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Simultaneous analysis of chlorophenols, alkylphenols, nitrophenols and cresols in wastewater effluents, using solid phase extraction and further determination by gas chromatography–tandem mass spectrometry

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Abstract

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Keywords: Phenols, wastewater, ~~solid phase extraction (SPE),~~ gas chromatography-mass spectrometry (GC-MS), standard addition calibration, matrix-matched calibration.

Formatted: None

1. Introduction

Phenolic compounds can be found in wastewater (WW) effluents via different sources. They can be detected in this type of samples because of their use in plastics [1], drug manufacturing, phytosanitary products or leather coloring [2], by anthropogenic emission [2] ~~or~~and by the use of treatments with aerobic or anaerobic microorganisms [4]. Some phenols show high toxicity, estrogenic [5] and anti-androgenic activity [6], and~~as well as~~ they can act as endocrine disrupters [7].

Phenols can be classified in a wide range of families. The most studied analytes in water are chlorophenols (CPs) [1] and alkylphenols (APs) [8]. However, the United States Environmental Protection Agency (US EPA) classifies CPs, nitrophenols and APs as priority pollutants [9] and it has established a maximum contamination level (MCL) for pentachlorophenol (PCP) of 1 μg L^{-1} in drinking waters [10]. On the other hand, the European Union (EU) has adopted a list of priority substances in the field of water policy, including 4-n-nonylphenol (4-n-NP), 4-tert-octylphenol (4-tertOP) and PCP [11]. Furthermore, maximum allowable concentrations (MAC) have been established for NP (2 μg L^{-1}) and PCP (1 μg L^{-1}) in inland and other surface waters [12]. However, it must be pointed out that legislation for WWs is still very scarce, and the values established in drinking water are usually used as guide in WWs. Bearing in mind these facts, the development of sensitive analytical methodologies for the simultaneous determination of phenols belonging to different groups, such as CPs, APs, nitrophenols (NTPs) and cresols (also known as methyl-phenols) with different polarity range (log K_{ow} 1.77-5.01) is needed in order to provide a complete overview of the occurrence of phenolic compounds in WW effluents.

Several extraction techniques have been applied for the extraction of phenols from aqueous samples, such as solid-phase extraction (SPE) [8,13-15] and liquid-liquid extraction (LLE) [16]. Recently, microextraction techniques, such as solid-phase microextraction (SPME) [17-19], stir bar sorptive extraction (SBSE) [19-21], liquid phase microextraction (LPME) [22] or dispersive liquid-liquid microextraction (DLLME) [23] have been appliedused. However, most of them have only been used for the simultaneous analysis of only one or few phenols belonging to the same family such as APs [18,20] and CPs [17]. It is well-known that SPE is the most used technique in water analysis [24] due to the lessreduced expositionure and contamination by organic solvents, the high pre-concentration factors that make possible to avoididing evaporation steps, the semi-automation of the extraction process, reducing the sample handling, and it allows the extraction of compounds with different physico-chemical properties. The application of microextraction techniques is increasing but several disadvantages, such as cost and lifetime of fibers and bars, or the limited scope for a wide polarity range can hinder their utilization.

For the determination of phenolic compounds, gas chromatography (GC) [13,25] or liquid chromatography (LC) [26,27] are the predominantthe most used techniques, mainly coupled to tandem mass spectrometry (MS/MS) [28-31]. When GC is used, a derivatization step is required in order to improve the chromatographic performance and sensitivity of the selected compounds, and several derivatizating reagents can be applied [32,33].

A well-known critical point in the analysis of WW is the matrix effect [34]. In order to minimize it, different calibration methods such as matrix-matched calibration

(MMC) [33,35], standard addition calibration (SAC) [34] and the use of isotope-labeled internal standards [36,37] have been employed for complex matrixes. Quantification based on isotope-labeled internal standards has disadvantages due to the expensiveness of these standards and their limited availability. MMC is often ~~the most~~ used quantification method in trace analysis. However, the lack of blank matrixes and the need for storing them can make this approach logistically onerous and not necessarily accurate. SAC is the most adequate technique to use when it is difficult to find ~~a~~ blank samples of the studied matrix ~~studied~~, but a calibration set is required for each sample, increasing the total number of injections and the time spent in data processing.

Another problem related to the determination of phenols in WW is that depending on the type of WW treatment, WW effluents can have different amounts of suspended particulate matter (SPM). This SPM is normally discarded during the extraction process by filtration in most of the analytical methods reported in literature [38]. However, a recent study [35] has demonstrated that certain analytes can be retained in the SPM, depending on its polarity. Therefore, it should be necessary to evaluate the presence of phenols in both phases in order to determine whether the SPM must be discarded or not.

Furthermore, it must be pointed out that many articles reporting simultaneous extraction and determination of different classes of phenols (including APs, CPs and, NTPs) in water [39,40] can be found. However, they have been developed for the analysis of this type of compounds in surface water, and they are not valid for the analysis in WW samples, due to they are more complex matrices with different physico-chemical characteristics (SPM levels, organic matter, etc.).

Therefore, ~~I~~in this study, a simultaneous SPE extraction ~~by SPE~~ and determination of different phenolic families ~~(, namely~~ CPs, NTPs, cresols and APs~~),~~ has been developed for WW effluent samples. In addition two novel aspects of this work must be pointed out: (i) a study of the presence of phenolic compounds in the SPM according to the strategy recently proposed by Barco-Bonilla et al [35], and (ii) a comparison of MMC and SAC in order to evaluate the best quantification strategy of phenolic compounds in complex matrices such as WWs. For that, ~~A study of the presence of phenolic compounds in the SPM has been carried out according to the strategy recently proposed by Barco-Bonilla et al [35]. Due to the complexity of the matrix and the difficulty to obtain blank samples, a study of quantification study using MMC and SAC was developed in order to evaluate the best quantification strategy. T~~two different WW effluents were studied individually: membrane bioreactor (MBR, low SPM content) and anaerobic pond (ANAP, high SPM content). The optimized SPE and quantification method was validated in both types of WWs effluent samples.

2. Experimental

2.1. Chemicals and materials

Phenolic compounds standards, 2-nitrophenol (2-NTP), 4-nitrophenol (4-NTP), 2,4-dimethylphenol (2,4-DMP), 2-CP, 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-diCP), 2,4,5-trichlorophenol (2,4,5-triCP), 2,4,6-trichlorophenol (2,4,6-triCP) and 4-n-NP were obtained from Fluka (Buchs, Switzerland). On the other hand, 3-nitrophenol (3-NTP), 4-chloro-3-methylphenol (4-C-3-MP), 4-tertOP and PCP were supplied by Supelco (Bellefonte, PA, USA). Purities were always >97%. A standard

140 solution (100 mg L^{-1}) of isotopically labeled PCP ([$^{13}C_6$]-PCP) was used as internal
141 standard (IS) and it was obtained from Dr. Erhenstofer (Augsburg, Germany). Stock
142 standard solutions of individual compounds (with concentrations ranging from 200 to
143 450 mg L^{-1}) were prepared by exact weighing of the powder or liquid and dissolution
144 in 50 mL of acetone. These solutions were then stored under refrigeration (T <5 ºC).
145 A working standard solution of the 13 phenolic compounds (2 mg L^{-1} of each
146 compound) was prepared by appropriate dilution of the stock solutions with acetone,
147 and it was stored under refrigeration (T <5 ºC). A working standard solution of [$^{13}C_6$]-
148 PCP (22 mg L^{-1}) was prepared by appropriate dilution of the standard solution with
149 acetone and stored under the aforementioned conditions. HPLC-grade methanol
150 (MeOH), anhydride acetic acid (AAA) (99.9%), and pyridine (Py) (99.8%) were
151 purchased from Sigma-Aldrich (Madrid, Spain). Acetone and hydrochloric acid (HCl)
152 were obtained from J.T. Baker (Deventer, Netherlands). Dichloromethane (DCM) was
153 purchased from Riedel-de Haën (Seelze-Hannover, Germany). Ultrapure water was
154 obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).
155 Thirty mm cellulose filters and 47-mm glass microfiber filters from Whatman
156 (Maidstone, England, UK) and 0.45-µm HNWP nylon membrane filters from
157 Millipore (Carrigtwohill, County Cork, Ireland) were also available for filtration
158 stages. For SPE, Oasis HLB (200 mg, 6 cm^3) cartridges were obtained from Waters
159 (Milford, MA, USA).

160

161 *2.2. Apparatus*

162 A GC system Varian 3800 (Varian Instruments, Sunnyvale, CA, USA) equipped with
163 electronic flow control was interfaced to a 1200L triple quadrupole (QqQ) mass

spectrometer. Samples were injected into an SPI/1079 split/splitless programmed-temperature injector using a Combi Pal (CTC Analytics AG, Zwingen, Switzerland) with a 100-µL syringe. A fused-silica untreated capillary column (2 m × 0.25 mm i.d.) from Supelco was used as pre-column connected to a VF-5 ms Factor Four capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) purchased from Varian. Helium was used as carrier gas (99.9999%) at a constant flow rate of 1 mL min^{-1}, and argon (99.999%) was used as collision gas. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV. The mass spectrometer was calibrated every four days with perfluorotributylamine. Varian Workstation software was used for instrument control and data analysis.

A Reax-2 rotary agitator from Heidolph (Schwabach, Germany) was used for agitation of the derivatization mixture. An analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland) and a rotary evaporator R-114 (Büchi, Flawil, Switzerland) were used during extraction and standard preparation. The horizontal shaker used in the distribution study was obtained from P-Selecta (Selecta, Barcelona, Spain).

2.3. Sampling

WW urban effluents from two different treatments, namely, MBR and ANAP, with low and high SPM content respectively, were collected from WW treatment plant (WWTP) of the foundation Centre for New Water Technologies ("Centro de las Nuevas Tecnologías del Agua", CENTA, Seville, Spain). This WWTP has 41,000 m^2 and it currently holds more than 20 systems with different technologies. Additional physicochemical data related to the treatments evaluated in this study can be found in

[35]. WW effluent samples were stored at 4 ºC and processed within 5 days after collection. In the MMC experiments, and ~~D~~due to the difficulty of finding ~~real~~WW effluent blank samples~~ of WWs effluents, during the optimization and validation stage~~, the corresponding signal of the blank was removed from the MMC plot in those samples where analyte signal was observed. ~~used as blank containing some of the analytes, the corresponding signal was removed from the MMC plot~~~~non spiked samples were used and they are named "blank" samples throughout the text, despite phenolic compounds could be found.~~

2.4. Distribution study

Non-filtered WW effluent samples were spiked with 0.5 µg L^{-1} of the studied phenolic compounds, and then, they were agitated overnight at a rate of 100 oscillations per min to allow a thoroughly interaction between the analytes and both phases of WW (aqueous phase and SPM). After this, samples were filtered to separate and analyze both phases. The aqueous phase was extracted by SPE, whereas for the analysis of the SPM, a method developed by Padilla-Sánchez et al. [33] for the extraction of phenolic compounds in agricultural soils was employed. The distribution of the compounds between both phases was determined as the percentage of them present in each phase.

2.5. GC-QqQ-MS/MS

Aliquots of 10 µL were injected into the GC system operating at a syringe injection flow rate of 10 µL s^{-1}. The injector temperature program was as follows: 70 ºC (hold

for 0.5 min) → 310 ºC (100 ºC min^{-1}, hold for 10 min). The injector split ratio was initially set at 10:1. Splitless mode was switched on at 0.5 min until 3.5 min. At 3.5 min, the split ratio was 100:1 and at 10 min, 20:1. The column oven program was as follows: 70 ºC (hold for 3.5 min) → 300 ºC (20 ºC min^{-1}) → 300 ºC (hold 4 min). Cryogenic cooling with CO_2 was applied when the injector temperature was 170 ºC. The total running time was 19 min.

The QqQ mass spectrometer was mainly operated in the selected reaction monitoring (SRM) mode, although selecting ion monitoring (SIM) mode was also used for confirmation purposes. The electron multiplier was set +200 V above the optimal value indicated by the software instrument. The temperatures of the transfer line, manifold and ionization source were set at 300, 40 and 265 °C, respectively. The optimal values for the scan time ranged from 0.132 to 0.240 s~~ec~~. Peak widths of *m/z* 2.0 and 1.5 were set in the first (Q1) and third quadrupole (Q3), respectively. The optimized MS/MS parameters are indicated in Table 1.

2.6. SPE extraction and derivatization procedure

~~250 mL of~~ WW effluent sample~~s~~ were filtered consecutively (250 mL) using two different pore-size filters (47-mm glass microfiber filters and 0.45-µm nylon membrane filters). The filtered WW effluents showed pH values between 7.7 and 8.3. Then, pH was adjusted to 2.5-2.7 with HCl (2 ~~N~~M) to ensure the protonated form of the phenolic compounds, facilitating the absorption into the solid phase, and an adequate ~~good~~ preservation of the samples. The Oasis~~ASIS~~ HLB cartridges were conditioned with 5 mL of acetone followed by 5 mL of MeOH and 3 x 5 mL of ultrapure water without allowing the cartridges to dry out. Then, the filtered WW

sample (250 mL) was passed through the cartridges under vacuum at a flow rate of 10 mL min^{-1}. The cartridges were dried for 2 h and the phenolic compounds were eluted sequentially with 3 mL of acetone and 2 mL of DCM. The extracts were collected into 5-mL volumetric flasks, adjusting the total volume with DCM, without any evaporation step. Then, the derivatization stage was performed according to the procedure described by Padilla-Sánchez et al. [33]. Briefly, 860 µL of the extract were transferred to a 2-mL vial and 20 µL of [$^{13}C_6$]-PCP (IS), 20 µL of Py and 100 µL of AAA were added to carry out the derivatization reaction. The mixture was shaking in a rotary agitator for 2 min and then injected directly to the GC-QqQ-MS/MS system.

3. Results and discussion

WWs can be submitted to different treatments, obtaining effluents with a variety of SPM contents, and thus, WW effluents can present different physico-chemical properties. When an analytical method is developed for this type of samples, this diversity should be taken into account. In order to cover a wide range of WW effluents, two types of them were evaluated, MBR and ANAP, which have low and high SPM content [35], respectively. The optimization of the extraction procedure as well as the quantification methods, were evaluated in both types of WW effluents. For that purpose, a GC-QqQ-MS/MS method recently developed [33] was applied.

3.1. Extraction method

For the optimization of the SPE procedure, a methodology reported by Pothitou et al. [8] was first considered. This study reported the determination of only one family of phenolic compounds, APs, using Oasis HLB cartridges and acetone as elution solvent. Besides, certain problems regarding the evaporation stages have been previously reported [33], and therefore, the extraction method was designed without any evaporation step. Since the families of phenolic compounds included in this study showed a wide polarity range, several elution solvents were tested to achieve a simultaneous extraction [8]. Acetone (5 mL), DCM (5 mL) and a sequential elution with acetone (3 mL) and DCM (2 mL) were tested. Bearing in mind that evaporation steps were not included in the extraction procedure and aliquots of the extracts are directly injected in the chromatographic system, the elution solvent could be partially retained in the solid phase or evaporated during the elution step. This can provoke an overestimation of the final concentration in relation to the theoretical value, obtaining high recovery values. In order to avoid this, 5-mL volumetric flasks were used to collect the extracts and the final volume was adjusted to 5 mL with the corresponding solvent used during the elution step. The obtained results are shown in Figure 1 and it can be observed that acetone provided adequate results for all compounds, except for 2,4-dMP and 4-n-NP. When DCM was used, recoveries higher than 120% were obtained for 2-CP, 2-NTP and 4-CP, although recovery for 4-n-NP was improved. Consequently, in order to obtain good recoveries for all the compounds, a sequential elution with acetone and DCM was tested. In general, this elution improved the recovery rates, especially for 2,4-dMP, 2-CP, 2-NTP and 4-CP. Nonetheless, recoveries between 50-60% may be accepted extraordinarily in environmental analysis whenever the precision values are adequate (<–30%). Therefore, further experiments were carried out using the sequential elution with acetone (3 mL) and

DCM (2 mL) as elution solvents. Finally, a total ion chromatogram (TIC) of an extracted spiked WW sample at 50 μg L^{-1} is showed in Figure 2.

3.2. Distribution study

Once the extraction method was optimized for the analysis of the aqueous phase of WW effluents samples, a distribution study is needed to verify whether the phenolic compounds are also present in the SPM. If phenolic compounds are present quantitatively in the SPM, the analysis of WW effluents should not be limited to the aqueous phase. The distribution study was therefore carried out, applying the approach described in Section 2.4 for both type of samples. It was observed that only the phenolic compounds with high log K_{ow} were found in the SPM, but at negligible percentages (<5 %). On the contrary, phenolic compounds with lower log K_{ow} where not found in the SPM (data not shown). Taking into account this result, further experiments were limited to the analysis of the target analytes in the aqueous phase, discarding the SPM phase. These results are in accordance with a previous study [35] reporting that polar compounds were not retained in the SPM.

3.3. Evaluation of the quantification method: comparison of MMC and SAC

Due to the complexity of the matrix and the difficulty to find blank WW samples, a study of the quantification of target compounds was proposed. For this aim, a comparison between SAC and MMC in WW effluents obtained by two different WW treatments (MBR and ANAP) was carried out. The study was performed using spiked and blank samples of WW effluents for SAC and MMC respectively, and calibration

curves were prepared in the range 10-150 µg L^{-1}, except for 2-NTP (10-300 µg L^{-1}), and 3-NTP and 4-NTP (100-300 µg L^{-1}). For SAC, a WW sample was spiked and the calibration levels were prepared after submitting the sample to the extraction procedure. For MMC, the calibration plot was prepared using blank extracts. Recoveries were evaluated using spiked samples at 0.5, 1 and 5 µg L^{-1}, taking into account the MCLs and MACs established by the EPA and the EU for these compounds [10,12]. Although, conventional criteria for the analysis of contaminants in foods demands an average recovery between 70% and 120%, bearing in mind the nature of the samples under study, it is possible to increase the recovery range to 60–120%, providing that the RSD values are <30% [35]. ~~Suitable r~~Recoveries were considered adequate when~~if~~ they ranged from 60 to 120%. Intra and inter-day precision was expressed as relative standard deviation (RSD, *n*=5), and they were determined by analyzing spiked samples during the same day and in different days, respectively. Good precision values were considered if RSDs were lower than 30%. The obtained results when both calibration procedures were applied are shown in Table 2 and 3 for the two types of WW effluents evaluated. –Figure 3 shows a comparison between SAC and MMC curves of 4-tertOP for ANAP (Figure 3a) and MBR (Figure 3b). It can be observed that for ANAP, which has high SPM content, the slope of the MMC curve was higher than the SAC slope. On the contrary, for MBR, which has lower SPM content, the slopes obtained by MMC and SAC were similar. This can be explained due to ANAP is a “dirty” WW effluent because of the high SPM content and this fact may affect the repeatability of the slopes in MMC curves, which may influence in the obtained results for ANAP when ~~applying~~ MMC is applied.

Recovery and precision were evaluated using both quantification approaches. It can be observed that in WW effluents with high SPM, such as ANAP, MMC did not provided adequate results for the lower spiked concentrations (0.5 and 1 μg L^{-1}). Recoveries and intra and inter-day precision of most of compounds were below 60% and over 30%, respectively for these two concentration levels. On the contrary, for 5 μg L^{-1}, recovery values were in the range 60-120%, except for 4-n-NP (51%) and intra and inter-day precision were <12%. These results (Table 2) suggested that MMC is not a suitable option for the adequate quantification of at very low concentrations of phenols in WWs effluents with high SPM. On the other hand, when SAC was used, recoveries of all compounds were in range 60-125%, except for 4-tertOP (135%) at the lowest fortification level (0.5 μg L^{-1}). Intra and inter-day precision values were <27% and <31% for all compounds, respectively. As it is shown in Table 2, the SAC approach is more appropriate for WW effluents with high SPM content. Linearity was studied in the range 10-150 μg L^{-1} (except for NTPs which was 100-300 μg L^{-1}) and the obtained determination coefficients (R^2) were in the range 0.9912 (3-NTP)-0.9999 (2-CP, 2,4,5-tTriCP, PCP and 4-n-NPvarious compounds) for ANAP (Table 4).

For WW effluents with low SPM, such as MBR (Table 3), the recoveries obtained when MMC was used for the three levels assayed ranged from 62-119%, except for 4-n-NP, with recoveries lower than 56%. Despite the adequate recovery results provided by MMC for all the studied fortification levels, in general, RSD values were <30% only for the highest spiked level studied (5 μg L^{-1}), as it can be observed in Table 3, whereas at the lowest concentration levels evaluated (0.5 and 1 μg L^{-1}), intra and inter-day precision ranged from 22 to 113%. On the other hand, the application of SAC on MBR WW samples yielded recovery values in the range 70-120%, except for 4-CP (125%) at 0.5 μg L^{-1}. Besides, RSD values were always <28% for intra-day

354 precision and <27% for inter-day precision in all cases, except for 2-NTP, which was
355 41% at 0.5 μg L^{-1}. In consequence, it can be concluded that for MBR treated WW
356 effluents, SAC was also the most suitable method for an adequate quantification of
357 WW effluents with low SPM content, such as MBR WW samples (Table 3).
358 Furthermore, linearity was also evaluated for MBR and R^2 values ranged from 0.9943
359 (4-NTP) to 0.9999 (2-CP, 4-CP, 2,4,6-triCP and 4-n-NP).

360 Considering these results, the SAC method should be applied for a reliable
361 quantification of phenols in WW effluents samples to compensate matrix effects on
362 the signal variation during detection and this does not depend on the SPM content of
363 the WW. The SAC methodology was therefore applied for the quantification of
364 phenols in real samples.

365

366 *3.4. Estimation of the lower limits of the methodology*

367 Despite of the estimation of the trueness and precision carried out in the previous
368 section, other performance characteristics of the method, such as limits of detection
369 (LODs) and quantification (LOQs) were studied. LODs and LOQs were determined
370 as the lowest concentration level that yielded a signal-to-noise (S/N) ratio of 3 and 10,
371 and they are shown in Table 4. LODs and LOQs were determined in WW sample
372 blanks for each phenolic compound studied. LODs were from 0.01 to 1 μg L^{-1} and
373 LOQs ranged from 0.03 μg L^{-1} to 2.5 μg L^{-1} for ANAP and MBR (Table 4). It must
374 be noticed that similar values were obtained for both types of WW effluents, except
375 for 2-NTP and 4-C-3-MP, which showed higher LOD and LOQ values in ANAP than
376 in MBR. This could be explained taking into account that the SPM content is higher

in ANAP, increasing the amount of co-extracted material and affecting the estimation of the lower limits of the method.

3.5. Application to the analysis of real WW effluent samples

The developed methodology was applied to the analysis of 8 WW effluent samples from the CENTA, obtained after the application of different WW treatments employed in this WWTP. To assure the quality of the results and avoid errors, the quantification of the phenolic compounds was achieved using the SAC approach. An internal quality control (IQC) was performed consisting of the analysis of spiked blank WW samples at 1 μg L^{-1} (except for 3-NTP and 4-NTP at 5 μg L^{-1}), which were used to assess the extraction efficiency and a SAC calibration curve to check linearity and sensitivity. Several phenolic compounds were found over the LOQs established by the method, showing the obtained results in Table 5. 2-CP and 2,4,6-triCP were found in six and five samples, respectively, with concentrations ranging from 0.04 to 0.20 μg L^{-1} for 2-CP and from 0.05 to 0.10 μg L^{-1} for 2,4,6-triCP. 4-CP and 4-tertOP were found in four samples, and the concentrations ranged from 0.04 to 0.08 μg L^{-1} and 0.04 to 0.16 μg L^{-1} respectively. 2-CP, 2,4-DiCP, 4-tertOP, PCP and 4-n-NP were found simultaneously in one of the samples (Table 5). It must be highlighted that phenolic compounds were not found over the MCLs and MACs established by the EPA and the EU for these compounds [10,12]. Finally, Figure 4 shows a positive sample of 4-tertOP detected in a WW effluent sample at 0.12 μg L^{-1}.

5. Conclusions

A single extraction method for the simultaneous extraction of CPs, APs, NTPs and cresols in WW effluent samples has been developed using SPE. A distribution study of the phenolic compounds between the aqueous phase and the SPM was carried out, verifying that the SPM could be in fact discarded during the extraction since only phenolic compounds with high log K_{ow} were found in the SPM at a negligible percentage. Due to the difficulty to find WW blank samples and to have good accuracy in the quantification, a study using MMC versus SAC was performed in two different treated WW effluent samples (ANAP and MBR) showing that SAC is the most suitable quantification approach. The method was validated studying recovery, intra and inter-day precision, lower limits (LODs and LOQs) and linearity. Determination of the analytes was carried out using GC-QqQ-MS/MS operating in SRM mode. The method was applied to WW effluent samples with satisfactory results, observing that phenols of several families were simultaneously detected in WW effluents, highlighting the potential of analytical methods that allows the simultaneous determination of several classes of phenolic compounds.

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506 **Figure Captions**

507 **Fig. 1.** Comparison of the recovery values obtained applying different elution solvents
508 for the extraction of spiked WW samples at 0.5 μg L^{-1}. Abbreviations: DCM:
509 dichloromethane; Sequential: sequential elution.

510 **Fig. 2.** Total ion chromatogram (TIC) of an extracted spiked WW sample (5 μg L^{-1})
511 obtained by GC-QqQ-MS/MS. For compound abreviations, see Table 1.

512 **Fig. 3.** Calibration curves in the range 10-150 μg L^{-1} for 4-tertOP when SAC and
513 MMC were used: a) ANAP; b) MBR. Abbreviations: ANAP: anaerobic pond; MBR:
514 membrane bioreactor; MMC: matrix-matched calibration; SAC: standard addition
515 calibration; 4-tertOP: 4-tertoctylphenol

516 **Fig. 4.** Selected-reaction monitoring (SRM) (a) chromatogram and (b) MS/MS
517 spectrum of 4-tertOP (0.12 μg L^{-1}) found in a real WW sample and (c) SRM
518 chromatogram and (d) MS/MS spectrum of a SAC standard (50 μg L^{-1}).

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Reviewer's comments:

1-Lines 67-68. Many articles can be found in the literatures which have reported simultaneous extraction and determination of different classes of phenols (including, APs, CPs, NTPs and alkyl phenols) in water. Some of them are as below:
1-I. Rodríguez et al, "Review: Solid phase extraction of phenols" J. Chromatogr. A, 885, (2000) 291-304.
2-B.O.Opeolu et al, International Journal of Physical Sciences 5 (2010) 576-581.
3-M. Saraji et al Anal Bioanal Chem 396 (2010) 2685-2693.
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5- P. Mußmann et al Fresenius' Journal of Analytical Chemistry 348 (1994) 654-659.
6-A. Peñalver et al, J. Chromatogr. A 953 (2002) 79-87.
7- S. Nakamura et al, Analyst, 126 (2001) 835-839.
8- M.E. Torres Padrón, et al, Journal of Chromatographic Science 46 (2008) 325-331.
9- K-K Chee et al Microchimica Acta 126 (1997) 97-104.
10- S. Angelino, et al Analytica Chimica Acta 346 (1997) 61-71.
11- J. L. Bernal et al Chromatographia 46 (1997) 295-300.

The articles cited by the reviewer are analytical methods developed for the analysis of phenolic compounds in water samples (surface water), but any of them is valid for wastewater (WW) samples. The difficulties to carry out analysis in WW samples are well known due to they are complex matrixes. Therefore, from our point of view, we believe that comparing surface water samples *vs* WW samples is not correct in terms of analytical methodology. Moreover, none of the aforementioned articles achieved a simultaneous analysis of the families APs, CPs, NTPs and alkylphenols, due to the different physico-chemical properties (wide range of polarity). This makes difficult the extraction of all the compounds with the same extraction procedure and the analysis with the same analytical instrument. Furthermore, only one of the papers referenced above (No 3) shows the analysis of phenols in WW samples, but using UV detection, not mass spectrometry detection. UV detection does not provide structural information of the compounds, and other detection approach (such as MS) is necessary for their confirmation. Then, the possibility of false positives increases. In conclusion, we believe that the proposed study is clearly different from those pointed out by the reviewer. This has been indicated in the revised version of the manuscript and some references have been included.

2-Lines 87-93. In my opinion, Standard addition is the most suitable method for compensating matrix effects and most of the quantification methods are based on standard addition method not MMC.

The aim of this research paper is not only the development of a method for the analysis of phenols in WW and their quantitation by MMC and SAC. The main purpose is carrying out a comparison between MMC and SAC for quantification, considering two different types of WW effluents. These effluents were obtained by two different treatments and, therefore, they show different contents in organic matter. It is important

to notice that the selected treatments are two examples of WW effluents that can be found in real WW treatment plants, depending on the technology applied.

According to the results showed in the manuscript, SAC was the best quantitation method for both treatments but, previously, we did not know if MMC could work for both or for one of them. However, it was clearly indicated in the manuscript that the validation of the method was carried out using SAC.

3-Lines 101-110. The novelty of the work was not clearly mentioned in the text.

We do not match the lines indicated by the reviewer in the text. In any case, we have tried to answer the question.

The novelty of the work relies on the development of a method for the simultaneous extraction and analysis of phenolic compounds from 4 different families: APs, NTPs, CPs and cresols. Another new aspect is the comparison of two different quantification methods, such as MMC and SAC. SAC is not usually applied in WW analysis but it was submitted to study since it is difficult to find WW blank samples for MMC. Another point is the distribution study, which was performed in order to ensure that the analytes are not retained in the suspended particulate matter of the WW samples during the filtration step. This study is rarely performed in general in WW analysis and the suspended particulate matter is normally discarded, which can cause underestimations.

Despite of these aspects were clearly mentioned thorough out the manuscript (page 5, lines 105-114; page 12, lines 264-275; page 15, lines 334-338; page 17, lines 374-288), the last paragraph of the introduction has been modified to state the main novelties of the current manuscript.

4-Lines 105-107. When sample matrix has effect on the detection system and there is suppression or, less frequently, enhancement of the analyte signals in the presence of matrix components (eg. in LC-ESI-MS); external calibration is not a suitable method for quantification.
To nullify the matrix effect another method such as matrix matched (MMC) or standard addition (SAC) should be used. In MMC, analytes are added in to the blank extract (or blank sample). So, if there is a matrix effect during the extraction step, MMC in not able to nullify that effect. MMC may eliminate matrix effects during the detection. On the other hand, standard addition method may compensate matrix effects during both sample preparation and detection steps. In fact, SAC is applied to eliminate matrix effect of an analytical method (including extraction and detection).
In my opinion, in the present study, is not suitable to compare SAC and MMC because the application filed of the two methods are different.

We do not match in the text the lines indicated by the reviewer. In any case, we have tried to answer to the question.

The reviewer comments that "**MMC may eliminate matrix effects during the detection"** and that **"standard addition method may compensate matrix effects during both sample preparation and detection steps".** We agree with these comments. However, from our point of view, this is not a reason for not testing SAC since it is not clear the origin of the observed matrix effect.

In consequence, we consider that a comparison of MMC and SAC for this type of analysis is possible. According to the results, SAC was the selected quantification technique for validation and quantification purposes. But, MMC was also tested because we did not know if the studied WW effluents, which belong to different treatments, could be quantified adequately by MMC, reducing the number of injected samples in routine analysis.

5-Lines 168-169. Non spiked samples have been used as "blanks" in MMC. Use of these blanks is no compatible with MMC basis. How did you quantify analytes in the real sample using such blanks in MMC?

The reviewer probably refers to lines 171-174 in the former version of the manuscript. As it is mentioned above, MMC was not the technique used for quantification purposes. The validation was carried out using SAC, and thus, the analysis of real samples was carried out using SAC. In order to avoid misunderstandings, this paragraph has been corrected in the revised version of the manuscript (Section 2.3)

6-Experimental section, sampling section. Chemical specification of the wastewater samples should be stated.

A number of physicochemical analyses to characterize the WW effluents were performed in preliminary experiments. The information related to physicochemical information can be found in a previous article (reference [35] in the revised manuscript)

7-Line 196. "to 0.240 sec."

There is not any mistake in this line. Probably, the reviewer refers to an old version of the manuscript. This mistake was corrected before the submission to Talanta.

9- Line 204. " HCl 2 M"

There is not anything in this line. Probably, the reviewer refers to an old version of the manuscript. We have revised this issue in line 209 of the submitted manuscript.

According to the reviewer's comment, HCl 2 N has been replaced by HCl 2 M.

10-Lines 212-213. Name of derivatization reagent should be mentioned and derivatization procedure should be explained briefly.

We do not match in the text the lines commented by the reviewer. The derivatization process is explained in Section 2.6, lines 222-225.

11-Lines 292-295. The criteria to chose suitable recoveries (60-120%) and precision (RSD%<30) should be explained.

From our point of view, 60% of recovery can be accepted whenever the relative standard deviation is adequate, that is, the procedure is repeatable. In these cases, a correction factor can be used to correct this recovery in the final result.

In relation to relative standard deviation, 25% is a typical value in residues and contaminants in food. In this type of environmental samples, we considered that a maximum value of 30% could be acceptable; besides, this is observed only in a few compounds. This has been indicated in the revised version of the manuscript.

12- Lines 301-308 and Fig. 3. The slopes in Fig 3a and 3b are very close. We can not judge about the slopes by a simple looking at the figures. You should considered RSD of the slopes and used a statistical test to compare them.

Although reviewer is right and statistical tests should be used in order to compare both slopes, we only tried to show in Figure 3 the differences when both calibration approaches are used with the two types of WW samples. However, we consider that the comparison of recovery and precision are enough data (Tables 2 and 3) to check if both calibration procedures provide reliable quantification data or not.

Simultaneous analysis of chlorophenols, alkylphenols, nitrophenols and cresols in wastewater effluents, using solid phase extraction and further determination by gas chromatography–tandem mass spectrometry

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Abstract

An analytical methodology has been developed for the simultaneous extraction of 13 phenolic compounds, including chlorophenols (CPs), nitrophenols (NTPs), cresols and alkylphenols (APs) in different types of wastewater (WW) effluents. A solid-phase extraction (SPE) method has been optimized prior to the determination by gas chromatography coupled to triple quadrupole tandem mass spectrometry (GC-QqQ-MS/MS). Due to the complexity of the matrix, a comparison study of matrix-matched-calibration (MMC) and standard addition calibration (SAC) was carried out for quantification purposes. The optimized procedure was validated using the SAC approach since it provided the most adequate quantification results (in terms of recovery and precision values). Recoveries were in the range 60–135% (0.5 μg L^{-1}), 70–115% (1 μg L^{-1}), and 78–120% (5 μg L^{-1}), with precision values (expressed as relative standard deviation, RSD) ≤ 30% (except for 2-nitrophenol) involving intra-day and inter-day precision studies. Limits of detection (LODs) and quantification (LOQs) were also evaluated, and LOQs ranged from 0.03 μg L^{-1} to 2.5 μg L^{-1}. The proposed method was applied to the analysis of 8 real WW effluent samples, finding some phenolic compounds (e.g. 2-chlorophenol, 2,4,6-trichlorophenol and 4-tert-octylphenol) at concentrations higher than the established LOQs.

Keywords: Phenols, wastewater, gas chromatography-mass spectrometry (GC-MS), standard addition calibration, matrix-matched calibration.

1. Introduction

Phenolic compounds can be found in wastewater (WW) effluents via different sources. They can be detected in this type of samples because of their use in plastics [1], drug manufacturing, phytosanitary products or leather coloring [2], by anthropogenic emission [2] and by the use of treatments with aerobic or anaerobic microorganisms [4]. Some phenols show high toxicity, estrogenic [5] and anti-androgenic activity [6], and they can act as endocrine disrupters [7].

Phenols can be classified in a wide range of families. The most studied analytes in water are chlorophenols (CPs) [1] and alkylphenols (APs) [8]. However, the United States Environmental Protection Agency (US EPA) classifies CPs, nitrophenols and APs as priority pollutants [9] and it has established a maximum contamination level (MCL) for pentachlorophenol (PCP) of 1 μg L^{-1} in drinking waters [10]. On the other hand, the European Union (EU) has adopted a list of priority substances in the field of water policy, including 4-n-nonylphenol (4-n-NP), 4-tert-octylphenol (4-tertOP) and PCP [11]. Furthermore, maximum allowable concentrations (MAC) have been established for NP (2 μg L^{-1}) and PCP (1 μg L^{-1}) in inland and other surface waters [12]. However, it must be pointed out that legislation for WWs is still very scarce, and the values established in drinking water are usually used as guide in WWs. Bearing in mind these facts, the development of sensitive analytical methodologies for the simultaneous determination of phenols belonging to different groups, such as CPs, APs, nitrophenols (NTPs) and cresols (also known as methyl-phenols) with different polarity range (log K_{ow} 1.77-5.01) is needed in order to provide a complete overview of the occurrence of phenolic compounds in WW effluents.

Several extraction techniques have been applied for the extraction of phenols from aqueous samples, such as solid-phase extraction (SPE) [8,13-15] and liquid-liquid extraction (LLE) [16]. Recently, microextraction techniques, such as solid-phase microextraction (SPME) [17-19], stir bar sorptive extraction (SBSE) [19-21], liquid phase microextraction (LPME) [22] or dispersive liquid-liquid microextraction (DLLME) [23] have been applied. However, most of them have been used for the simultaneous analysis of only one or few phenols belonging to the same family such as APs [18,20] and CPs [17]. It is well-known that SPE is the most used technique in water analysis [24] due to the reduced exposition and contamination by organic solvents, the high pre-concentration factors avoiding evaporation steps, the semi-automation of the process, and it allows the extraction of compounds with different physico-chemical properties. The application of microextraction techniques is increasing but several disadvantages, such as cost and lifetime of fibers and bars, or the limited scope for a wide polarity range can hinder their utilization.

For the determination of phenolic compounds, gas chromatography (GC) [13,25] or liquid chromatography (LC) [26,27] are the predominant techniques, mainly coupled to tandem mass spectrometry (MS/MS) [28-31]. When GC is used, a derivatization step is required in order to improve the chromatographic performance and sensitivity of the selected compounds, and several derivatizating reagents can be applied [32,33].

A well-known critical point in the analysis of WW is matrix effect [34]. In order to minimize it, different calibration methods such as matrix-matched calibration (MMC) [33,35], standard addition calibration (SAC) [34] and the use of isotope-labeled internal standards [36,37] have been employed for complex matrixes. Quantification based on isotope-labeled internal standards has disadvantages due to the expensiveness of these standards and their limited availability. MMC is often used

quantification method in trace analysis. However, the lack of blank matrixes and the need for storing them can make this approach logistically onerous and not necessarily accurate. SAC is the most adequate technique to use when it is difficult to find blank samples of the studied matrix, but a calibration set is required for each sample, increasing the total number of injections and the time spent in data processing.

Another problem related to the determination of phenols in WW is that depending on the type of WW treatment, WW effluents can have different amounts of suspended particulate matter (SPM). This SPM is normally discarded during the extraction process by filtration in most of the analytical methods reported in literature [38]. However, a recent study [35] has demonstrated that certain analytes can be retained in the SPM, depending on its polarity. Therefore, it should be necessary to evaluate the presence of phenols in both phases in order to determine whether the SPM must be discarded or not.

Furthermore, it must be pointed out that many articles reporting simultaneous extraction and determination of different classes of phenols (including APs, CPs and, NTPs) in water [39,40] can be found. However, they have been developed for the analysis of this type of compounds in surface water, and they are not valid for the analysis in WW samples, due to they are more complex matrices with different physico-chemical characteristics (SPM levels, organic matter, etc.).

Therefore, in this study, a simultaneous SPE extraction and determination of different phenolic families (CPs, NTPs, cresols and APs) has been developed for WW effluent samples. In addition two novel aspects of this work must be pointed out: (i) a study of the presence of phenolic compounds in the SPM according to the strategy recently proposed by Barco-Bonilla et al [35], and (ii) a comparison of

MMC and SAC in order to evaluate the best quantification strategy of phenolic compounds in complex matrices such as WWs. For that, two different WW effluents were studied individually: membrane bioreactor (MBR, low SPM content) and anaerobic pond (ANAP, high SPM content). The optimized SPE and quantification method was validated in both types of WWs effluent samples.

2. Experimental

2.1. Chemicals and materials

Phenolic compounds standards, 2-nitrophenol (2-NTP), 4-nitrophenol (4-NTP), 2,4-dimethylphenol (2,4-DMP), 2-CP, 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-diCP), 2,4,5-trichlorophenol (2,4,5-triCP), 2,4,6-trichlorophenol (2,4,6-triCP) and 4-n-NP were obtained from Fluka (Buchs, Switzerland). On the other hand, 3-nitrophenol (3-NTP), 4-chloro-3-methylphenol (4-C-3-MP), 4-tertOP and PCP were supplied by Supelco (Bellefonte, PA, USA). Purities were always >97%. A standard solution (100 mg L^{-1}) of isotopically labeled PCP ([$^{13}C_6$]-PCP) was used as internal standard (IS) and it was obtained from Dr. Erhenstofer (Augsburg, Germany). Stock standard solutions of individual compounds (with concentrations ranging from 200 to 450 mg L^{-1}) were prepared by exact weighing of the powder or liquid and dissolution in 50 mL of acetone. These solutions were then stored under refrigeration (T <5 ºC). A working standard solution of the 13 phenolic compounds (2 mg L^{-1} of each compound) was prepared by appropriate dilution of the stock solutions with acetone, and it was stored under refrigeration (T <5 ºC). A working standard solution of [$^{13}C_6$]-PCP (22 mg L^{-1}) was prepared by appropriate dilution of the standard solution with acetone and stored under the aforementioned conditions. HPLC-grade methanol

(MeOH), anhydride acetic acid (AAA) (99.9%), and pyridine (Py) (99.8%) were purchased from Sigma-Aldrich (Madrid, Spain). Acetone and hydrochloric acid (HCl) were obtained from J.T. Baker (Deventer, Netherlands). Dichloromethane (DCM) was purchased from Riedel-de Haën (Seelze-Hannover, Germany). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). Thirty mm cellulose filters and 47-mm glass microfiber filters from Whatman (Maidstone, England, UK) and 0.45-µm HNWP nylon membrane filters from Millipore (Carrigtwohill, County Cork, Ireland) were also available for filtration stages. For SPE, Oasis HLB (200 mg, 6 cm^3) cartridges were obtained from Waters (Milford, MA, USA).

2.2. Apparatus

A GC system Varian 3800 (Varian Instruments, Sunnyvale, CA, USA) equipped with electronic flow control was interfaced to a 1200L triple quadrupole (QqQ) mass spectrometer. Samples were injected into an SPI/1079 split/splitless programmed-temperature injector using a Combi Pal (CTC Analytics AG, Zwingen, Switzerland) with a 100-µL syringe. A fused-silica untreated capillary column (2 m × 0.25 mm i.d.) from Supelco was used as pre-column connected to a VF-5 ms Factor Four capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) purchased from Varian. Helium was used as carrier gas (99.9999%) at a constant flow rate of 1 mL min^{-1}, and argon (99.999%) was used as collision gas. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV. The mass spectrometer was calibrated every four days with perfluorotributylamine. Varian Workstation software was used for instrument control and data analysis.

A Reax-2 rotary agitator from Heidolph (Schwabach, Germany) was used for agitation of the derivatization mixture. An analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland) and a rotary evaporator R-114 (Büchi, Flawil, Switzerland) were used during extraction and standard preparation. The horizontal shaker used in the distribution study was obtained from P-Selecta (Selecta, Barcelona, Spain).

2.3. Sampling

WW urban effluents from two different treatments, namely, MBR and ANAP, with low and high SPM content respectively, were collected from WW treatment plant (WWTP) of the foundation Centre for New Water Technologies ("Centro de las Nuevas Tecnologías del Agua", CENTA, Seville, Spain). This WWTP has 41000 m^2 and it currently holds more than 20 systems with different technologies. Additional physicochemical data related to the treatments evaluated in this study can be found in [35]. WW effluent samples were stored at 4 ºC and processed within 5 days after collection. In the MMC experiments, and due to the difficulty of finding WW effluent blank samples, the corresponding signal of the blank was removed from the MMC plot in those samples where analyte signal was observed.

2.4. Distribution study

Non-filtered WW effluent samples were spiked with 0.5 µg L^{-1} of the studied phenolic compounds, and then, they were agitated overnight at a rate of 100 oscillations per min to allow a thoroughly interaction between the analytes and both

phases of WW (aqueous phase and SPM). After this, samples were filtered to separate and analyze both phases. The aqueous phase was extracted by SPE, whereas for the analysis of the SPM, a method developed by Padilla-Sánchez et al. [33] for the extraction of phenolic compounds in agricultural soils was employed. The distribution of the compounds between both phases was determined as the percentage of them present in each phase.

2.5. GC-QqQ-MS/MS

Aliquots of 10 µL were injected into the GC system operating at a syringe injection flow rate of 10 µL s^{-1}. The injector temperature program was as follows: 70 ºC (hold for 0.5 min) → 310 ºC (100 ºC min^{-1}, hold for 10 min). The injector split ratio was initially set at 10:1. Splitless mode was switched on at 0.5 min until 3.5 min. At 3.5 min, the split ratio was 100:1 and at 10 min, 20:1. The column oven program was as follows: 70 ºC (hold for 3.5 min) → 300 ºC (20 ºC min^{-1}) → 300 ºC (hold 4 min). Cryogenic cooling with CO_2 was applied when the injector temperature was 170 ºC. The total running time was 19 min.

The QqQ mass spectrometer was mainly operated in the selected reaction monitoring (SRM) mode, although selecting ion monitoring (SIM) mode was also used for confirmation purposes. The electron multiplier was set +200 V above the optimal value indicated by the software instrument. The temperatures of the transfer line, manifold and ionization source were set at 300, 40 and 265 °C, respectively. The optimal values for the scan time ranged from 0.132 to 0.240 s. Peak widths of *m/z* 2.0 and 1.5 were set in the first (Q1) and third quadrupole (Q3), respectively. The optimized MS/MS parameters are indicated in Table 1.

206 *2.6. SPE extraction and derivatization procedure*

207 WW effluent samples were filtered consecutively (250 mL) using two different pore-
208 size filters (47-mm glass microfiber filters and 0.45-µm nylon membrane filters). The
209 filtered WW effluents showed pH values between 7.7 and 8.3. Then, pH was adjusted
210 to 2.5-2.7 with HCl (2 M) to ensure the protonated form of the phenolic compounds,
211 facilitating the absorption into the solid phase, and an adequate preservation of the
212 samples. The Oasis HLB cartridges were conditioned with 5 mL of acetone followed
213 by 5 mL of MeOH and 3 x 5 mL of ultrapure water without allowing the cartridges to
214 dry out. Then, the filtered WW sample (250 mL) was passed through the cartridges
215 under vacuum at a flow rate of 10 mL min^{-1}. The cartridges were dried for 2 h and the
216 phenolic compounds were eluted sequentially with 3 mL of acetone and 2 mL of
217 DCM. The extracts were collected into 5-mL volumetric flasks, adjusting the total
218 volume with DCM, without any evaporation step. Then, the derivatization stage was
219 performed according to the procedure described by Padilla-Sánchez et al. [33].
220 Briefly, 860 µL of the extract were transferred to a 2-mL vial and 20 µL of [$^{13}C_6$]-
221 PCP (IS), 20 µL of Py and 100 µL of AAA were added to carry out the derivatization
222 reaction. The mixture was shaking in a rotary agitator for 2 min and then injected
223 directly to the GC-QqQ-MS/MS system.

224

225 **3. Results and discussion**

226 WWs can be submitted to different treatments, obtaining effluents with a variety of
227 SPM contents, and thus, WW effluents can present different physico-chemical
228 properties. When an analytical method is developed for this type of samples, this
229 diversity should be taken into account. In order to cover a wide range of WW

effluents, two types of them were evaluated, MBR and ANAP, which have low and high SPM content [35], respectively. The optimization of the extraction procedure as well as the quantification methods, were evaluated in both types of WW effluents. For that purpose, a GC-QqQ-MS/MS method recently developed [33] was applied.

3.1. Extraction method

For the optimization of the SPE procedure, a methodology reported by Pothitou et al. [8] was first considered. This study reported the determination of only one family of phenolic compounds, APs, using Oasis HLB cartridges and acetone as elution solvent. Besides, certain problems regarding the evaporation stages have been previously reported [33], and therefore, the extraction method was designed without any evaporation step. Since the families of phenolic compounds included in this study showed a wide polarity range, several elution solvents were tested to achieve a simultaneous extraction [8]. Acetone (5 mL), DCM (5 mL) and a sequential elution with acetone (3 mL) and DCM (2 mL) were tested. Bearing in mind that evaporation steps were not included in the extraction procedure and aliquots of the extracts are directly injected in the chromatographic system, the elution solvent could be partially retained in the solid phase or evaporated during the elution step. This can provoke an overestimation of the final concentration in relation to the theoretical value, obtaining high recovery values. In order to avoid this, 5-mL volumetric flasks were used to collect the extracts and the final volume was adjusted to 5 mL with the corresponding solvent used during the elution step. The obtained results are shown in Figure 1 and it can be observed that acetone provided adequate results for all compounds, except for 2,4-dMP and 4-n-NP. When DCM was used, recoveries higher than 120% were

obtained for 2-CP, 2-NTP and 4-CP, although recovery for 4-n-NP was improved. Consequently, in order to obtain good recoveries for all the compounds, a sequential elution with acetone and DCM was tested. In general, this elution improved the recovery rates, especially for 2,4-dMP, 2-CP, 2-NTP and 4-CP. Nonetheless, recoveries between 50-60% may be accepted extraordinarily in environmental analysis whenever the precision values are adequate (<30%). Therefore, further experiments were carried out using the sequential elution with acetone (3 mL) and DCM (2 mL) as elution solvents. Finally, a total ion chromatogram (TIC) of an extracted spiked WW sample at 50 μg L^{-1} is showed in Figure 2.

3.2. Distribution study

Once the extraction method was optimized for the analysis of the aqueous phase of WW effluent samples, a distribution study is needed to verify whether the phenolic compounds are also present in the SPM. If phenolic compounds are present quantitatively in the SPM, the analysis of WW effluents should not be limited to the aqueous phase. The distribution study was therefore carried out, applying the approach described in Section 2.4 for both type of samples. It was observed that only the phenolic compounds with high log K_{ow} were found in the SPM, but at negligible percentages (<5%). On the contrary, phenolic compounds with lower log K_{ow} where not found in the SPM (data not shown). Taking into account this result, further experiments were limited to the analysis of the target analytes in the aqueous phase, discarding the SPM phase. These results are in accordance with a previous study [35] reporting that polar compounds were not retained in the SPM.

278 *3.3. Evaluation of the quantification method: comparison of MMC and SAC*

279 Due to the complexity of the matrix and the difficulty to find blank WW samples, a
280 study of the quantification of target compounds was proposed. For this aim, a
281 comparison between SAC and MMC in WW effluents obtained by two different WW
282 treatments (MBR and ANAP) was carried out. The study was performed using spiked
283 and blank samples of WW effluents for SAC and MMC respectively, and calibration
284 curves were prepared in the range 10-150 μg L^{-1}, except for 2-NTP (10-300 μg L^{-1}),
285 and 3-NTP and 4-NTP (100-300 μg L^{-1}). For SAC, a WW sample was spiked and the
286 calibration levels were prepared after submitting the sample to the extraction
287 procedure. For MMC, the calibration plot was prepared using blank extracts.
288 Recoveries were evaluated using spiked samples at 0.5, 1 and 5 μg L^{-1}, taking into
289 account the MCLs and MACs established by the EPA and the EU for these
290 compounds [10,12]. Although, conventional criteria for the analysis of contaminants
291 in foods demands an average recovery between 70% and 120%, bearing in mind the
292 nature of the samples under study, it is possible to increase the recovery range to 60–
293 120%, providing that the RSD values are <30% [35]. Recoveries were considered
294 adequate when they ranged from 60 to 120%. Intra and inter-day precision was
295 expressed as relative standard deviation (RSD, n=5), and they were determined by
296 analyzing spiked samples during the same day and in different days, respectively.
297 Good precision values were considered if RSDs were lower than 30%. The obtained
298 results when both calibration procedures were applied are shown in Table 2 and 3 for
299 the two types of WW effluents evaluated. Figure 3 shows a comparison between SAC
300 and MMC curves of 4-tertOP for ANAP (Figure 3a) and MBR (Figure 3b). It can be
301 observed that for ANAP, which has high SPM content, the slope of the MMC curve
302 was higher than the SAC slope. On the contrary, for MBR, which has lower SPM

303 content, the slopes obtained by MMC and SAC were similar. This can be explained
304 due to ANAP is a "dirty" WW effluent because of the high SPM content and this fact
305 may affect the repeatability of the slopes in MMC curves, which may influence in the
306 obtained results for ANAP when MMC is applied.

307 Recovery and precision were evaluated using both quantification approaches. It can
308 be observed that in WW effluents with high SPM, such as ANAP, MMC did not
309 provide adequate results for the lower spiked concentrations (0.5 and 1 μg L^{-1}).
310 Recoveries and intra and inter-day precision of most of compounds were below 60%
311 and over 30%, respectively for these two concentration levels. On the contrary, for 5
312 μg L^{-1}, recovery values were in the range 60-120%, except for 4-n-NP (51%) and
313 intra and inter-day precision were <12%. These results (Table 2) suggested that MMC
314 is not a suitable option for the adequate quantification at very low concentrations of
315 phenols in WWs effluents with high SPM. On the other hand, when SAC was used,
316 recoveries of all compounds were in range 60-125%, except for 4-tertOP (135%) at
317 the lowest fortification level (0.5 μg L^{-1}). Intra and inter-day precision values were
318 <27% and <31% for all compounds, respectively. As it is shown in Table 2, the SAC
319 approach is more appropriate for WW effluents with high SPM content. Linearity was
320 studied in the range 10-150 μg L^{-1} (except for NTPs which was 100-300 μg L^{-1}) and
321 the obtained determination coefficients (R^2) were in the range 0.9912 (3-NTP)-0.9999
322 (2-CP, 2,4,5-triCP, PCP and 4-n-NP) for ANAP (Table 4).

323 For WW effluents with low SPM, such as MBR (Table 3), the recoveries obtained
324 when MMC was used for the three levels assayed ranged from 62-119%, except for 4-
325 n-NP, with recoveries lower than 56%. Despite the adequate recovery results provided
326 by MMC for all the studied fortification levels, in general, RSD values were <30%

only for the highest spiked level studied (5 μg L^{-1}), as it can be observed in Table 3, whereas at the lowest concentration levels evaluated (0.5 and 1 μg L^{-1}), intra and inter-day precision ranged from 22 to 113%. On the other hand, the application of SAC on MBR WW samples yielded recovery values in the range 70-120%, except for 4-CP (125%) at 0.5 μg L^{-1}. Besides, RSD values were always <28% for intra-day precision and <27% for inter-day precision in all cases, except for 2-NTP, which was 41% at 0.5 μg L^{-1}. In consequence, it can be concluded that for MBR treated WW effluents, SAC was also the most suitable method for an adequate quantification of WW effluents with low SPM content, such as MBR WW samples (Table 3). Furthermore, linearity was also evaluated for MBR and R^2 values ranged from 0.9943 (4-NTP) to 0.9999 (2-CP, 4-CP, 2,4,6-triCP and 4-n-NP).

Considering these results, the SAC method should be applied for a reliable quantification of phenols in WW effluent samples to compensate matrix effects on the signal variation during detection and this does not depend on the SPM content of the WW. The SAC methodology was therefore applied for the quantification of phenols in real samples.

3.4. Estimation of the lower limits of the methodology

Despite of the estimation of the trueness and precision carried out in the previous section, other performance characteristics of the method, such as limits of detection (LODs) and quantification (LOQs) were studied. LODs and LOQs were determined as the lowest concentration level that yielded a signal-to-noise (S/N) ratio of 3 and 10, and they are shown in Table 4. LODs and LOQs were determined in WW sample blanks for each phenolic compound studied. LODs were from 0.01 to 1 μg L^{-1} and

LOQs ranged from 0.03 μg L^{-1} to 2.5 μg L^{-1} for ANAP and MBR (Table 4). It must be noticed that similar values were obtained for both types of WW effluents, except for 2-NTP and 4-C-3-MP, which showed higher LOD and LOQ values in ANAP than in MBR. This could be explained taking into account that the SPM content is higher in ANAP, increasing the amount of co-extracted material and affecting the estimation of the lower limits of the method.

3.5. Application to the analysis of real WW effluent samples

The developed methodology was applied to the analysis of 8 WW effluent samples from the CENTA, obtained after the application of different WW treatments employed in this WWTP. To assure the quality of the results and avoid errors, the quantification of the phenolic compounds was achieved using the SAC approach. An internal quality control (IQC) was performed consisting of the analysis of spiked blank WW samples at 1 μg L^{-1} (except for 3-NTP and 4-NTP at 5 μg L^{-1}), which were used to assess the extraction efficiency and a SAC calibration curve to check linearity and sensitivity. Several phenolic compounds were found over the LOQs established by the method, showing the obtained results in Table 5. 2-CP and 2,4,6-triCP were found in six and five samples, respectively, with concentrations ranging from 0.04 to 0.20 μg L^{-1} for 2-CP and from 0.05 to 0.10 μg L^{-1} for 2,4,6-triCP. 4-CP and 4-tertOP were found in four samples, and the concentrations ranged from 0.04 to 0.08 μg L^{-1} and 0.04 to 0.16 μg L^{-1} respectively. 2-CP, 2,4-DiCP, 4-tertOP, PCP and 4-n-NP were found simultaneously in one of the samples (Table 5). It must be highlighted that phenolic compounds were not found over the MCLs and MACs established by the

374 EPA and the EU for these compounds [10,12]. Finally, Figure 4 shows a positive
375 sample of 4-tertOP detected in a WW effluent sample at 0.12 μg L^{-1}.

376

377 **5. Conclusions**

378 A single extraction method for the simultaneous extraction of CPs, APs, NTPs and
379 cresols in WW effluent samples has been developed using SPE. A distribution study
380 of the phenolic compounds between the aqueous phase and the SPM was carried out,
381 verifying that the SPM could be in fact discarded during the extraction since only
382 phenolic compounds with high log K_{ow} were found in the SPM at a negligible
383 percentage. Due to the difficulty to find WW blank samples and to have good
384 accuracy in the quantification, a study using MMC versus SAC was performed in two
385 different treated WW effluent samples (ANAP and MBR) showing that SAC is the
386 most suitable quantification approach. The method was validated studying recovery,
387 intra and inter-day precision, lower limits (LODs and LOQs) and linearity.
388 Determination of the analytes was carried out using GC-QqQ-MS/MS operating in
389 SRM mode. The method was applied to WW effluent samples with satisfactory
390 results, observing that phenols of several families were simultaneously detected in
391 WW effluents, highlighting the potential of analytical methods that allows the
392 simultaneous determination of several classes of phenolic compounds.

393

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Figure Captions

Fig. 1. Comparison of the recovery values obtained applying different elution solvents for the extraction of spiked WW samples at 0.5 μg L^{-1}. Abbreviations: DCM: dichloromethane; Sequential: sequential elution.

Fig. 2. Total ion chromatogram (TIC) of an extracted spiked WW sample (5 μg L^{-1}) obtained by GC-QqQ-MS/MS. For compound abreviations, see Table 1.

Fig. 3. Calibration curves in the range 10-150 μg L^{-1} for 4-tertOP when SAC and MMC were used: a) ANAP; b) MBR. Abbreviations: ANAP: anaerobic pond; MBR: membrane bioreactor; MMC: matrix-matched calibration; SAC: standard addition calibration; 4-tertOP: 4-tertoctylphenol

Fig. 4. Selected-reaction monitoring (SRM) (a) chromatogram and (b) MS/MS spectrum of 4-tertOP (0.12 μg L^{-1}) found in a real WW sample and (c) SRM chromatogram and (d) MS/MS spectrum of a SAC standard (50 μg L^{-1}).

Cover Letter

UNIVERSIDAD DE ALMERÍA
Departamento de Hidrogeología y Química Analítica

Almeria, 12 July 2011

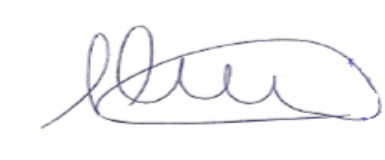
Dear Editor:

Please, find enclosed the revised version of the manuscript entitled "*Simultaneous analysis of chlorophenols, alkylphenols, nitrophenols and cresols in wastewater effluents, using solid phase extraction and further determination by gas chromatography–tandem mass spectrometry*", Manuscript No. TAL-D-11-00529.

We revised our manuscript taking into account the reviewer's comments (changes are indicated by using "Track Changes"). Although we tried to clarify the reviewer's comments, we have considered that it was also necessary to indicate some aspects, which can be seen in the document uploaded as "*Response to Reviewers*".

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

Yours Sincerely,



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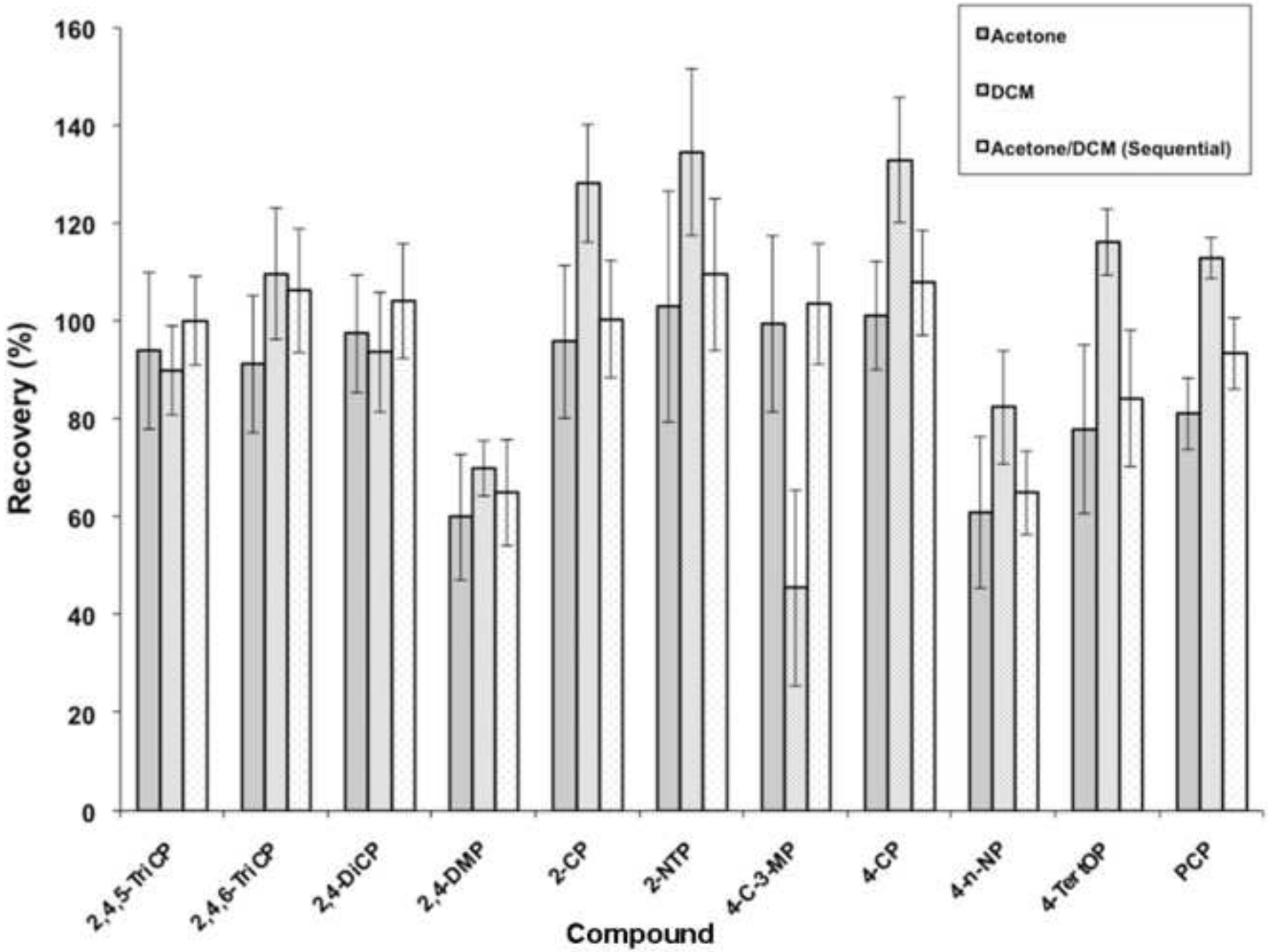

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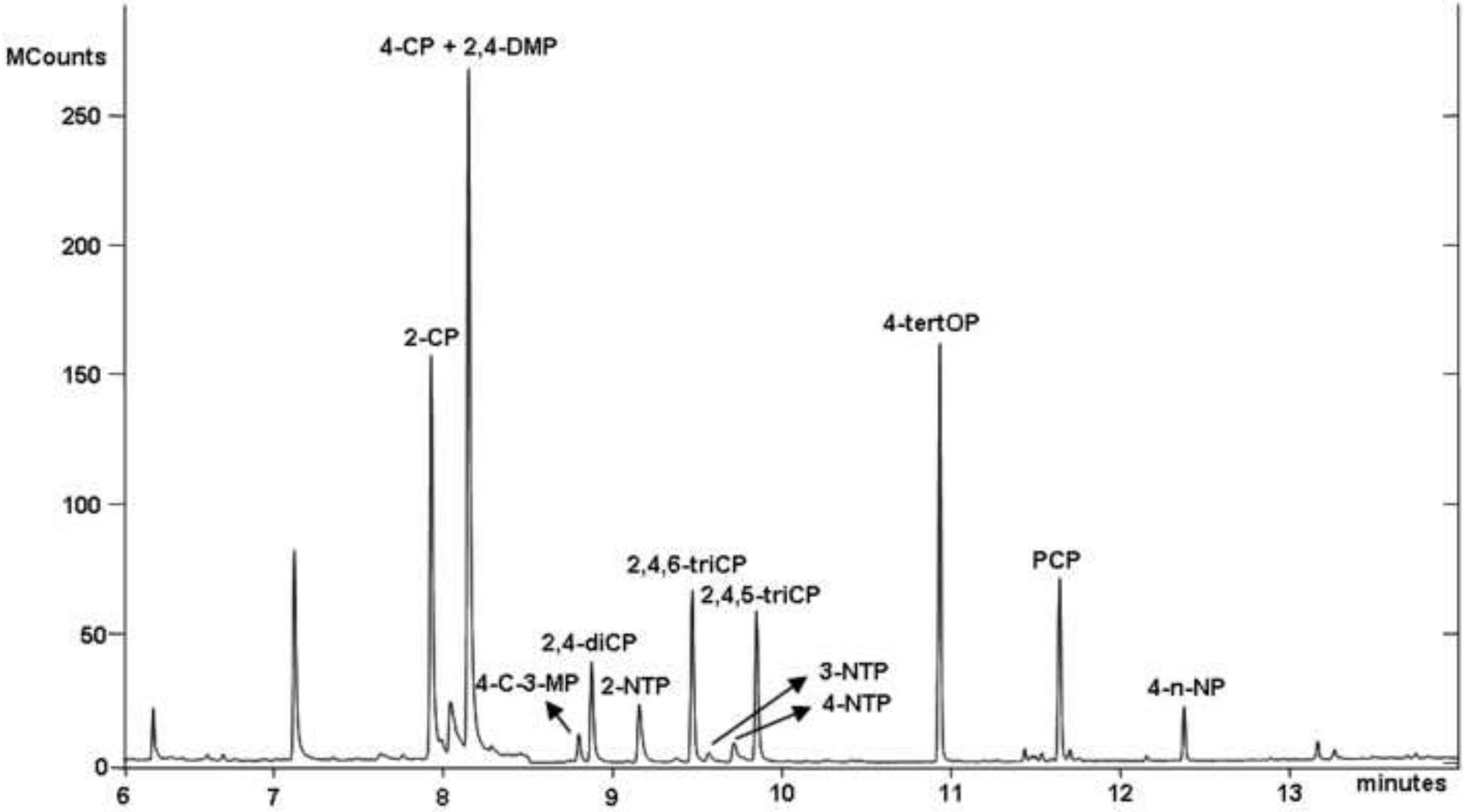

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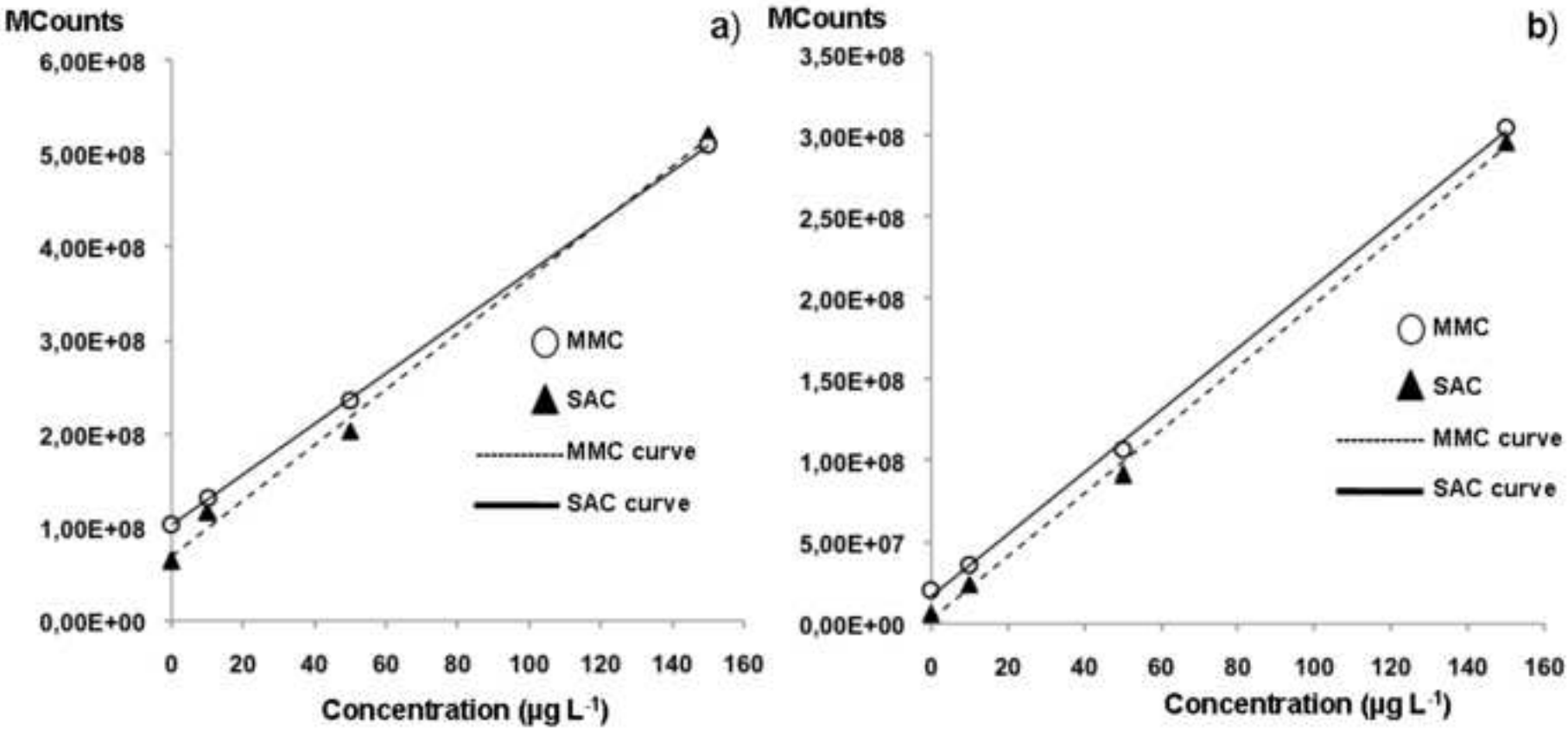

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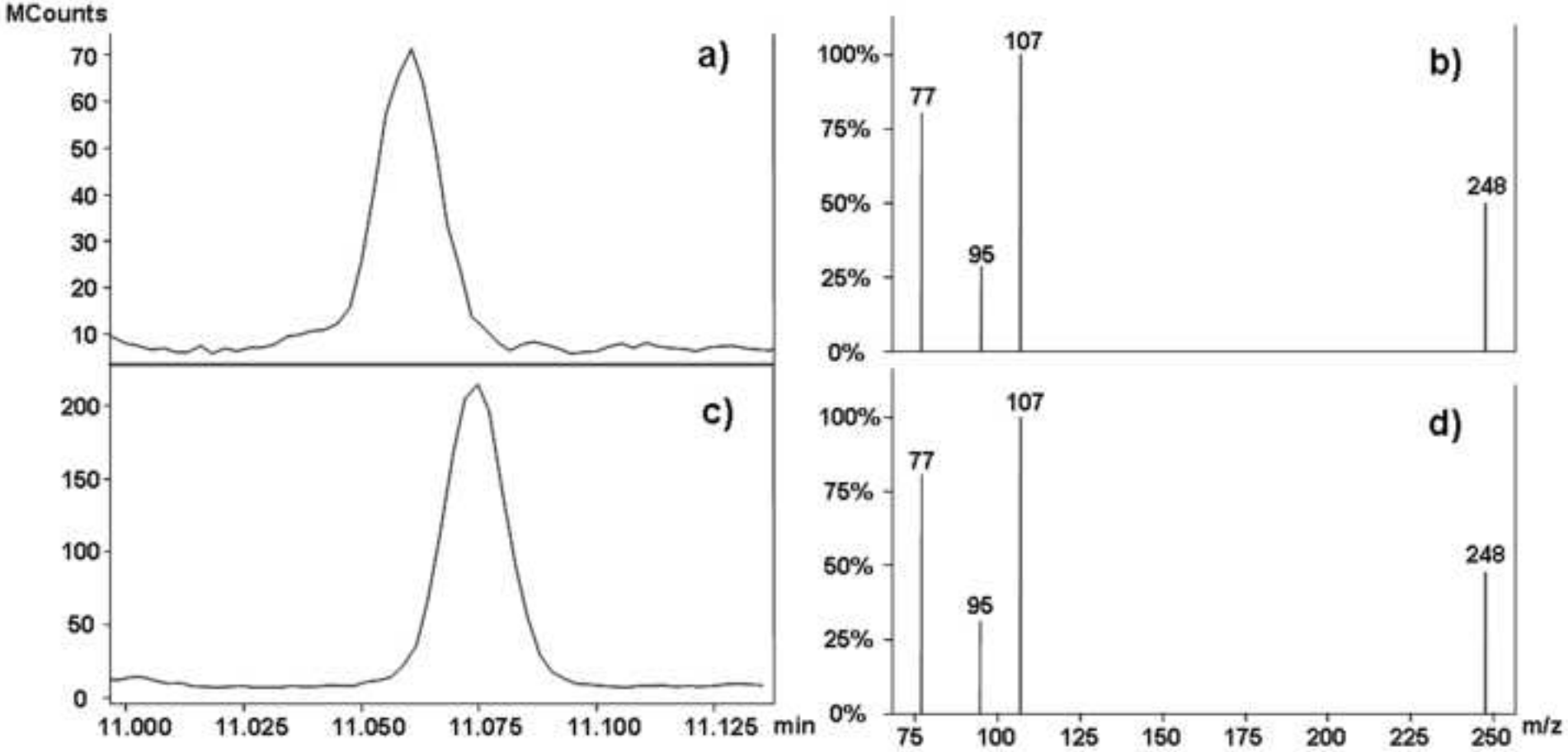

Table 1
GC-QqQ-MS/MS conditions for the derivatized phenols

Compound	Abbreviations	Family	M.W.[a] (amu)	Log K_{ow}	RTW[b] (min)	Precursor ion (*m/z*)	Product ions, *m/z* (collision energy, eV)
2-Chlorophenol	2-CP	Chlorophenol	128.5	2.17	7.80-7.88	128	92 (10), 100 (5), 170
4-Chlorophenol	4-CP	Chlorophenol	128.5	2.36	8.06-8.15	128	65 (15), 100 (5), 170
2,4-Dimethylphenol	2,4-DMP	Cresol	122.0	2.42	8.08-8.15	122	77 (20), 107 (5), 164
4-Chloro-3-methylphenol	4-C-3-MP	Cresol	142.5	3.10	8.80-8.84	142	77 (10), 79 (5), 184
2,4-Dichlorophenol	2,4-DiCP	Chlorophenol	163.0	3.08	8.88-8.92	162	98 (15), 126 (10), 205
2-Nitrophenol	2-NTP	Nitrophenol	139.0	1.89	9.17-9.21	139	81 (10), 109 (10), 181
2,4,6-Trichlorophenol	2,4,6-TriCP	Chlorophenol	197.5	3.38	9.52-9.55	196	132 (15), 160 (10), 239
3-Nitrophenol	3-NTP	Nitrophenol	139.0	2.00	9.58-9.62	139	81 (5), 93 (5), 111 (10), 181
4-Nitrophenol	4-NTP	Nitrophenol	139.0	1.85	9.74-9.77	139	93 (15), 109 (5), 181
2,4,5-Trichlorophenol	2,4,5-TriCP	Chlorophenol	197.5	4.1	9.91-9.94	196	97 (25), 132 (15), 160 (5), 239
4-Tertoctylphenol	4-TertOP	Alkylphenol	206.0	4.12	11.04-11.10	135	77 (20), 95 (10), 107 (5), 248
Pentachlorophenol	PCP	Chlorophenol	266.5	5.15	11.74-11.78	266	167 (20), 202 (10), 230 (10), 308
4-n-Nonylphenol	4-n-NP	Alkylphenol	220.0	4.48	12.48-12.52	107	77 (30), 81 (15), 95 (10), 262

[a]Molecular weight
[b]Retention time window

Table 2
Study of recoveries and intra- and inter-day precision in ANAP treated WWs effluents samples using MMC and SAC.[a]

Phenolic compound	ANAP											
	SAC						MMC					
	% Recovery (RSD %)[b]			Inter-day precision (RSD %)[c]			% Recovery (RSD %)[b]			Inter-day precision (RSD %)[c]		
	0.5 µg L^{-1}	1µg L^{-1}	5 µg L^{-1}	0.5 µg L^{-1}	1µg L^{-1}	5 µg L^{-1}	0.5 µg L^{-1}	1µg L^{-1}	5 µg L^{-1}	0.5 µg L^{-1}	1 µg L^{-1}	5 µg L^{-1}
2-CP	**123**[d] (7)	115 (8)	101 (8)	11	13	12	**47** (**82**)	62 (**33**)	70 (4)	**124**	**50**	7
4-CP	**125** (14)	98 (12)	113 (7)	21	18	10	64 (**114**)	70 (**111**)	108 (4)	**172**	**168**	6
2,4-DMP	95 (21)	111 (12)	116 (6)	20	19	10	**32** (**50**)	**59** (25)	97 (6)	**76**	**37**	10
4-C-3-MP	100 (17)	97 (9)	120 (5)	26	15	6	**53** (**35**)	70 (**104**)	87 (3)	**53**	**158**	4
2,4-diCP	96 (13)	89 (8)	109 (4)	20	12	6	115 (**56**)	98 (26)	98 (5)	**84**	**39**	8
2-NTP	60 (27)	110 (5)	103 (6)	30	9	8	**46** (**60**)	**48** (29)	**64** (5)	**91**	**44**	12
2,4,6-triCP	89 (12)	93 (7)	111 (5)	18	11	9	**57** (**38**)	70 (22)	86 (5)	**58**	**33**	6
3-NTP	N.D.[e]	N.D.	93 (8)	----	----	16	N.D.	N.D.	93 (5)	----	----	9
4-NTP	N.D.	N.D.	110 (7)	----	----	13	N.D.	N.D.	88 (4)	----	----	7
2,4,5-triCP	87 (9)	85 (5)	103 (5)	14	11	8	**41** (30)	62 (19)	80 (5)	**46**	**29**	10
4-tertOP	**135** (8)	70 (4)	101 (3)	12	6	5	61 (**38**)	**56** (18)	72 (3)	**57**	**27**	5
PCP	120 (8)	76 (7)	90 (5)	12	11	8	**47** (29)	64 (**38**)	88 (6)	**44**	**57**	9
4-n-NP	85 (12)	93 (6)	109 (4)	18	9	6	62 (**56**)	**30** (24)	**51** (7)	**84**	**37**	11

[a] Abbreviations: ANAP: anaerobic pond; SAC: standard addition calibration. MMC: matrix-matched calibration
[b] Intra-day precision, expressed as RSD, is given in brackets (n = 5 for each concentration level).
[c] n = 5 for each concentration level.
[d] Figures in bold indicate that the values are outside the limits (recovery and precision) established in the validation requirements (recoveries ranging from 60 to 120% and RSD values < 30%).
[e] ND: Not detected.

Table 3
Study of recoveries and intra and inter-day precision in MBR treated WW effluent samples using MMC and SAC.[a]

Phenolic compound	MBR											
	SAC						MMC					
	Recovery (%)[b]			Inter-day precision[c]			Recovery (RSD %)[b]			Inter-day precision[c]		
	0.5 µg L^{-1}	1µg L^{-1}	5 µg L^{-1}	0.5 µg L^{-1}	1µg L^{-1}	5 µg L^{-1}	0.5 µg L^{-1}	1µg L^{-1}	5 µg L^{-1}	0.5 µg L^{-1}	1 µg L^{-1}	5 µg L^{-1}
2-CP	113 (12)	98 (11)	93 (5)	19	17	13	96 (**45**)	95 (17)	109 (9)	**68**	25	13
4-CP	**125**[d] (14)	90 (11)	95 (8)	21	19	16	119 (16)	91 (20)	81 (2)	24	22	7
2,4-DMP	84 (9)	90 (7)	105 (7)	21	13	11	103 (**74**)	97 (**38**)	95 (6)	**113**	**57**	11
4-C-3-MP	87 (10)	111 (10)	98 (9)	16	15	13	96 (**34**)	101 (20)	99 (13)	**45**	29	19
2,4-DiCP	90 (16)	99 (9)	103 (11)	24	13	17	104 (**55**)	106 (**42**)	104 (11)	**83**	**63**	15
2-NTP	110 (27)	98 (15)	102 (9)	**41**	23	14	62 (**51**)	80 (**45**)	103 (4)	**77**	**68**	6
2,4,6-TriCP	105 (17)	94 (7)	85 (9)	26	11	15	95 (**57**)	92 (**37**)	94 (10)	**63**	**55**	15
3-NTP	N.D.[e]	N.D.	81 (13)	----	----	20	N.D.	N.D.	103 (23)	----	----	29
4-NTP	N.D.	N.D.	78 (10)	----	----	19	N.D.	N.D.	81 (14)	----	----	22
2,4,5-TriCP	88 (15)	97 (8)	88 (12)	23	12	18	100 (**37**)	92 (**42**)	89 (7)	**44**	**63**	11
4-TertOP	98 (14)	78 (13)	83 (5)	21	14	8	77 (17)	70 (22)	71 (2)	24	**35**	3
PCP	92 (17)	77 (12)	93 (7)	26	17	11	79 (28)	70 (**35**)	83 (5)	**34**	**31**	10
4-n-NP	79 (15)	85 (16)	90 (6)	24	19	9	**40** (**43**)	**46** (**50**)	**55** (5)	**64**	**61**	7

[a] Abbreviations: ANAP: anaerobic pond; SAC: standard addition calibration. MMC: matrix-matched calibration
[b] Intra-day precision, expressed as RSD, is given in brackets (n = 5 for each concentration level).
[c] n = 5 for each concentration level.
[d] Figures in bold indicate that the values are outside the limits (recovery and precision) established in the validation requirements (recoveries ranging from 60 to 120% and RSD values < 30%).
[e] ND: Not detected.

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Table 4
Validation study in both types of WW effluent samples using SAC.[a]

Phenolic compound	Linearity range (µg L^{-1})	ANAP			MBR		
		Linearity (R^2)	LOD[b] (µg L^{-1})	LOQ[c] (µg L^{-1})	Linearity (R^2)	LOD[b] (µg L^{-1})	LOQ[c] (µg L^{-1})
2-CP	10-150	0.9999	0.01	0.03	0.9999	0.01	0.03
4-CP	10-150	0.9998	0.01	0.03	0.9999	0.01	0.03
2,4-DMP	10-150	0.9993	0.01	0.05	0.9990	0.03	0.05
4-C-3-MP	10-150	0.9990	0.30	2.50	0.9992	0.10	0.50
2,4-DiCP	10-150	0.9994	0.01	0.03	0.9996	0.01	0.03
2-NTP	10-300	0.9979	0.30	2.50	0.9989	0.10	0.25
2,4,6-triCP	10-150	0.9999	0.01	0.05	0.9999	0.01	0.03
3-NTP	100-300	0.9912	1.00	2.00	0.9943	1.00	2.00
4-NTP	100-300	0.9966	1.00	2.00	0.9990	1.00	2.00
2,4,5-TriCP	10-150	0.9999	0.01	0.03	0.9999	0.01	0.03
4-TertOP	10-150	0.9997	0.01	0.03	0.9998	0.01	0.03
PCP	10-150	0.9999	0.01	0.03	0.9997	0.01	0.03
4-n-NP	10-150	0.9999	0.03	0.05	0.9999	0.03	0.05

[a] Abbreviations: ANAP: anaerobic pond; MBR: membrane bioreactor
[b] LOD calculated in the sample
[c] LOQ calculated in the sample

Table 5
Concentration (μg L^{-1}) found after the application of the proposed method in real WW samples.

Compound	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
2-CP		0.04	0.04	0.12	0.04	0.20	<LOQ[a]	0.12
4-CP	0.04	<LOQ		0.04		0.08	0.08	<LOQ
2,4-DMP	0.06		0.04		<LOQ			
4-C-3-MP						<LOQ		
2,4-diCP	<LOQ	<LOQ	<LOQ		<LOQ	0.04		0.04
2-NTP	<LOQ		<LOQ					
2,4,6-triCP	0.06	0.10	0.06	<LOQ	<LOQ	0.05	0.10	
3-NTP								
4-NTP								
2,4,5-triCP						0.04		<LOQ
4-tertOP	0.16	0.06					0.04	0.12
PCP	<LOQ	<LOQ	<LOQ	<LOQ		<LOQ	<LOQ	0.04
4-n-NP				<LOQ		<LOQ		0.08

[a] Values under the LOQ established by the method validation.