response was linear from the analyte LOQ to 500 ng g⁻¹ d.w. for all analytes, but for ACR, CDM, DCF, LMG, and VSA, for which the linearity range extended only up to 400 ng g⁻¹ d.w. or even 300 ng g⁻¹ d.w. in the case of VSA. Coefficients of determination (r^2) higher than 0.998 were obtained for all calibration curves. All curves were constructed by linear weighted least-squares regression using 1/x as weighting factor, and including at least 6 calibration points, for which the predicted concentration did not differ more than 20% (exceptionally 25%) from the theoretical concentration.

Recoveries of the overall method were calculated by analysing lettuce leave samples (n=6) fortified at 50 and 400 ng g⁻¹ d.w. As it can be observed in Table 3, absolute and relative analyte recoveries obtained at both concentration levels were, in general, in good agreement. The lowest overall method absolute recoveries were observed for IBU and VSA (around 30%). These low recoveries can be explained by pronounced ionization suppression effects observed in the case of IBU (81%) (see Table 3) and the low recoveries provided by the SPE cartridge for VSA (30%) (see Figure 6). However, extraction losses and matrix effects can be well compensated by the use of isotopically labelled surrogates as indicated by relative recovery values, which were between 81 and 119% in all cases, but for ACR, OxCBZ, and CDM. The deviations observed in the relative recoveries of the aforementioned compounds can be attributed to the fact that isotopically labelled analogues were not available for these compounds, and thus those of structurally related compounds (e.g., CBZ-d₁₀ for OxCBZ) or closely eluting compounds with similar absolute recoveries (e.g., CBZ-d₁₀ for ACR and MDZ-d₃ for CDM) were used. Method repeatability was satisfactory for all target compounds, with RSD values below 9 % for all analytes. The on-line SPE clean-up process contributed to improve the precision of the method because it reduced sample handling. This parameter was negatively affected when applying hexane-washing of the extracts to remove undesired matrix components (see Figure 8).

All target analytes but CDM, MTD, and VSA were subject to ionization suppression effects, as it is shown in Table 3. MS signal was observed to get reduced between 12% (TMP) and 81 % (IBU). In addition to IBU, ionization of ACR, LMG, and OxCBZ was seriously affected by lettuce matrix components, by more than 50%. Besides correcting for analyte losses, isotopically labelled compounds added as surrogate standards also contributed to compensate for matrix-derived ionization effects.

593 As for method sensitivity, the methodology developed allowed to detect and quantify most of the
594 target analytes in lettuce leaves at concentrations as low as 0.1 ng g⁻¹ d.w. and 0.5 ng g⁻¹ d.w.,
595 respectively (this is the case for DCF). The highest MDL and MQL were obtained for LMG, with
596 12.6 ng g⁻¹ d.w. and 42.0 ng g⁻¹ d.w., respectively.

597

598

599 **Figure 8.** Analyte recovery (%) in hexane-washed extracts obtained with the different extraction
600 techniques and solvents at a concentration of 100 ng g⁻¹ d.w.

601 (SLE: Solid-liquid extraction (n=2, lettuce leaves fortified with the target analytes), PLE (Blank)
602 (n=1, hydromatrix fortified with the target analytes) and PLE (n=2, lettuce leaves fortified with the
603 target analytes); USE: ultrasound assisted extraction (n=2, lettuce leaves fortified with the target
604 analytes))

605

606

Table 3. Performance of the method for the different target analytes in lettuce leave samples: linearity (coefficients of determination (r^2)), extraction (USE) recoveries, absolute (AR) and relative (RR) method recoveries, method repeatability (RSD), matrix effects, and method limits of detection (MDL) and quantification (MQL).

Compound	Linearity (r^2)	USE recovery ^a (%)	SPE recovery ^b (%)	Method recovery ^c		Repeatability ^d RSD (%)	Matrix effect ^e (%)	MDL ^f (ng g ⁻¹ d.w.)	MQL ^g (ng g ⁻¹ d.w.)
				AR(%)	RR(%)				
ACR*	0.9990	76	56	56/57	58/74	4/5	-70	3.2	10.0
CBZ	0.9986	88	70	78/75	81/97	1/1	-47	0.2	1.0
<i>CBZ-d₁₀</i>				96		4			
CDM**	0.9994	85	78	43/57	99/146	4/4	0	0.2	0.8
COC	0.9992	87	82	53/59	103/119	1/1	-29	3.6	12.0
<i>COC-d₃</i>				51		5			
DCF	0.9992	75	140	97/85	105/110	2/2	-25	0.1	0.5
<i>DCF-d₄</i>				92		5			
IBU	0.9999	83	136	29/25	94/91	8/3	-81	4.8	15.8
<i>IBU-d₃</i>				31		7			
LMG	0.9986	75	53	45/45	102/105	4/2	-57	12.6	42.0
<i>LMG-¹³C₃</i>				44		3			
MDZ	0.9998	66	61	43/43	103/111	1/2	-20	0.6	2.0
<i>MDZ-¹³C₆</i>				42		5			
MTD	0.9990	93	91	55/68	91/119	1/3	13	0.6	2.0
<i>MTD-d₃</i>				60		4			
OxCBZ*	0.9994	83	68	69/57	72/74	2/5	-53	1.4	4.0
TMP	0.9998	82	63	70/63	98/106	2/1	-12	2.2	8.0
<i>TMP-d₃</i>				72		8			
VSA	0.9996	85	30	31/19	96/111	2/9	11	2.0	10.0
<i>VSA-d₄</i>				32		4			
VST	0.9999	77	46	96/61	97/104	1/1	-25	0.4	2.0
<i>VST-d₃</i>				98		3			

*Compound quantified using CBZ-d₁₀ as IS

**Compound quantified using MDZ-¹³C₆ as IS

^a Evaluated at 50 ng g⁻¹ d.w. by comparing peak areas obtained in the on-line-SPE-LC-MS/MS analysis of lettuce extracts fortified before and after the USE extraction.

^b Evaluated at 0.1 ng mL⁻¹ by comparing peak areas obtained in the on-line SPE-LC-MS/MS analysis of HPLC-grade water and LC-MS/MS of a standard solution at equivalent concentration.

^c AR: absolute recovery and RR: relative recoveries calculated at 50 and 400 ng g⁻¹ d.w. (corresponding values separated by “/”) by comparing peak areas obtained in the on-line SPE-LC-MS/MS analysis of lettuce extracts fortified before USE extraction and LC-MS/MS analysis of a standard solution at equivalent concentration.

^d Calculated as the relative standard deviation (RSD) of the analyte recoveries.

^e Calculated at 200 ng g⁻¹ d.w. by comparing peak areas obtained in the on-line SPE-LC-MS/MS analysis of lettuce extracts fortified after USE extraction and on-line SPE-LC-MS/MS analysis of HPLC-grade water spiked at an equivalent concentration (1 ng mL⁻¹).

^f Method limit of detection (MDL): estimated from MS signals observed in the real samples or the recovery samples at 50 ng g⁻¹ d.w.

^g Method limit of quantification (MQL): estimated from MS signals observed in the real samples or the recovery samples at 50 ng g⁻¹ d.w.

3.4. Uptake of selected drugs by lettuce plants

The optimized and validated analytical method was applied to determine the occurrence of selected drugs in the leaves of the lettuce plants participating in the uptake study. The leaves of each individual were analysed in triplicate. Leaves of control plants, i.e., lettuces that were not irrigated with the target drugs, were found to be free of drugs; however, those of experimental plants accumulated to some extent the investigated drugs. The concentrations measured are summarized in Figure 9. All analytes were detected in all investigated samples, but IBU and VSA that were detected only in few plants at concentrations below the MQL. The compounds that accumulated the most were CBZ followed by MTD, CDM, and COC, presenting average concentrations above 100 ng g⁻¹ d.w. in the lettuces analysed in all cases (1497, 475, 215 and 170 ng g⁻¹ d.w., respectively). Since CBZ concentrations exceeded the method linearity range for this compound, samples were diluted and re-analysed. CBZ has been reported by other authors to potentially move inside the plant, and thus it accumulates preferably in plant leaves than in roots (36). The lowest concentrations were observed for ACR, DCF, IBU, VSA and VST with average values below 30 ng g⁻¹ d.w. This could be attributed to a low potential for uptake, translocation or accumulation of these compounds by lettuce plants, due to plant physiology itself or compound degradation in the soil. In the case of DCF, these low concentrations are in agreement with previous studies that reported slightly higher amounts of DCF in plant roots than in leaves (34, 36, 37). Results from this study clearly showed that lettuce is capable to take pharmaceuticals up from soil and water, and to accumulate them in edible parts.

649 **Figure 9.** Average concentration (ng g^{-1} d.w.) of the target analytes after triplicate analysis the six
650 lettuces grown for the uptake study (n=18).

651

652

653 **4. Conclusions**

654 After comparing different techniques to extract drugs from lettuce leaves, a novel analytical method
655 based on USE and SPE clean-up of the extract connected in series to the LC-ESI-MS/MS
656 instrument was optimized and validated for the reliable quantification of 13 drugs. The drugs
657 selected have been reported to be commonly present in wastewater treatment plant effluent water,
658 and consequently they are likely to be taken up into and accumulated by crops. Satisfactory relative
659 recoveries (72-103%) were obtained for all compounds but those for which isotopically labelled
660 analogues were not available acridone, cis-diltiazem and oxcarbazepine, with RSD values below
661 9% in all cases. The validated methodology allows the detection and quantification of the selected
662 compounds in lettuce leaves in the low ng g^{-1} d.w. Thus, it was applied to analyse drug uptake by
663 lettuces grown in soil pots. Results revealed that all investigated drugs could enter lettuce after
664 being irrigated with water fortified (contaminated) with the selected compounds. This work
665 represents the first step to provide novel knowledge on the behaviour of the selected compounds in
666 the water-soil-plant system, as some of the selected compounds are investigated for the first time in
667 plant tissues (i.e., cocaine, methadone, midazolam, cis-diltiazem, valsartan, and valsartan acid)
668 Future work is directed to further investigate the fate of the selected compounds in the plant root
669 system and in the soil, and the effect that a contaminated soil may have on new plant individuals.
670 This future work will contribute to identify the main mechanisms driving the uptake and
671 accumulation of these drugs in the plant, and thereby, to correctly assess the potential risk of
672 consuming edible plants contaminated with these products to human health.

673

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683

684

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688

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