Graphene-derivatized silica as an efficient solid-phase extraction sorbent for pre-concentration of Fluoroquinolones from water samples followed by liquid-chromatography coupled to fluorescence detection

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Abstract

This work presents a novel analytical method based on Graphene for the determination of five widely used Fluoroquinolones (FQs) in aqueous matrices. The procedure entails solid-phase extraction (SPE) on Graphene-derivatized silica (200 mg), followed by liquid chromatography with fluorescence detection. Monolayer graphene oxide (GO) flakes were covalently-bonded onto aminopropyl silica microparticles, and then treated with aqueous hydrazine to obtain the reduced GO (RGO). The final material (RGO-silica) was characterized by thermogravimetric analysis (TGA), scanning electron microscopy (SEM) and BET analysis, and for the first time evaluated as mixed-mode sorbent for the SPE of FQs from natural waters. Accuracy was studied on tap and raw river water spiked at concentrations in the range 10-10000 ng L^{-1}, obtaining mean absolute recoveries from 73 to 118%. The inter-day precision was good, showing RSDs in the range 5-15%. Sample volumes up to 1 L provided enrichment factors up to 200, achieving accurate quantification of concentrations as low as 10 ng L^{-1}. The analytes were simultaneously and quantitatively eluted from the RGO-silica cartridge in a single fraction by using acetonitrile combined to aqueous tetrabutyl ammonium hydroxide. The batch-to-batch reproducibility was verified on independently prepared RGO-silica samples. RGO-silica was advantageous in terms of adsorption capacity and reusability with respect to commercial sorbents; the cartridge proved to be reusable for 10 consecutive extractions, with no significant loss of efficiency (recovery >70%). The analytical procedure was applied to the determination of FQs in actual environmental waters.

Keywords: Emerging pollutants; Fluoroquinolones; Graphene; Sample preparation; Solid-phase extraction; Surface water

1. Introduction

Since the first experimental evidence of its unique electronic properties in 2004 [1], Graphene (GN) has become the most intensively studied material. GN represents a new form of carbonaceous material, consisting of single-layer or few-layer thickness of sp^2-hybridized carbon atoms forming a honeycomb structure. Due to the high surface area and outstanding physical-chemical properties, GN has attracted great interest in the recent years in many research fields, including Analytical Chemistry [2]. In particular, GN appears as the ideal candidate to prepare novel sorbent materials for solid-phase extraction (SPE), a technique that has gained great popularity for trace determination of a variety of analytes in view of its low cost, high enrichment factors and reduced consumption of organic solvents [2]. As described in the recent review by Sitko et al. [2], graphene oxide (GO) and reduced graphene oxide (RGO) – obtained by exfoliation of graphite and from GO reduction, respectively – have been tested for pre-concentration of metal ions and organic compounds, due to the possibility to establish various interplays with the target species, *viz.* π-π stacking, cation-π bonding, electron donor-acceptor interaction, hydrogen bond, hydrophobic interaction etc. With regard to organic analytes (i.e. chlorophenols, phthalate esters and sulfonamides) better performance was observed in respect of carbon nanotubes, graphitic carbon, and commercial sorbents [3-6], owing to the special morphology of GN wherein both sides of the planar sheets are easily accessible for molecular adsorption, enabling fast adsorption equilibrium and analyte elution [4].

A recent work [7] investigated in batch the sorption affinity of RGO/magnetite for two Fluoroquinolone (FQ) antibiotics, reporting adsorption capacities in water around 20 mg g^{-1}. However, to the best of our knowledge, no analytical methods focusing on the use of GN or GN-based materials for SPE of FQs from water are currently available in the literature.

FQs are antibiotics for human and veterinary medicine, endowed with broad activity spectrum against Gram bacteria and good oral absorption. Their occurrence in the environment is issue of great concern and has been assessed in the last years, both in aquatic [8] and soil [9] compartments at concentrations in the ranges

ng-μg L^{-1} and μg-mg kg^{-1}, respectively. The environmental diffusion of these anthropogenic compounds is due to various reasons. First, FQs are excreted in pharmacologically active form [10]; moreover, their removal in wastewater treatment plants (WWTPs) is only partial [11,12], consequently variable quantities of drugs are regularly released into aquatic basins, affecting FQs transformation and removal rates. On this account, FQs can be defined as ‘‘pseudo-persistent’’ emerging contaminants [13]. Additional sources of contamination are the custom of recycling liquid manure from livestock farming and sewage sludge from WWTPs as fertilizers [10], animal medication in fish farming [14] and recycling of sludge for the production of compost, widely employed as soil conditioner/fertilizer [15]. Although photodegradation represents the major natural FQs decontamination pathway both in water [16,17] and soil systems [18,19], the presence of these antibiotics in the environment involves serious threats to the ecosystem and human health due to their ability to stimulate bacterial resistance [20,21], and due to the formation of pharmacologically active photoproducts contributing to the environmental impact [21,22]. For these reasons, FQs environmental monitoring is an important task, requiring accurate and sensitive analytical methods. With regard to water systems, the low concentrations usually detected (from tens ng L^{-1} to few μg L^{-1}) necessitate a pre-concentration step prior analysis, usually performed by reversed-phase SPE on hydrophobic or mixed-mode sorbents such as C18-silica and HLB (hydrophilic/lipophilic-balanced polymer), respectively [8].

On the basis of the current state of the art, we deemed interesting to test GN for SPE of FQs from natural waters. A hybrid material consisting of silica microparticles modified with GN was prepared, characterized by scanning electron microscopy (SEM), thermogravimetric analysis (TGA) and BET measurement, and tested as a fixed-bed SPE sorbent for the simultaneous pre-concentration of five widely employed FQs, *viz.* Ciprofloxacin (CIP), Enrofloxacin (ENR), Levofloxacin (LEV), Marbofloxacin (MAR) and Norfloxacin (NOR), from tap water and not tampered river water. After SPE, separation and quantification was performed by high performance liquid chromatography coupled with fluorescence detection (HPLC-FD). The main figures of merit of the proposed analytical method have been explicated. Cartridge reusability and batch-to-batch reproducibility have been assessed. The analytical procedure has also been applied to the determination of FQs in actual environmental water samples.

2. Experimental

84 *2.1. Chemicals*

85 All the chemicals employed were reagent grade or higher in quality. Graphene oxide (flake size 0.5-5 μm,
86 thickness 1.1 ± 0.2 nm, 80% atomic layer, C 79% O 20%) was purchased from Graphene Laboratories Inc.
87 (New York, USA). Silica (<63 μm, 230 mesh) from Merck (Darmstadt, Germany) was used. CIP, ENR,
88 LEV, MAR, NOR, tetrabutyl ammonium hydroxide (TBAH), anhydrous N,N-dimethylformamide (99.8%)
89 and aminopropyltriethoxysilan (99%) were supplied by Sigma–Aldrich (Milan, Italy). Oasis HLB (200 mg)
90 cartridges were purchased from Sigma-Aldrich and Waters (Milan, Italy), respectively. HPLC gradient grade
91 acetonitrile (ACN) and methanol (MeOH) were purchased by VWR (Milan, Italy). H_3PO_4 (85%, w/w), HCl
92 (37%, w/w), anhydrous NaOH (97%) and hydrazine (≥99%) were obtained from Carlo Erba Reagenti
93 (Milan, Italy). Ultra-pure water (resistivity 18.2 MΩ cm^{-1} at 25°C) was produced by a Millipore Milli-Q
94 system. pH was measured by an Orion 420A pH meter (Thermo Electron, Rodano, Italy). FQs stock
95 solutions of 200 μg mL^{-1} were prepared in MeOH containing 0.1% (v/v) 1 M NaOH and stored in the dark at
96 4°C. FQs working solutions of 0.1-1 μg mL^{-1} in 25 mM H_3PO_4 were renewed weekly. All the laboratory
97 operations involving use of standard solutions were conducted under red light.

98

99 *2.2. Synthesis of RGO-silica*

100 Silica microparticles were firstly silanized to obtain aminopropyl silica (APS). Silica (5 g) was stirred
101 overnight in 300 mL 3% v/v aminopropyltriethoxysilan toluene solution and then refluxed at 100°C for 2 h.
102 Large part of the solvent was removed under vacuum and the remaining suspension was filtered by means of
103 a Büchner funnel on a paper filter; the solid was washed with 200 mL ethanol and then dried at 60°C for 4 h.
104 GO was covalently grafted on silica by a coupling reaction between the amino groups of APS and the
105 carboxyl groups of GO using DCC as the coupling agent, similarly to the procedure by Liu et al. [23]. GO
106 (250 mg) was dispersed in 150 mL anhydrous DMF by sonication (10 min) and then 2.5 g of APS and 100
107 mg DCC were added. The mixture was stirred at 60°C for 24 h. The suspension was filtered and the solid
108 material (GO-silica) was washed with 200 mL ultrapure water followed by 100 mL MeOH to remove any
109 residual coupling agent or unbound GO, and finally dried at 60°C for 4 h. RGO-silica was prepared by
110 reduction of GO-silica with aqueous hydrazine. GO-silica (2 g) was stirred at 90°C for 3 h in 200 mL

aqueous hydrazine (1%, v/v). The solid was collected by filtration, washed with ultrapure water (200 mL) followed by MeOH (100 mL), and finally dried at 60°C for 4 h.

2.3. Characterization of RGO-silica

SEM images were acquired by a Cambridge S360 microscope; powders were inserted in special stubs and coated with gold under low vacuum (2×10^{-2} mbar) to obtain a conductive material (Au layer thickness 10 nm). For BET analyses, carried out using an ASAP 2010 physisorption analyzer (Micromeritics Instrument Corporation), samples were heat-treated at 200°C under vacuum before measurement.

TGA measurements have been performed with TGA 1 Star System Mettler Toledo. Heating rate was 10°C min^{-1} and the range of temperature was from 25°C to 1000°C. Reactive gas was air at the flux of 100 ml min^{-1}. For each experiment about 10 mg of materials were weight, and put in an alumina crucible. TGA furnace was conditioned for 2 h to provide a suitable atmosphere for combustion.

2.4. Water samples

Tap water from the Pavia municipal waterworks (pH 7.7, conductivity at 20°C 271 µS cm^{-1}, Ca^{2+} 35 mg L^{-1}, Mg^{2+} 10 mg L^{-1}, Cl^- 5 mg L^{-1}, NO_3^- 0.6 mg L^{-1}, SO_4^{2-} 5 mg L^{-1}) and surface water collected in July 2014 from Stàffora River (pH 7.5, conductivity at 20°C 430 µS cm^{-1}, Ca^{2+} 62 mg L^{-1}, Mg^{2+} 12 mg L^{-1}, Na^+ 7 mg L^{-1}, K^+ 2 mg L^{-1}, Cl^- 15 mg L^{-1}, NO_3^- 3 mg L^{-1}, SO_4^{2-} 20 mg L^{-1}) were used for recovery tests.

Samples from Po River (pH 7.7, conductivity at 20°C 350 µS cm^{-1}, Ca^{2+} 40 mg L^{-1}, Mg^{2+} 10 mg L^{-1}, Cl^- 12 mg L^{-1}, NO_3^- 8 mg L^{-1}, SO_4^{2-} 30 mg L^{-1}), Ticino River (pH 7.6, conductivity at 20°C 385 µS cm^{-1}, Ca^{2+} 42 mg L^{-1}, Mg^{2+} 8 mg L^{-1}, Cl^- 10 mg L^{-1}, NO_3^- 9 mg L^{-1}, SO_4^{2-} 35 mg L^{-1}), Terdoppio River (pH 7.1, conductivity at 20°C 204 µS cm^{-1}, Ca^{2+} 28 mg L^{-1}, Mg^{2+} 7 mg L^{-1}, Cl^- 8 mg L^{-1}, NO_3^- 6 mg L^{-1}, SO_4^{2-} 27 mg L^{-1}) and a ditch located downstream a swine farm located near Pavia (pH 7.2, conductivity at 20°C 263 µS cm^{-1}, Ca^{2+} 47 mg L^{-1}, Mg^{2+} 9 mg L^{-1}, Cl^- 13 mg L^{-1}, NO_3^- 19 mg L^{-1}, SO_4^{2-} 20 mg L^{-1}) were collected in July 2014 at 30-50 cm depth, directly in amber glass bottles. Samples were stored in the dark at 4°C and analyzed within 24 h.

2.5. SPE procedure

138 The cartridges (6-mL polypropylene tubes) were prepared by placing 200 mg RGO-silica between two
139 polyethylene frits, and were washed under vacuum with 20 mL MeOH followed by 50 mL ultrapure water to
140 remove impurities and minimize void/channeling effect. For the extraction procedure, the cartridge was
141 conditioned with 5 mL MeOH, 5 mL ultrapure water; the water sample (0.1-1 L) was fed to the column at a
142 flow rate of ca. 10 mL min^{-1}, and then the cartridge was dried under vacuum for 5 min. The analytes were
143 simultaneously eluted with 5 mL ACN-50 mM TBAH (30:70), at a flow rate of 1 mL min^{-1}. The extract was
144 acidified with 1 M HCl and injected into the HPLC system. For reusability tests, 5 mL extracting solution
145 were fed to the sorbent between each extraction to avoid any potential carry over.
146
147 *2.6. Chromatography*
148 The HPLC-FD system consisted of a pump Series 200 equipped with vacuum degasser and interfaced with a
149 programmable fluorescence detector (FD) (Perkin Elmer, Monza, Italy). A Supelco 4.6 × 250 mm, 5 μm
150 Ascentis RP-Amide coupled with a similar guard-column was the analytical column. After an equilibration
151 period of 5 min, 50 μL of each sample were injected into the HPLC system. The FD excitation/emission
152 wavelengths selected were 297/507 nm for MAR, 280/450 nm for CIP, ENR, NOR and 280/500 nm for
153 LEV. Isocratic elution was performed in 25 mM H_3PO_4-ACN (87:13), flow rate 1 mL min^{-1}.
154
155 *2.7. RGO-silica SPE followed by HPLC-FD: method validation*
156 Selectivity was evaluated on the basis of the HPLC-FD chromatograms of the SPE extracts obtained by pre-
157 concentration of not tampered blank river water [24-26].
158 Linearity was determined by three independent five-point calibration curves generated for each analyte in the
159 range 1-50 μg L^{-1}; the linearity range is indeed wider [24], but here limited to 1-50 μg L^{-1} to accurately
160 evaluate recovery at the concentrations expected after SPE [8].
161 Matrix effects were investigated by comparing the slopes of the calibration curves obtained in ACN-50 mM
162 TBAH (30:70) to those observed in the SPE extracts obtained from pre-concentration of 500 mL blank river
163 water (matrix-matched calibration), according to literature [26].
164 Method quantification limits (MQLs) were fixed at the lowest concentration that provided acceptable
165 recovery (≥70%) and precision (RSD<20%) [26].

Recovery was evaluated on tap water fortified with different amounts of FQs, in the range 10-10000 ng L^{-1} (n=4), and on blank river water – previously analyzed by a validated procedure [24] – spiked at 10-20 ng L^{-1} (n=3).

The intraday precision (repeatability) was evaluated on independently fortified tap water samples (500 ng L^{-1}, n=5). The inter-day precision (within-laboratory reproducibility) was assessed on tap water (10-10000 ng L^{-1}, n=4) and also on river water spiked at 10-20 ng L^{-1} (n=3).

The batch-to-batch reproducibility was assessed by recovery tests on tap water spiked with 500 ng L^{-1} of MAR and ENR, using two RGO-silica samples independently synthesized following the entire preparation procedure (Section 2.2).

3. Results and Discussion

3.1 Characterization of RGO-silica

The morphology of RGO-silica is shown in Fig. 1. As it is apparent from the SEM images, the semitransparent GN flakes tightly enfold the silica particles, that have irregular shape. Some corrugations typical of monolayer GN sheets are evident (indicated by arrows). These features are consistent with those reported by Liu et al. [23], and were also observed on the second batch of RGO-silica (Fig. 1b), accounting for good reproducibility in the preparation procedure. The SEM images evidence that GN is lying on the silica microparticles, that effectively serve as the support for the GN flakes. Being the material designed for SPE application, this apparent good distribution over silica grains is certainly advantageous because aggregation of GN sheets would otherwise lower the number of active sites available for adsorption.

Surface area values of 243 m^2 g^{-1} and 268 m^2 g^{-1} were determined by BET analysis for the two RGO-silica batches, respectively.

Results from TGA showed the amount of immobilized GN. As reported in Table 1, the weight loss from the GN support (APS) was 12.5 wt%. Taking into account this weight loss, we calculated the amount of GO immobilized on APS, that resulted 8.6 wt%. For RGO-silica a loss weight of 18 wt% was noticed, that corresponds to 5.5 wt% GN. To justify the quantification of GN obtained by the thermal program applied, we verified that GN is quantitatively combusted. Reproducibility of the synthesis has been assessed by

193 replicating the measurements, under the same conditions, on a second batch of RGO-silica (see results for
194 Batch 2).
195
196 *3.2 Solid-phase extraction on RGO-silica*
197 GO is a polar normal-phase sorbent, as it possesses large quantities of epoxy, hydroxyl and carboxyl groups;
198 on the contrary RGO can be applied in reversed-phase SPE, being a non-polar, hydrophobic sorbent with
199 strong affinity for carbon-based ring structures [2]. However, differently from typical hydrophobic sorbents,
200 the presence of residual hydrophilic groups, even after chemical reduction, makes RGO a mixed-mode
201 hydrophilic/lipophilic sorbent with great potentiality for adsorption of a wide polarity-range compounds
202 [2,3].
203 In view of this rationale, we reasoned to evaluate RGO as a SPE sorbent for pre-concentration of FQs,
204 amphoteric molecules consisting of an aromatic quinolone core bearing polar ionisable groups, that in
205 principle are prone to interact with the RGO flakes grafted onto the silica support.
206 The first part of the experimental work was focused to explore the feasibility of using RGO-silica for SPE of
207 FQs from water. The amount of RGO-silica (200 mg) was chosen basing on the most used SPE sorbents
208 commercially available for enrichment of trace contaminants from surface waters [8], and considering the
209 extraction procedures designed for FQs [8]. Larger amounts were not tested because of the high surface area
210 of GN and, consequently, the greater number of adsorption sites compared to commercial phases [3,5,6,23].
211 GN was immobilized on silica microparticles to easily prepare/use typical SPE cartridges, thus avoiding high
212 column pressure or GN losses that can occur when neat GN is used [2].
213 Preliminary recovery tests were carried out on tap water (100-250 mL) spiked with 500-10000 ng L^{-1} of each
214 FQ (for the highest spike the SPE extract was diluted before analysis). The sample pH was initially corrected
215 with HCl to pH ca. 6 due to the highest sorption affinity of RGO as reported by Tang et al. [7]. This however
216 was not a crucial point in the procedure, as the analytes at the environmentally significant concentration
217 explored were as well retained working at the native pH of actual samples (7.0-7.7). Quantitative adsorption
218 of the five analytes was observed on the RGO-silica cartridge, as FQs were not detected (<IDL) in the
219 percolated sample. Since FQs are zwitterionic molecules, acidic or alkaline mixtures have been proposed for
220 their desorption from reversed-phase or mixed-mode sorbents [8]. In this context, differently from the results

previously found on HLB (the most used phase) and C18-silica [24], desorption was only partial (<25%) by using 1-3 × 2.5 mL ACN-25 mM H_3PO_4 (20:80); likewise, ACN-0.01 M NaOH (30:70) failed in desorbing FQs, as recovery did not exceed 40% even collecting 4 eluting fractions. Recovery was lowered by increasing ACN% (data not shown), and not significantly improved in going from 0.01 to 0.5 M NaOH, suggesting this type of eluting mixture was not efficient.

FQs were not eluted by MeOH or MeOH-NH_3 (1-5% v/v) (30:70), solutions frequently used for HLB [8]. These findings, together with the quantitative adsorption of the analytes on the cartridge, indicate that FQs are more strongly retained on RGO-silica than on the two commercial materials. This is reasonably due to the multiple interactions RGO is able to establish with the FQ molecule. First of all an aspecific interaction (Van der Waals forces) with the FQ apolar portions, but especially π-π stacking with the FQ aromatic ring, electrostatic interactions (hydrogen bond) with the polarized FQ groups and cation-π bonding with the protonated FQ side-chain amino group [2,7].

Recovery was strongly improved by using an organic base (TBAH) instead of NaOH or NH_3. TBAH was chosen because able to desorbs FQs from clay minerals [27], matrices showing strong binding affinity toward these drugs. FQs were quantitatively eluted by 2 × 4 mL ACN-50 mM TBAH (30:70), obtaining recovery higher than 73% (see Table 2). Desorption of the target analytes was only partial (<50%) using higher or lower ACN concentrations; omitting ACN, FQs elution was very poor (<10%), highlighting the role of the organic modifier in desorbing such amphoteric compounds. TBAH concentration affected recovery. Indeed, by using ACN-25 mM TBAH (30:70) good recovery was obtained only with 4 elutions; TBAH concentrations higher than 50 mM were discarded because of the excessive viscosity of the extract to be analyzed.

Basing on these results, additional recovery tests were performed at lower concentrations (10-100 ng L^{-1}) on 0.1-1 L samples, achieving recoveries greater than 82% for 10 ng L^{-1} spike (n=4). As an example, a typical chromatogram obtained after SPE on RGO-silica is reported in Fig. 2.

Quantitative recovery and suitable sensitivity were gained also with a single elution (5 mL), thus achieving. pre-concentration factors up to 200. These, higher than those reported in our previous work [24], are suitable for determination of FQs in surface water [28]. Recovery as high as 91% was achieved by SPE of 1 L river

248 water spiked with 10 ng L^{-1} FQs followed by a single elution with 5 mL ACN-50 mM TBAH (30:70), with
249 good interday precision (see Tables 2,4).
250 The quantitative recovery of the five drugs gained also for volumes up to 1 L (10-10000 ng L^{-1} spikes)
251 evidenced a large breakthrough volume for the RGO-silica bed, due to the high affinity of GN for FQs [7].
252 Indeed, it was demonstrated that, for various analytes, e.g. chlorophenols [3], phthalate esters [4,5],
253 sulfonamides [6], GN has higher capacity in comparison with commercial sorbents. As here verified, also for
254 FQs GN showed higher adsorption capabilities than the HLB phase (200 mg). In fact, considerable amounts
255 of analytes were not retained on this commercial sorbent, namely 33%, 21%, 64%, 54% and 46% for CIP,
256 ENR, LEV, MAR, and NOR, respectively (500 mL tap water pH 3, 10 μg L^{-1} FQs). Instead, on RGO-silica
257 adsorption was quantitative, allowing to gain full recoveries (92-106%, n=3).
258 Immobilization of GN onto silica microparticles seemed useful to obtain a hybrid material endowed with
259 good performance in terms of enrichment factor, with no need for evaporation to dryness of the SPE extract.
260 Indeed, 200 mg RGO-silica 5.5 wt% (≡ 11 mg GN) consented to process up to 1 L river water spiked with
261 FQs at concentrations ranging from 10 to 10000 ng L^{-1}, achieving enrichment factors up to 200. Using neat
262 GN (20 mg), Liu et al. found out that for chlorophenols the maximum volume loadable on the cartridge was
263 50 mL, with enrichment factors up to 50 [3]. This may be due to a decrease of available active sites due to
264 aggregation of GN sheets, that instead may result better distributed by immobilization onto a micrometric
265 support.
266 Due to the good chromatographic traces and the negligible matrix effect observed (see Section 3.3), no
267 washing step to remove co-adsorbed matrix compounds from the RGO-silica cartridge was applied before
268 elution of the analytes, also in the case of complex matrices such as not tampered river water. Differently, it
269 is known that the mixed-mode HLB phase does not provide a suitable sample cleanup of actual matrices,
270 despite the quantitative recovery [8]. As discussed by Tamtam et al. [28], using HLB recovery was enhanced
271 in river water working at pH 2, but at the same time considerable co-extraction of matrix constituents was
272 noticed; better selectivity was gained at pH 7 but recovery at the low concentrations levels was not
273 quantitative for most FQs (10-46%). As a matter of fact, to couple recovery and selectivity anion exchanger
274 polymeric sorbents (WAX, SAX) were needed for removing matrix interferences prior pre-concentration of

275 FQs on HLB [24,29,30]. On the other hand, C18-silica was shown to possess the highest affinity for natural
276 organic matter [24], as a proof of its non-selectivity.
277 The high chemical stability of GN, joined to its peculiar characteristics favorable to easy
278 adsorption/desorption processes [3,4], allowed the RGO-silica cartridge to be reused for various day-to-day
279 extractions. Recovery higher than 70% for all analytes was observed for 10 extractions, indicating that also
280 in the case of FQs the efficiency of the GN-based sorbent was not affected by sorbent drying, as evidenced
281 by Liu et al. for chlorophenols [3]. This is advantageous with respect to traditional SPE based on C18-silica,
282 that suffer from a marked decrease of recovery, especially for polar compounds, due to hydrophobic collapse
283 of the C18-alkyl chains on the silica surface [31].
284
285 *3.3 Analytical figures of merit*
286 *3.3.1 Selectivity*
287 Being FQs polar and highly fluorescent molecules, reversed-phase liquid chromatography coupled to
288 fluorimetric detection has habitually been adopted for their determination [8,32]. In fact, considering that
289 FQs are detected as native compounds (not as derivates), information provided by fluorescence spectra is
290 valuable in terms of selectivity [24].
291 Method selectivity was evaluated from the chromatograms of blank water extracts. Fig. 3 shows the profile
292 obtained for a blank river water SPE extract and that of the SPE extract obtained from the same river water
293 sample spiked with 10 ng L^{-1} of each FQ prior pre-concentration (enrichment factor 200). As it is apparent,
294 FQs peaks were well resolved and quantifiable also at these low concentrations; no peaks were noticed at the
295 retention times of the five FQs. This excluded the interference of matrix substances accountable for false-
296 positive signals; moreover, organic matter (retention time < 4 min) did not interfere in the separation of the
297 five drugs.
298 Although FD is not powerful as MS detection in terms of identification, it has been recommended for
299 residues analysis [32] and widely adopted for FQs [8] but also sulfonamides [6] determination in
300 environmental waters as it offers higher selectivity (and sensitivity) than UV detection, that nonetheless is
301 used for trace determination of drugs and pollutants [3,5,33]. Specific detection would be provided by
302 HPLC-MS after SPE.

303

304 *3.3.2 Linearity and matrix effect*

305 To assess potential matrix effects, calibration curves were prepared for each FQ in the SPE eluting solution
306 and in the SPE extracts obtained from pre-concentration of blank river water (matrix-matched calibration), as
307 described in Section 2.7. The linear regression equations, mean of three independent calibration lines,
308 showed good linearity in the range 1-50 μg L^{-1} for the five FQs (R^2>0.995), with slopes not significantly
309 different (p=0.05) in the two matrices (see Table 3).

310 The enhancement or suppression of the FD signal was calculated according to the following equation [26]:

311 $$signal\frac{enhancement}{suppression} = \left[1 - \frac{S_m}{S_s}\right] \times 100$$

312 where S_m is the slope of the calibration curve in the matrix-matched solution, and S_s is the slope found in
313 ACN-50 mM TBAH (30:70) standard solution. Values between 2-9% were observed, therefore matrix effect
314 can be considered negligible, being included in the range of the method precision [34].

315 Optimal linear regression coefficients (R^2>0.99) were obtained by duplicate spiking (in the range 2-10 μg L^{-1})
316 on the SPE extracts.

317 These findings, together with the quantitative absolute recovery found also in raw river water, make SPE on
318 RGO-silica followed by HPLC-FD a suitable analytical procedure for FQs determination in complex
319 matrices, not requiring internal standard correction [26].

320

321 *3.3.3 Sensitivity*

322 In order to correctly value method sensitivity, the MQL was fixed at 10 ng L^{-1}, a concentration that provided
323 accurate determination [26]; consequently, MDLs were estimated around 3 ng L^{-1}. These values are similar
324 to those reported in literature for determination of FQs in river water by SPE on HLB followed by UPLC-
325 MS [28]. Indeed, considering the typical concentrations of FQs in surface waters [8], the method sensitivity
326 is suitable for the determination of FQs in actual samples, also at the low ng L^{-1} levels. For instance, the
327 concentrations measured after SPE of 1 L sample containing 10 ng L^{-1} FQs are around 2 μg L^{-1}, that is a
328 concentration included in the calibration line. Indeed, the present enrichment factors allowed to accurately
329 quantify FQs concentrations as low as 10 ng L^{-1}, obtaining recovery in the range 74-91%, and RSDs<15%.

330

331 *3.3.4 Recovery*

332 All the mean absolute recoveries obtained in water samples spiked at environmentally significant
333 concentrations (10-10000 ng L^{-1}) are gathered in Table 2. As it can be seen, recovery was generally between
334 73% and 118% in tap water; quantitative extraction was also observed for all the five FQs in untreated river
335 water spike with 10-20 ng L^{-1}, with recoveries higher than 74% (n=3).

336

337 *3.3.5. Precision*

338 Precision was evaluated by calculating the residual standard deviations (RSDs) associated to the mean
339 recovery obtained for each concentration level (see Table 4).

340 The intra-day precision (repeatability), calculated for 500 ng L^{-1} spike, showed RSDs <9% (n=5); as shown
341 in Table 4, the inter-day precision (within-laboratory reproducibility) showed RSDs <15% (n=4), for spikes
342 in the range 10-10000 ng L^{-1}; suitable reproducibility was also observed on untreated river water spiked with
343 10-20 ng L^{-1} FQs, obtaining RSDs<15% (n=3).

344

345 *3.3.6. Batch-to-batch reproducibility*

346 The batch-to-batch reproducibility was assessed on two independently synthesized RGO-silica batches
347 (starting from the pristine silica), by comparing recoveries obtained from the pre-concentration of tap water
348 samples spiked with 500 ng L^{-1} of MAR and ENR (Table 5). As apparent, good accordance was found
349 between the mean recoveries observed on the two batches, indicating that the preparation procedure of RGO-
350 silica is reproducible. This is also uphold by the characterization report (Section 3.1).

351

352 *3.4. Analysis of actual samples*

353 The procedure was applied to the determination of FQs in contaminated river/ditch water collected in the
354 Pavia county (Italy). As it is shown in Table 6, variable amounts of drugs were determined, ranging from few
355 tens to hundreds ng L^{-1}.

356 In particular, the highest concentrations of ENR and MAR, the two veterinary drugs among the selected FQs,
357 were found in the ditch sample collected downstream swine farms, and in the Terdoppio River, sampled in

an agricultural zone devoted to intensive cattle breeding. FQs for human use were determined at concentrations of tens ng L^{-1} in Po and Ticino Rivers; typical chromatograms are shown in Fig. 4.

The obtained results evidence that such xenobiotics are effectively released into water basins, in some cases reaching concentrations up to the μg L^{-1} level. Once again this draw attention to the failure of actual WWTPs in removing quantitatively these emerging pollutants, and poses serious concern due to adverse effects of FQs, that is ecotoxicity [35] and promotion of bacterial resistance [20,21].

4. Conclusions

A simple and robust method has been developed for the determination of five widely used FQs in natural waters, using a GN-based SPE sorbent (RGO-silica). This is easily prepared in laboratory with good reproducibility. RGO-silica was packed in conventional SPE columns and exhibited excellent analytical performance, allowing pre-concentration of sample volumes up to 1 L, and elution in a single fraction. Quantitative recovery and suitable reproducibility were observed both in tap and river water, over three-magnitude order concentrations. No washing step before analytes elution was required. Superior adsorption capacity and reusability were observed in comparison with commercial SPE sorbents. RGO-silica maintained its extraction efficiency for various pre-concentrations, and was successfully employed for the analysis of actual surface water samples. The results obtained in the pre-concentration of these emerging pollutants from complex matrices widen the application of GN in sample preparation, and highlight the potentiality of this new member of carbon materials for analytical purposes.

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525 **Figure captions**

526 **Fig. 1.** SEM images of RGO-silica acquired on (a) batch 1 and (b) batch 2; dimension 10.0 μm,
527 magnification 3K, ETH 20.0 KV.

528

529 **Fig. 2.** HPLC-FD chromatogram obtained from pre-concentration of 250 mL tap water spiked with 100 ng L^{-1}
530 of each FQ (SPE sorbent: 200 mg RGO-silica 5.5 wt%).

531

532 **Fig. 3.** HPLC-FD chromatograms overlay of a blank river water (1 L) SPE extract (a) and the SPE extract of
533 the same river water sample spiked with 10 ng L^{-1} of each FQ prior pre-concentration (b); SPE sorbent: 200
534 mg RGO-silica 5.5 wt%.

535

536 **Fig. 4.** HPLC-FD chromatograms obtained from pre-concentration of actual water samples: (a) Ticino River
537 (500 mL), b) Po River (250 mL); SPE sorbent: 200 mg RGO-silica 5.5 wt%.

538

539

540 **Table captions**

541 **Table 1** Results obtained from TGA on two independently synthesized RGO-silica batches.

542

543 **Table 2** Mean absolute recoveries (%) obtained by SPE of tap and river water samples spiked with 10-10000
544 ng L^{-1} FQs.

545

546 **Table 3** Calibration curves obtained for each FQ in SPE extracting solution and blank river water SPE
547 extract (matrix-matched calibration).

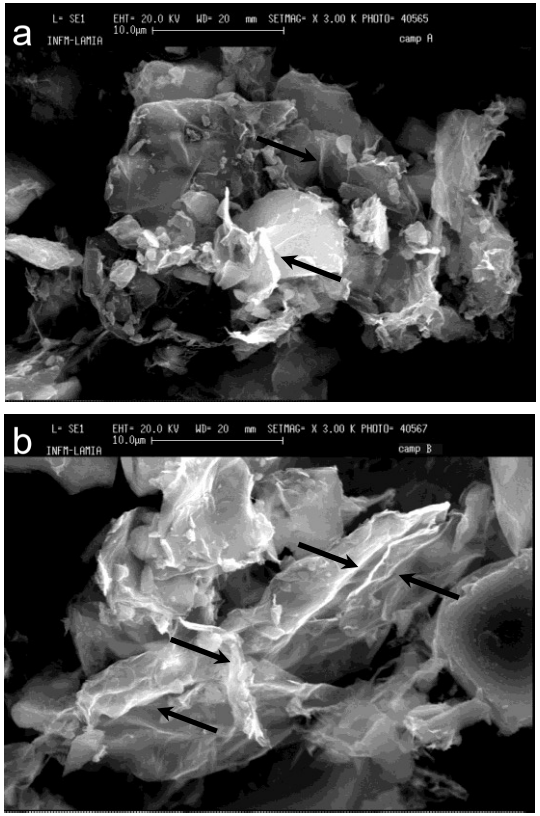
548

549 **Table 4** Values of RSD (%) for the inter-day precision (within-laboratory reproducibility) obtained by SPE
550 of tap and river water samples spiked with FQs.

551

552 **Table 5** Mean absolute recoveries obtained on two RGO-silica batches by SPE of tap water samples spiked
553 with MAR and ENR (500 ng L^{-1}).
554
555 **Table 6** FQs amounts determined in actual surface water samples by SPE on RGO-silica (200 mg, 5.5 wt%)
556 followed by HPLC-FD.

Figure 1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INFM-LAMIA
camp B

Figure 2

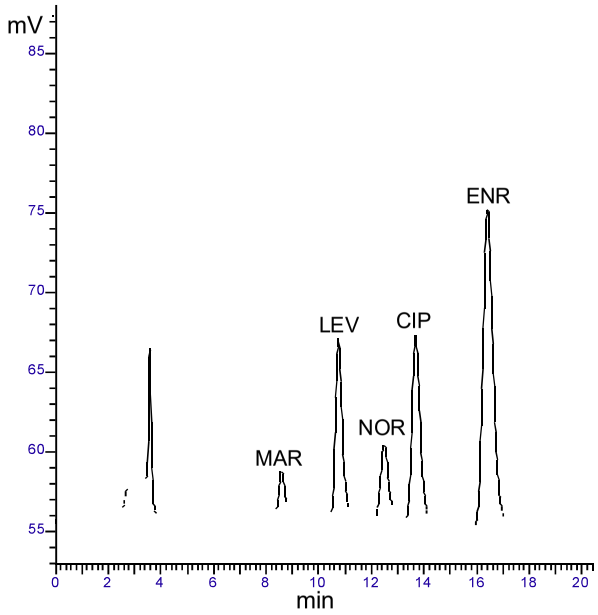
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Figure 3

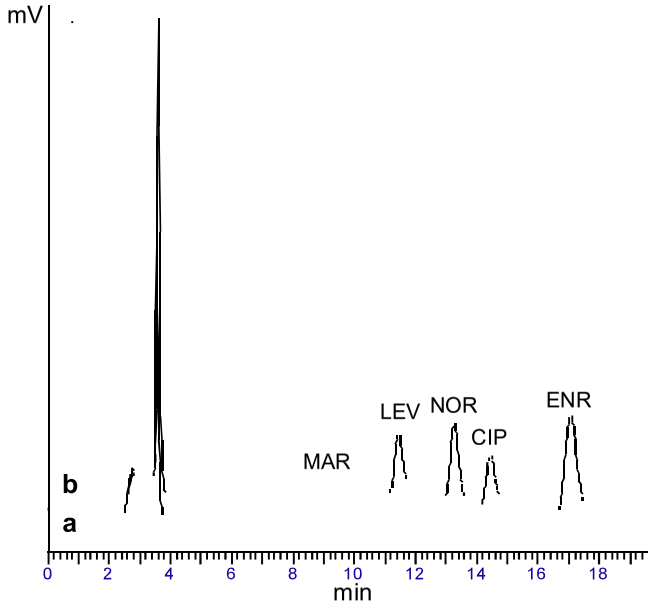

Figure 4

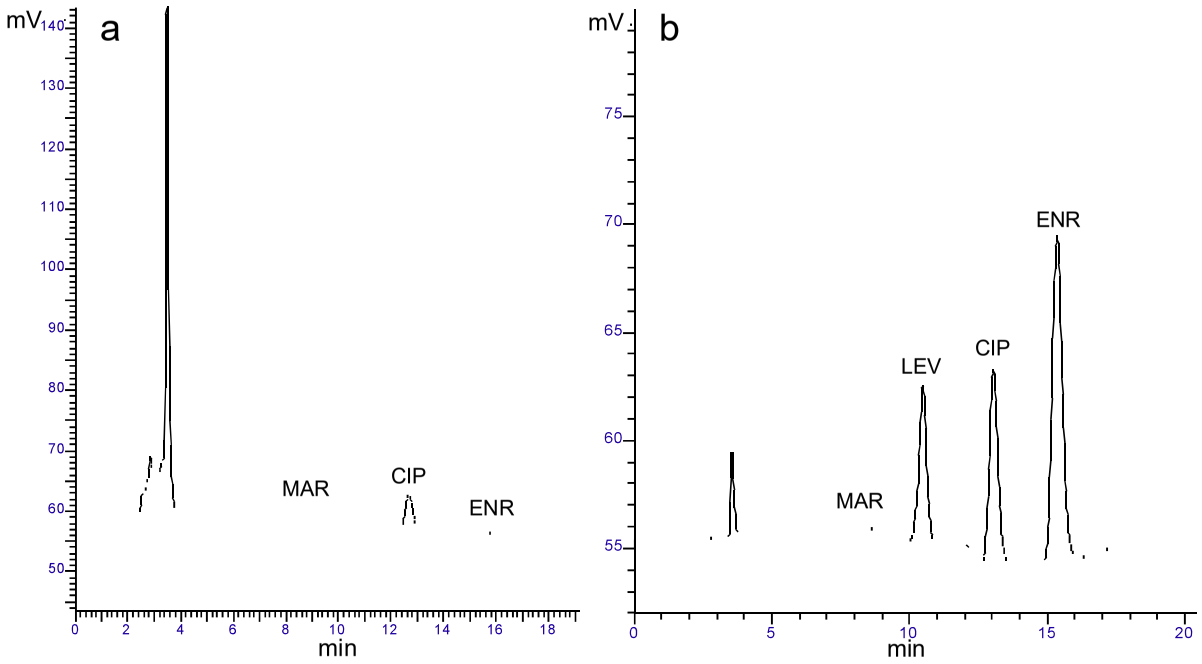
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Table 1

	Batch 1		Batch 2	
	Weight loss (%)	% GN	Weight loss (%)	% GN
APS	12.5	-	12.5	-
GO/silica	21.1	8.6	20.1	7.6
RGO/silica	18.0	5.5	18.0	5.5
GO	99.7	100	99.7	100

Table 2

	Mean absolute recovery (%) obtained on RGO/silica							
	Tap water (n=4)						River water (n=3)	
Spike (ng L^{-1})	*10000*	*1000*	*500*	*100*	*20*	*10*	*20*	*10*
CIP	108	89	73	73	77	82	79	74
ENR	113	92	83	85	100	83	99	87
LEV	118	95	84	98	110	93	99	88
MAR	94	86	79	81	76	98	88	89
NOR	118	94	87	103	107	92	109	91

Table 3

	ACN-50 mM TBAH (30:70)		Matrix-matched solution	
	Equation[a]	R^2	Equation[a]	R^2
CIP	y=85455(1824) x + 29221(20920)	0.997	y=93696(5796) x - 12610(76741)	0.996
ENR	y=150268(4558) x + 58284(52269)	0.998	y=154712(11202) x - 121187(148330)	0.995
LEV	y=74963(1245) x + 4126(14275)	0.999	y=71335(2269) x - 20383(30040)	0.999
MAR	y=13588(356) x + 1863(4083)	0.999	y=14463(919) x - 10495(12169)	0.996
NOR	y=102200(5783) x + 45183(66322)	0.995	y=104323(6353) x - 38822(84127)	0.996

[a] Calculated as peak area (y) vs. FQs concentration (x); in parentheses slope and intercept uncertainties obtained by OLLSR.

Table 4

	RSD (%) for the inter-day precision (within-laboratory reproducibility) obtained on RGO/silica							
	Tap water (n=4)						River water (n=3)	
Spike (ng L^{-1})	*10000*	*1000*	*500*	*100*	*20*	*10*	*20*	*10*
CIP	9	7	10	9	15	11	12	14
ENR	10	10	7	10	11	14	12	11
LEV	5	9	6	12	14	10	13	15
MAR	6	8	5	13	7	7	10	12
NOR	8	6	5	13	13	11	11	13

Table 5

	Mean absolute recovery (%), p=0.05	
	Batch 1[a]	*Batch 2*[b]
ENR	97 ± 7	83 ± 8
MAR	94 ± 9	81 ± 5

[a] (n=8).
[b] (n=5).

Table 6

Sample	Mean FQs concentrations (ng L^{-1}) [a]				
	CIP	ENR	LEV	MAR	NOR
Po River	39	40	42	29	<MQL
Terdoppio River	28	504	<MQL	298	<MQL
Ticino River	18	<MQL	<MQL	13	<MQL
Farm ditch	<MDL	4675	<MDL	870	<MDL

[a] RSDs<13% (*n*=3); duplicate spikes were performed on each SPE extract.