OMW spillage control tool based on tracking *p*-Coumaric acid degradation by HPLC

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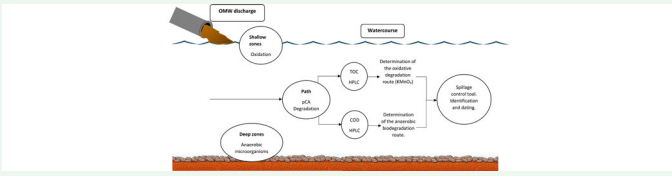
ABSTRACT

Olive mill wastewater (OMW) is a major watercourse pollutant agent with a high concentration of phenolic compounds. It is estimated that 30 million OMW m^3 are released into rivers every year. Protecting the health of these courses against the uncontrolled discharges implies establishing an adequate legislation, where spillage control tools play a fundamental role. In this paper, a new tool for OMW spillage control is discussed. It is based on the use of a RP-HPLC-UV protocol to track p-Coumaric acid (pCA), a characteristic OMW phenolic compound, and its derivative compounds through their chemical oxidation and biological anaerobic degradation. Laboratory assays and real-life experiences allowed to determine degradation routes and apparition times for every pCA derivative, making it possible to detect an OMW spill and assess its age. Moreover, this RP-HPLC-UV introduces solid advantages over previous detection procedures, namely, quicker response times and smaller costs than HPLC methods and superior specificity than colorimetric methods. Finally, this tool was put to test in an actual OMW-polluted watercourse. In all scenarios, the tool demonstrated solid reliability.



KEYWORDS

HPLC;
pCA;
olive mill wastewater;
spill;
control

1. Introduction

Olive mill wastewater (OMW) is a major waste stream resulting from numerous operations that occur during the production stages of olive oil. OMW effluent contains several organic and inorganic contaminants and its environmental impact is notably serious [1], mainly due to high concentrations of phenolic compounds (PC), whose concentrations reach levels between 0.5 and 24 g L^{-1} [2]. Moreover, OMW is chemically characterized by high chemical oxygen demand (COD) 40–220 g L^{-1}, also high biochemical oxygen demand (BOD) 35–110 g L^{-1} and a pH of 3–6. All these factors result in a toxic effect to microorganisms, plants and marine organisms [3] and soils, inhibiting seed germination and plant growth, changing the physicochemical and biological soil properties and eventually making its treatment immensely difficult [4–7].

OMW spillage is a largely widespread practice in the Mediterranean region, where more than 98% of the annual worldwide olive oil production takes place [8]. The high OMW/olive ratio (about 7–8 m^3 OMW/$T_{oliveoil}$) causes about 30 million OMW m^3 to be discharged every year, and, to make matters worse, its production is seasonal – the whole annual 30 million m^3 outlet occurs during the winter months alone. This leads to the natural regeneration capacity of rivers in the area being exhausted, provoking oxygen concentration to drastically drop and thus giving rise to anoxic/anaerobic conditions even in shallow waters. In this scenario, uncontrolled growth of anaerobic bacteria occurs and water becomes unavoidably putrefied.

This severe aggression to watercourses poses a huge and growing environmental concern. Protecting the health of these courses against the uncontrolled OMW spills that, unfortunately, continue happening year after

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year implies establishing an adequate legislation [9] where spillage control devices play a fundamental role. These tools should be able to identify unequivocally the presence of OMW along the course of the river while remaining affordable and easily portable.

p-Coumaric acid (4-hydroxycinnamic acid, pCA from now on) is a biorecalcitrant PC typically found in OMW [10]. Therefore, pCA and its derivatives by degradation draw a reliable evidence of the presence of OMW as long as no other industrial spill is considered (urban wastewater feeds do not contain this molecule). Finding the presence of pCA and its derivatives indicates a very highly probable OMW discharge [11].

Hydroxytyrosol [4-(2-hydroxyethyl)-1,2-benzenediol,2] (HTyr) is the major polyphenol present in OMW with antioxidant potential very superior to that of pCA, hence its industrial value. HTyr is characterized by a high antioxidant activity, similar to that of the main synthetic antioxidants. The antioxidant and antibacterial properties of HTyr [12] and their other beneficial effects on human health were confirmed by a large number of studies, encouraging its commercialization. HTyr being such a valuable substance, it is an increasingly common procedure in olive mill production facilities to carry out a recovery of HTyr and Tyrosol (Tyr can be transformed into HTyr through bacterial conversion and is also present in OMW [13]) prior to releasing OMW into the environment. This is not the case of pCA, a substance generally not so appreciated and most often released into watercourses.

The recovery of these compounds is quickly gaining popularity. Specialized literature on this subject is continuously developing, OMW becoming increasingly valorized through processes that enable the recovery of valuable substances – most importantly, HTyr and Tyr [14]. Selective recovery of HTyr and Tyr is currently being investigated via treatment of OMW with commercial sorbents [14]. Molecularly Imprinted Polymers is a promising technology in this field [15].

HTyr is not commercially available because its production in large quantities is an expensive process and involves the use of toxic reagents [16]. A solution might rely on the bioconversion of Tyr from OMW to HTyr [1].

The very status of HTyr as a high-value molecule justifies why our protocol follows pCA in the study of OMW instead of their major polyphenolic compound – in most cases, HTyr has been almost totally removed from the OMW before the spill takes place, hence rendering any protocol based on HTyr monitoring unreliable.

pCA in oil is quantified mainly by the Folin–Ciocalteau method, based on the reductive properties of phenols in an alkaline medium. This is a nonspecific colorimetric method – thus, additional analytical methodology is necessary to determine changes in the pCA content in wastewater throughout the degradation process. Several high-performance analytical methods have been developed to characterize the complex phenolic content of OMW [5,17]. The main tool used in the characterization and quantitative analysis of pCA is liquid chromatography coupled with mass spectrometry [18]. However, this method has two great disadvantages: its steep price and the fact that the poor mobility of the necessary equipment renders it unusable for field analysis.

The aim of this work is to develop an analytic tool for OMW spillage control based on the detection and monitoring of pCA and its derivatives inasmuch as compounds that indicate OMW presence.

This system has proved to be 100% reliable in OMW spillages where pCA is present – that is to say, the very vast majority of OMW spillage cases. In fact, even though HTyr is found in olive oil in larger quantities than pCA, previous studies point at pCA as an ever-present PC in olive oil, whereas HTyr is not always present [19]. Hence, please note that given the case of a pCA-free OMW spill, the present tool would not be of any use – a scenario where HTyr-tracking-based tools would be an interesting line of research.

To attain this objective we used the Reversed-Phase High-Performance Liquid Chromatography (HPLC) method [20] as a starting point for the design of an innovative protocol for the determination of pCA and its reaction intermediates in wastewaters. Since the treatment requirements for the samples are minimal, the overall process is uncomplicated to reproduce – the mobile phase composition is simple, allowing good separation and resolution. These advantages reveal this method as suitable for analysis. Working with a wavelength of 280 nm with calibrated intervals between 10 and 500 mg L^{-1} yielded optimal linear adjustments.

Once the method was set up and fully enhanced, its performance as a spill control tool was put to test by using it to monitor pCA degradation routes through chemical oxidation and biological anaerobic degradation. This emulates the conditions of the spill when entering the watercourse, with anaerobic degradation taking place in deep zones and oxidation in shallow zones.

The present work had the following specific goals: (i) designing an innovative protocol for the determination of pCA and its derivatives by the RP-HPLC-UV, besides (ii) the application of such methods to pCA degradation routes via oxidation (as it occurs in the shallow zones of the river) as well as via biologic anaerobic degradation (as it occurs in the deep zones of the river), and

therefore, the method proves suitable for the reliable detection of OMW spills from the olive industry and (iii) testing the protocol in an actual OMW-affected watercourse.

This tool has been developed to face the challenge that resistant and periodical spills from the olive industry represent in the Mediterranean countries, where our research group has a working experience that dates back to the year 1998 [11,21].

2. Materials and methods

2.1. Materials

2.1.1. pCA and its derivate compounds

Considering that pCA is a characteristic compound of OMW, not present in urban wastewaters and difficult to biodegrade, it is possible to make a simplification of olive waters – which are widely varied but share the presence of pCA as a common feature – by using this molecule to follow degradation routines and to compare their results without interferences that could misrepresent the behavior of the different systems.

pCA degradation by chemical oxidation can happen in the three following ways [11], as shown in Figure 1.

Route a: Oxygen attacking the exocyclic double bond of pCA gives rise to glyoxylic acid and *p*-hydroxybenzaldehyde acid. The latter can be oxidized and by means of decarboxylation (Route c) it can originate hydroquinone and phenol.

Route b: The decarboxylation of pCA gives rise to *p*-vinylphenol, which is an intermediary that cannot be detected due to its high reactivity. By adding catalyzed water to *p*-vinylphenol, 4-(1 hydroxyethyl) phenol is

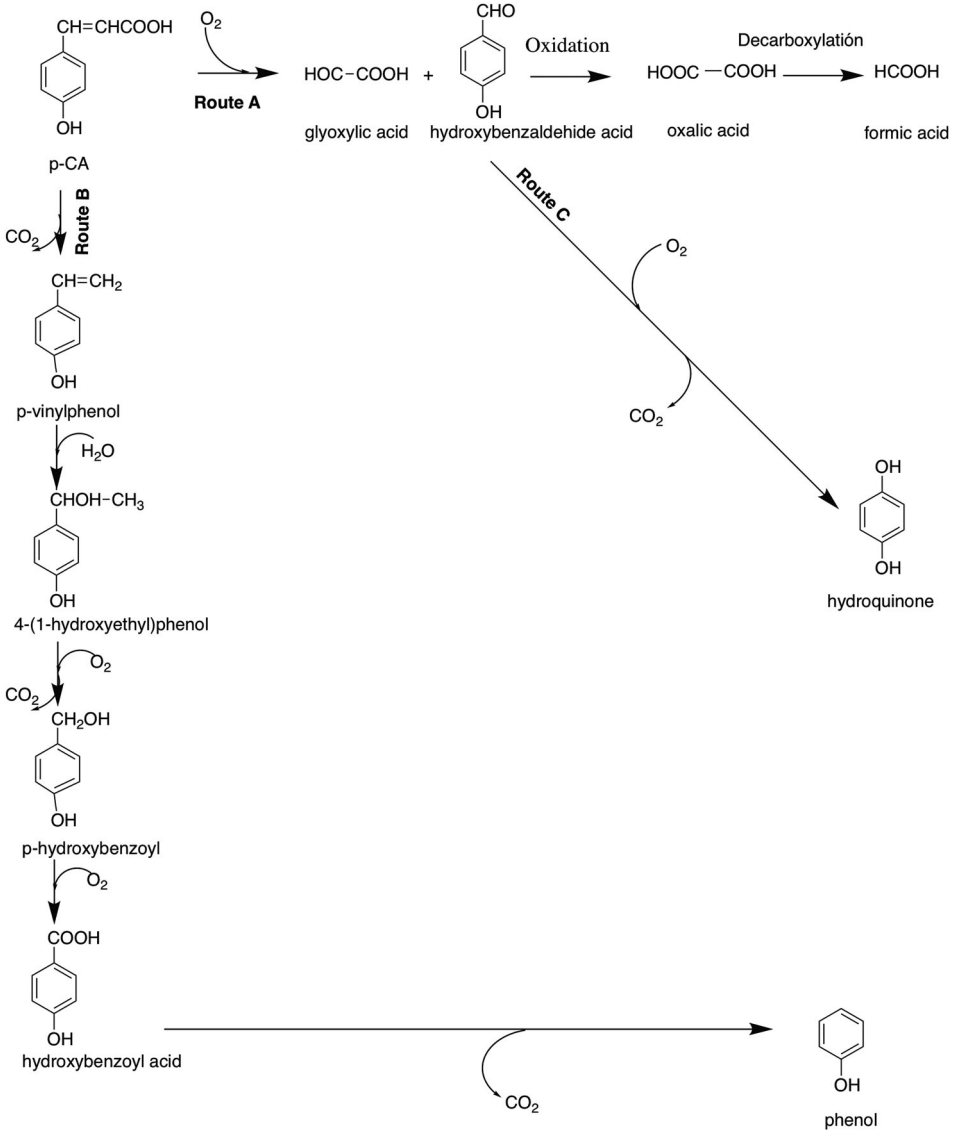

Figure 1. pCA degradation routines.

formed, which can produce *p*-hydroxybenzoyl by decarboxylation. This alcohol can be oxidized to *p*-hydroxybenzoyl acid. The latter, which can also be produced by the oxidation of the *p*-vinylphenol, would give rise to phenol through decarboxylation.

Derivate compounds of pCA by anaerobic biological degradation.

In anaerobic conditions, pCA is fully degraded to *p*-hydroxybenzoic acid in 3 days, to *p*-hydroxybenzaldehyde in 4 days and to Hydroquinone and Phenol in 7 days [22,23].

Therefore, *p*-hydroxybenzoic acid, hydroquinone, hydroxybenzaldehyde and Phenol can be defined as representative molecules to be determined when verifying pCA degradation routes.

2.1.2. OMW spill

The protocol was put to test in the Alcarayón stream, an affluent of the river Guadiamar (Seville, Spain). Despite its headwaters being clean, it suffers from OMW spills downstream. An experimental study was carried out throughout six months, four of which coinciding with the olive oil production season. Water samples of an OMW spill were taken in the very point where the discharges take place – an area that has been suffering from periodic OMW discharges for the last 25 years. This point will be referred to as S1. Also, water samples were taken both before and after the point where the industrial spill occurs. The location where these samples were collected will be referred to as US (for upstream) and DS (for downstream), respectively. Samples from urban sewerages and OMW were also taken into study. Each sample had its physicochemical parameters determined and was subject to chromatographic analysis. Spatiotemporal variations in pollutant concentrations were closely monitored.

The sampling was carried out following the conserving, transporting and storing instructions of the Standard Methods for the examination of Water and Wastewater, 160 B and C sections [24].

The following parameters were measured in all samples – pH: the activity of H+ ions measured with a Hamilton pH C101 potentiometer with an accuracy of ±0.1 pH units. Electric conductivity: measured with a WTW@Multiline P4 in accordance to the UNE-EN 27888-93 normative. Dissolved Oxygen (DO): measured with an Intellical LDO luminescent/optical DO field sensor with an accuracy of ±0.05 mV. Temperature: measured via the temperature probes from the DO and pH measuring equipment, with an accuracy of ±0.1°C. COD: in accordance to the APHA, 5220 C, 1992 normative. Polyphenols (pCA and derivatives): by means of the protocol discussed in this paper (RP-HPLC-UV).

2.2. Methods

2.2.1. pCA chemical oxidation: addition of potassium permanganate ($KMnO_4$)

Organic acids, alcohols, aldehydes, ketones, phenols, cresols and a wide range of nitrogen compounds and aromatic hydrocarbons are all subject to oxidation by $KMnO_4$.

$KMnO_4$ doses might vary depending on the structure and molecular size of the organic compounds present in the water. Typically, $KMnO_4$ behaves partially, breaking organic molecules in smaller units with less pollutant potential. Previous studies point at the capacity of $KMnO_4$ to fully oxidate certain hardly biodegradable compounds to CO_2 and H_2O. [25].

The treatment of pCA and its derivatives with different doses of $KMnO_4$ was assayed in order to verify pCA degradation routes through chemical oxidation by $KMnO_4$. Three groups of samples were prepared: pCA, hydroquinone and phenol, all of 50 mL and with a concentration of 500 mg L^{-1}. Thirty samples were analyzed, 10 of each compound and treated with $KMnO_4$ quantities ranging from 1 to 10 mL.

Biodegradability measurements were calculated considering the remaining organic matter in the supernatant as Total Organic Carbon (TOC). Products obtained by chemical oxidation were analyzed using the RP-HPLC-UV.

2.2.2. Assays on anaerobic biodegradability

2.2.2.1. Microdigesters. A set of seven microdigesters was constructed. Each microdigester consisted of a 125 mL flask with a lid that incorporated a reverse valve allowing the outlet of gas but preventing any inlet [26].

A nitrogen current was applied to the culture for 15 min in order to obtain anaerobic conditions. The nitrogen was inserted through the sampling aperture with the aid of a needle so as to allow air outlet through the valve. After the aforementioned 15 min, the aperture was sealed with paraffin.

This kind of reactors permit taking samples periodically and are especially indicated for extended periods of incubation. Methods that do not allow biogas dissipation cause an overpressure that affects the bacterial culture.

2.2.2.2. Mediums and conditions of the culture. The microdigesters were prepared by adding 10 mL of inoculum to the mineral medium. This inoculum consisted of biomass originally from the anaerobic digesters in a conventional urban wastewater treatment plant (WTP) located in Seville (Spain). It was chosen due to it being faithfully representative of the conditions that can be

found in the deep waters of a course – populated by bacteria similar to those of urban wastewaters, carrying out anaerobic digestion as imposed by low O_2 concentrations.

The dose of inoculum that was used equals 0.7 gVSS L^{-1} – its concentration is usually expressed as volatile suspended solids (VSS) in the sludge. This offers sufficient data for our purposes and makes it easier to analyze than considering the concentrations of several bacterial groups separately.

The minimum concentration in the sludge lies within an interval of 0.8–8 g VSS L^{-1}. Nonetheless, as far as sludge from urban WTP are concerned – whose activity is 50% higher than in pure cultures – a concentration of 0.5 VSS L^{-1} is enough [25].

The mineral medium was composed of: Yeast extract 0.20 g L^{-1}, Meat concentrated extract 15.00 g L^{-1}, Sodium acetate 30.00 g L^{-1}, Sodium lactate 8.00 g L^{-1} and Ethanol 0.50 g L^{-1}.

Seven microdigesters were constructed in order to test different toxic compounds, namely hydroxybenzoic acid (1), hydroxybenzaldehyde (2), pCA (3), hydroquinone (4), phenol (5), equal parts standards mixture of all previous compounds (6) and OMW (7) (5 mL, 500 mg L^{-1}). The operative method for the assembly of anaerobic micro-reactors is described as it follows.

Firstly, 90 mL of the mineral culture medium was inserted into a flask. Then 500 mg L^{-1} of the toxic compound was added prior to the inclusion of the 10 mL inoculum. pH was adjusted between 7.0 and 7.1. Then the microdigester was closed with a reverse valve lid and a nitrogen current was applied. After 15 min, the microreactor is sealed and incubation is proceeded using an orbital incubator (New Brunswick Scientific) at 150 rpm. Finally, the microdigester is thermostatized at 35 ± 1°C.

Every reactor was sampled periodically with the aid of a syringe through the sealed sampling aperture. After each sample was taken, the aperture was re-sealed in order to maintain the anaerobic conditions. The samples were centrifuged, filtered and frozen at 20°C. They were analyzed using liquid chromatography and their biodegradability was determined by monitoring COD in the digesters during the incubation lapse.

COD was determined by the potassium dichromate volumetric quantification method as indicated in UNE 77-004-02, ISO 6060-86. The gas composition was analyzed by forcing the gas through a bath of NaOH in order to remove CO_2 and so, the experimental values of CH_4 were obtained [27].

2.2.3. HPLC in reverse phase (UV)

The systems used in the experience consisted of a Gilson HPLC system (Gilson Medical Electronics, Middleton, WI, USA) and a Dynamax UV-1 absorbance detector (Rainin Instrument, Woburn, MA, USA), set at 280 nm. The chromatograms were monitored with an Apple Macintosh Classic II computer.

Chromatographic separations were achieved using a 15 cm × 4.6 or 4.0 mm i.d. Nucleosil C18 (5 µm, 120 Å) at room temperature, preceded by a pre-column that prevents impurities from entering the system. A reverse-phase HPLC assay was carried out using an isocratic system with a flow rate of 1 mL/min, a mobile phase of acetonitrile.

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The instrumental equipment and material that were employed consisted of 25 µL glass micro-syringes, Eppendorf vessels, HPLC filters with 0.45 µm pore diameter, a centrifugal machine and MILI-Q Plus 50 equipment to produce analytical grade water for critical applications (HPLC) and a HPLC.

The employed reagents include liquid chromatographic eluents ultra-pure water to prepare the mobile phase, reactive and stock solutions of analyzed compounds and standard solutions of pCA, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, hydroquinone and phenol. All chemicals were of analytical grade.

The HPLC equipment was calibrated with aid of standards, thus guaranteeing the reliability of its analysis and rendering LCMS double-check techniques unnecessary. This way, the appliance of the protocol remains within an affordable price range – a price range a larger number of control institutions can reach than if expensive LCMS techniques were involved [20,28].

2.2.3.1. Calibration curves. The calibration curves were made by using different concentrations standards (0–500 mg L^{-1}) of stock solutions prepared for each compound: pCA, hydroxybenzoic acid, hydroquinone, *p*-hydroxybenzaldehyde and phenol. The injections in the HPLC were carried out in triplicate and yielded a calibration curve for each compound. These curves were plotted by comparing their areas to the standards' concentrations expressed in mg L^{-1}.

The optimal analysis of the samples demanded two fundamental problems to be solved: In the first place, to achieve a sufficient resolution permitting the observation of the complete separation of the compounds (thus allowing for a correct integration), and in the second place, to define accuracy limits and optimal detection conditions.

The determination procedures of pCA and its degradation derivatives are described hereinafter.

Samples preparation: samples were centrifuged at a speed of 400 rpm during 10 min to separate suspended particles. The aqueous phase was filtered through a 0.450 µm filter, labeled and frozen until the HPLC analysis.

Results evaluation: the chromatographic determination is carried out by the external standard method. Using the height and area of each peak, and its corresponding calibration line, the concentration of each of the analyzed compounds is calculated. The area and peak height are both directly proportional to analyte concentration in the sample. The detection and quantification limits were determined by the signal/noise ratio method. The software employed was Star Chromatography Workstation Varian with the statistics pack PRESTA.

2.2.4. Advanced organic matter analysis: TOC and TC combustion-infrared method

To quantify the amount of organic carbon, organic molecules must be broken into simpler carbon units. For this analysis, The combustion-infrared method was used, whose accuracy depends on the reduction of the molecular size, and it is appropriate for samples with TOC ≥ 1 mg L^{-1}. The measure of TOC was performed by measuring total carbon (TC) and inorganic carbon (IC) separately. The difference between these two reveals the TOC value.

TC: Combustion inside a muffle furnace set at 680°C in the presence of a platinum catalyst. CO_2 was measured by means of an infrared analyzer.

$$TC\ (Organic + Inorganic) + Pt + O_2 \longrightarrow CO_2 + H_2O.$$

IC: inorganic carbon at room temperature becomes CO_2 in the acidification chamber.

$$IC + H_3PO_4 \longrightarrow CO_2 + H_2O.$$

Unlike BOD and COD, TOC is independent of the oxidation state of the organic matter.

To determine the organic dissolved carbon, the samples were centrifuged and filtered through a 45 µm filter.

3. Results and discussion

3.1. Results

3.1.1. RP-HPLC-UV method for pCA determination and its derivate compounds

The optimal conditions for the analysis of pCA and its derivatives by degradation are described hereafter.

The ultraviolet region absorption spectrum of pCA revealed that it presents its maximal absorption wavelength at 280 nm. The chromatograms displayed maximum sensibility at this wavelength, making it optimal for the study of pCA and pCA derivatives by degradation.

The best chromatographic separation was attained for an ACN:H_2O (25:75%, v/v) mobile phase, with retention times below 7 min with an isocratic flow of 1 mL min^{-1}.

3.1.1.1. Calibration curves. Concentrations were calculated using calibration curves.

Good linear adjustments were obtained in the 4.6 mm column, with an r^2 between 0.970 and 0.999 (CV <15%) for hydroquinone, *p*-hydroxybenzaldehyde and phenol and r^2 between 0.995 and 0.999 (CV <15%) for *p*-hydroxybenzoic acid and pCA. In the 4.0 mm diameter column, similar adjustments with CV < 20% (Table 1) were obtained for all compounds.

Detection and quantification limits were determined by the signal/noise method with a ratio of $S/N = 3$ for L_D and 10 for L_Q. Table 2 shows the obtained results. The calibration curves allowed determining L_D and L_Q by linear or r^2 adjustment methods.

3.1.2. Simulation of shallow, aerobic zones of a watercourse: TOC variation analysis as a response to $KMnO_4$ addition.

A clear TOC decrease was observed in pCA as the $KMnO_4$ dose increased. Hydroquinone behaved similarly, while phenol was nonetheless barely affected by the $KMnO_4$ addition. This is due to the very limited degradability of phenol.

The results obtained are displayed in Table 3.

3.1.3. Simulation of shallow, aerobic zones of a watercourse. pCA monitorization by RP-HPLC-UV

Measurement conditions are described hereinafter.

Chromatographic column: 5 µm nucleosil C-18, 15 cm long and 4 mm inner diameter.

Table 1. Calibration curves and adjustments.

Compound	Equation	r^2	CV (%)
150 × 4.0 mm			
p-hydroxybenzoic acid	$y = 9.913 \times 10^2x - 4.422 \times 10^4$	0.998	18
pCA	$y = 4.962 \times 10^3x$	0.950	19
Hydroquinone	$y = 4.735 \times 10^3x$	0.998	6.3
p-hydroxybenzaldehyde	$y = 6.322 \times 10^3x$	0.970	12
Phenol	$y = 3.499 \times 10^2x$	0.970	7.3
150 × 4.6 mm			
p-hydroxybenzoic acid	$y = 3.556 \times 10^2x$	0.995	6.8
pCA	$y = 4.994 \times 10^3x$	0.999	4.9
Hydroquinone	$y = 4.833 \times 10^2x$	0.990	13
p-hydroxybenzaldehyde	$y = 5.508 \times 10^3x$	0.991	12
Phenol	$y = 3.878 \times 10^2x$	0.999	5.3

Table 2. Detection and quantification limits. Signal-to-noise ratio method.

Compound	L_D (mg L^{-1})	CV (%)	L_Q (mg L^{-1})	CV (%)
Signal/noise method (150 × 4.0 mm)				
p-hydroxybenzoic acid	4.2 ± 1.0	24	14.0 ± 3.0	24
pCA	1.05 ± 0.24	23	3.50 ± 0.80	23
Hydroquinone	0.70 ± 0.20	32	2.30 ± 0.70	32
p-hydroxybenzaldehyde	0.70 ± 0.16	22	2.30 ± 0.50	22
Phenol	12.0 ± 3.0	32	40 ± 10	32
Signal/noise method (150 × 4.6 mm)				
p-hydroxybenzoic acid	13.0 ± 3.0	23	44 ± 10	23
pCA	0.90 ± 0.30	32	2.80 ± 0.90	32
Lineal or quadratic adjustment method (150 × 4.0 mm)				
p-hydroxybenzoic acid	21.2 ± 1.6	7.6	31 ± 50	17
pCA	4.21 ± 0.10	24	14 ± 30	23
Hydroquinone	17.00 ± 0.60	3.3	27.4 ± 1.9	6.8
p-hydroxybenzaldehyde	10.10 ± 0.24	2.4	11.70 ± 0.80	6.9
Phenol	20.0 ± 4.0	19	80.0 ± 1.4	17
Lineal or quadratic adjustment method (150 × 4.6 mm)				
p-hydroxybenzoic acid	18.3 ± 1.5	8.3	39.0 ± 6.0	15
pCA	2.30 ± 0.40	16	18.7 ± 1.2	6.7

Measured wavelength: λ = 280 nm.

Mobile phase: ACN = 25% and H_2O = 75%.

The obtained calibration curves can be consulted in Section 3.1.1. The linear regressions for the area vs. concentration and height vs. concentration charts were calculated.

Figure 2 displays the obtained chromatograms as well as the products of the chemical degradation. The results of the chromatographic analysis are shown in Table 4.

The compounds identified and quantified during the oxidation process of pCA were *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid.

The maximum concentration of aromatic reaction intermediates was obtained during the addition of $KMnO_4$ quantities ranging between 0.50 and 1.50 mL. *p*-Hydroxybenzoic acid reaches its peak concentration when the $KMnO_4$ addition equaled 0.5 mL while p-hydroxybenzaldehyde reaches its peak concentration (110 mg L^{-1}) when the $KMnO_4$ addition was of 1.5 mL.

The pCA sample, usually colorless upon $KMnO_4$ addition, took a yellowish color in the addition interval between 0.5 and 1.5 mL. This color can be attributed to *p*-hydroxybenzaldehyde presence as well as that of other undetected compounds such as p-benzoquinone, originated as a result of hydroquinone oxidation.

These results reveal that pCA oxidation occurred in two different paths. One consists of oxygen attacking the double exocyclic ring and originating *p*-hydroxybenzaldehyde, whose oxidation by decarboxylation gives rise to hydroquinone and phenol. The other, which occurs simultaneously, consists of the decarboxylation of pCA, which forms *p*-hydroxybenzoic acid and phenol.

3.1.4. Simulation of deep, anaerobic zones of a watercourse: COD variation analysis in microdigesters

Figure 3 and Table 5 show the results obtained in COD measurements of the analytes from microdigesters during the one-month-long incubation lapse. With the aim to follow the evolution of organic load subsequent to the pCA discharge, COD variation percentage was monitored for each compound specifically over a period of 30 days.

COD was observed to decrease in all analytes during the first 5 days. Up to this point, the highest decrease rate was found in OMW and phenol samples (44%) and

Table 3. TOC variation and COD evolution (expressed in elimination percentage) in function of $KMnO_4$ additions.

$KMnO_4$ (1N) *V* (mL)	pH	TC (mg L^{-1})	IC (mg L^{-1})	TOC (mg L^{-1})	%COD removal
pCA					
1	8.08	283.0 ± 2.3	9.30 ± 0.20	273.6 ± 2.4	14.5
2	8.62	286 ± 15	9.50 ± 0.20	277 ± 15	13.4
4	8.62	228 ± 15	20.5 ± 1.1	208 ± 16	35.2
6	8.56	243.0 ± 9.0	56.8 ± 1.7	186 ± 10	41.8
8	8.75	252 ± 11	94.80 ± 0.80	157 ± 12	50.9
9	9.03	253 ± 13	84.10 ± 0.10	169 ± 23	47.2
10	9.02	266 ± 20	161.9 ± 2.1	104 ± 22	67.5
Hidroquinone					
1	6.72	294.0 ± 10.0	1.2890 ± 0.0020	294 ± 10	11.3
2	7.30	247.0 ± 21.0	9.8 ± 1.6	237 ± 31	28.1
4	7.72	305.0 ± 0.3	22.60 ± 0.40	282.60 ± 0.70	14.4
6	8.18	274.0 ± 13	48.4 ± 1.0	226 ± 14	31.6
8	8.54	249.0 ± 2.0	109.8 ± 1.1	139.0 ± 3.0	57.8
9	9.03	200.0 ± 11.0	118.2 ± 1.5	82 ± 13	75.2
10	8.83	193.0 ± 9.0	120.9 ± 1.2	72.0 ± 9.0	78.2
Phenol					
1	8.10	353 ± 25	21.40 ± 0.20	333 ± 25	12.7
2	8.14	283.0 ± 5.0	60.30 ± 0.10	314.0 ± 5.0	17.4
4	8.28	300.0 ± 9.0	28.10 ± 0.10	346.0 ± 9.0	8.9
6	8.03	340 ± 15	47.7 ± 1.3	326 ± 16	14.1
8	8.35	353 ± 13	104.9 ± 8.0	269 ± 21	29.1
9	8.58	314 ± 17	91.0 ± 5.0	285 ± 22	25.4
10	8.62	330 ± 30	102.9 ± 1.6	271.0 ± 5.0	28.6

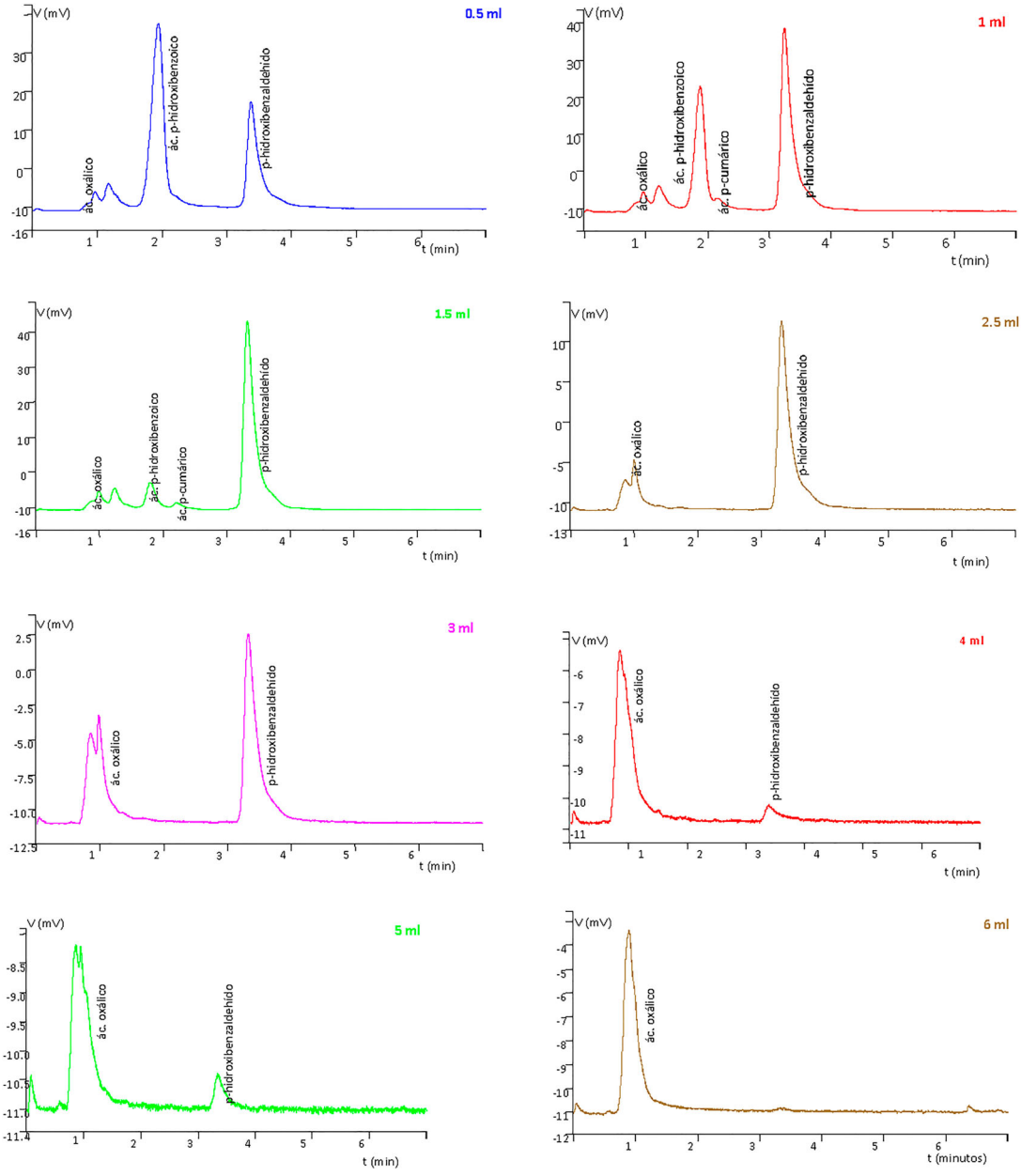

Figure 2. Chromatograms of pCA and its degradation derivatives.

the lowest corresponded to the standards mixture and benzaldehyde samples (13%). Nonetheless, after 18 days of incubation, in OMW, hydroquinone, phenol and standards mixture samples, COD rose instead of continuing to decline as expected. This is explained by the fact that a significant part of the microorganism population had died and liberated its cellular content into the medium, thus increasing the total available substrate. This circumstance could have been avoided or attenuated by using an inoculum from OMW anaerobic digesters instead of biomass from WTP anaerobic digesters. This inoculum was finally chosen because pCA degradations occur indistinctively whether the bacterial population is adapted or not. Watercourses long and periodically suffering from OMW spillage present adapted bacterial populations, but those only occasionally polluted by OMW do not. The organic matter disposal performance is higher when the bacteria population is adapted to OMW.

In spite of this, and because the goal of this research wass not optimal organic matter disposal but the development of a protocol as reliable and widely applicable as possible – even in unfavorable situations where OMW enters the ecosystem for the first time and adapted bacteria is not available – we opted for WTP inoculums.

Table 4. Results of the chromatographic analysis of 50 mL pCA samples (500 mg L^{-1}) oxidized by $KMnO_4$ (1 N).

	pCA samples 50 ml (C_0= 500 ppm)									
Identified compound	Added volume $KMnO_4$ (1 N) (mL)									
% C_O	0.50	1.00	1.50	2.50	3.00	4.00	5.00	6.00	7.00	10.00
p-hydroxybenzoic acid										
C (mg L^{-1})		380	116							
$u^a(C)$ (mg L^{-1})		70	21							
% C_O		75	23							
p-hydroxybenzaldehyde										
C (mg L^{-1})	51	104	110	51	31.0	1.59	1.29			1.26
u(C) (mg L^{-1})	7	13	14	6	4.0	0.19	0.16			0.16
C_O (%)	10	21	22	10	5.2	0.31	0.25			0.25
pCA										
C (mg L^{-1})		3.90	2.40							
u(C) (mg L^{-1})		0.70	0.50							
% C_O		77	48							

[a]u: measurement uncertainty.

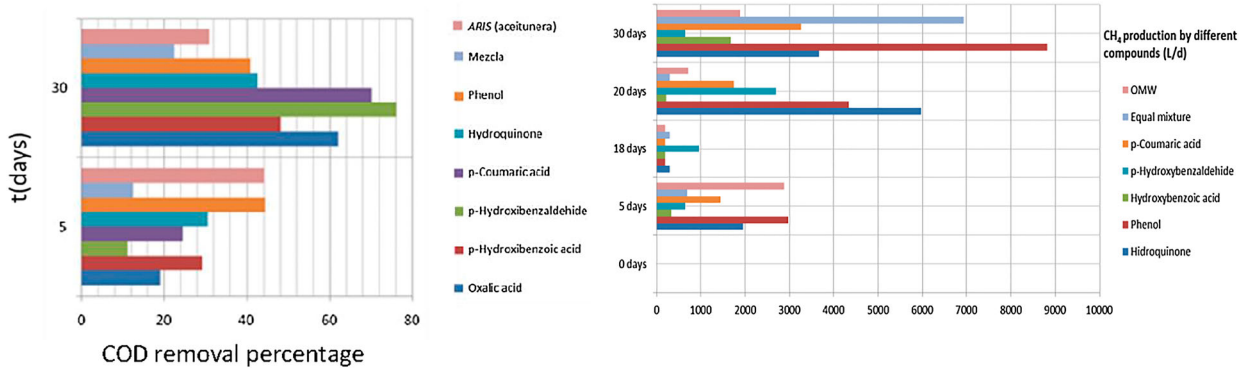

Figure 3. COD evolution (removal percentage) + CH_4 production by different compounds.

Our assays aimed at reproducing the real-life conditions of the shallow and deep waters of a (periodically or unprecedentedly) polluted watercourse. Concretely, the inoculum designed to emulate deep water pCA degradation will be typically anaerobic. The use of unadapted microbial population seeks to emulate the conditions of a previously unpolluted by OMW stream. The protocol was proved reliable even in this unfriendly configuration.

The results obtained reveal that the most toxic compounds were phenol (whose COD increase rate was of 125%), followed by hydroquinone (COD increase rate 85%), the standards mixture (COD increase rate 15%) and OMW (COD increase rate 3%). After 13 days, COD decrease had started in hydroquinone and OMW cultures, already reaching levels below their initial values. At the same time, a COD decrease was also observed in the phenol culture although its COD was still 71.3% above its initial value. Only in the standards mixture, sample COD continued to rise, reaching an increment of 83% above its initial value. Cultures inoculated with pCA, 4-hydroxybenzoic acid and *p*-hydroxybenzaldehyde showed a COD decrease

Table 5. COD evolution over time in pCA and its degradation derivatives.

	0 days	5 days			18 days			20 days			30 days		
Compound	COD mg L^{-1}	COD/$COD_{removal}$ mg L^{-1}	%$_r$	CH_4 L	$COD/COD_{removal}$ mg L^{-1}	%$_r$	CH_4 L	$COD/COD_{removal}$ mg L^{-1}	%$_r$	L	$COD/COD_{removal}$ mg L^{-1}	%$_r$	L
p-hydroxybenzoic	27,104	19,152	29	1670	20,640	24	102	19,800	27	218	14,040	48	1680
p-hydroxybenzaldehyde	26,136	23,184	11	649	19,200	27	956	8400	68	2700	6240	76	648
pCA	28,072	21,168	25	1450	25,920	8	82	19,200	32	1747	8320	70	3264
Hydroquinone	29,040	20,168	31	1952	53,760	−85	0	28,800	1	5971	16,640	43	3672
Phenol	29,040	16,128	44	2970	65,280	−125	0	49,800	−71	4334	17,160	41	8813
Equal mixture	24,200	21,168	13	697	27,840	−15	0	44,400	−83	0	18,720	23	6934
OMW	27,104	15,120	44	2876	27,840	−3	0	25,200	7	713	18,720	31	1879

straight from day one, although it must be noted that the pCA culture suffered a severe destabilization that provoked a dramatic drop in the elimination rate from 25% in day 5 to 8% in day 19.

Finally, at day 30, the decreasing tendency is stabilized and generalized. All cultures had experienced a COD decline in comparison to their original values. Final lowest COD measurements were found in the standards mixture sample (23% lower) and OMW sample (31% lower). Final highest COD measurements correspond to the *p*-hydroxybenzaldehyde sample (76% lower) and the pCA sample (70% lower).

COD variation percentages over time were subjected to a regression analysis. No satisfactory adjustment was obtained for hydroquinone, *p*-hydroxybenzaldehyde and the standards mixture ($r^2 < 0.9$). Meanwhile, phenol and OMW presented optimal square adjustments. The OMW sample had the highest r^2.

The COD variations led to an increase in biogas production, measured as CH_4 concentration. Figure 3 and Table 5 show also the registered methane production.

It is apparent that CH_4 production also decays according to the decrease in COD removal performance.

3.1.5. Simulation of deep, anaerobic zones of a watercourse. Analysis of samples from microdigesters by HPLC

Measurement conditions are described hereinafter.

Chromatographic column: 5 µm nucleosil C-18, 15 cm long and 4.6 mm inner diameter, Measured wavelength: λ = 280 nm, Mobile phase: ACN = 25% and H_2O = 75%.

A close monitoring of the anaerobic degradation that occurs in the microdigesters containing pCA and OMW was performed with aim of not only to determining the degradation compounds, but also to follow the evolution of their apparition over time (and so their stability) during a month. The objective behind this is developing a spillage control tool that does not just detect the presence of the waste, but is also able to date the discharge depending on the measured compounds. Moreover, the equal parts mixture microdigester was also monitored to study the possible behavior of a watercourse that is periodically affected by OMW spills – a scenario where pCA and several of its derivatives may coexist.

3.1.5.1. OMW anaerobic degradation.

The concentration of hydroxybenzoic acid rose during the first days due to the partial degradation of the sample, but eventually, after 30 days, the presence of this phenol compound was no longer detected. Hydroquinone was the only compound to be detected (6.5 mg L^{-1}), originated by pCA anaerobic degradation (Table 6, Figure 4).

3.1.5.2. pCA anaerobic degradation.

After the 30-day incubation process, the detected compounds were hydroquinone (due to degradation of pCA) and residual amounts of pCA. During the degradation process, hydroxybenzoic acid and phenol were detected (Table 7, Figure 6).

3.1.5.3. Anaerobic degradation of the equal parts standards mixture.

pCA degradation provokes a general increase in *p*-hydroxybenzoic acid and also gives rise to hydroquinone before disappearing. After 30 days, hydroquinone is the only significantly detected compound in the mixture. A phenol increase is also significantly noted (together with hydroquinone it represents the final stages of pCA degradation) as time advances (Table 8, Figure 6).

Following the evolution of the different compounds over time allows estimate how long ago a spill occurred. In this regard, the following was observed.

After a day, pCA was identified in a 0.5–1.1 mg L^{-1} concentration, making it the highest value detected in the OMW reactor. *p*-hydroxybenzoic acid was also measured in concentrations between 7 and 12 mg L^{-1}, as well as hydroquinone (6–19 mg L^{-1}), indicating that the degradation of pCA and *p*-hydroxybenzoic acid had started. Eventually, phenol was detected in minor concentrations and only in the equal parts standards mixture reactor.

After 2 days, phenol and hydroquinone were detected in all microdigesters. *p*-hydroxybenzoic acid was found in the equal parts standards mixture reactor.

After 3 days, pCA was still detected in the OMW reactor and there was a certain quantity of hydroquinone

Table 6. Results of the OMW microdigester HPLC analysis.

Microdigester 8: OMW					
	Time (days)				
Compound (mg L^{-1})	1	2	3	7	30
p-hydroxybenzoic acid	7.10 ± 0.50	–	14.3 ± 1.0	25.4 ± 1.7	–
pCA	0.530 ± 0.030	–	0.850 ± 0.041	–	–
Hydroquinone	6.10 ± 0.81	6.12 ± 0.82	5.50 ± 0.70	8.1 ± 1.0	6.50 ± 0.81
Phenol	–	–	–	16.20 ± 0.91	–

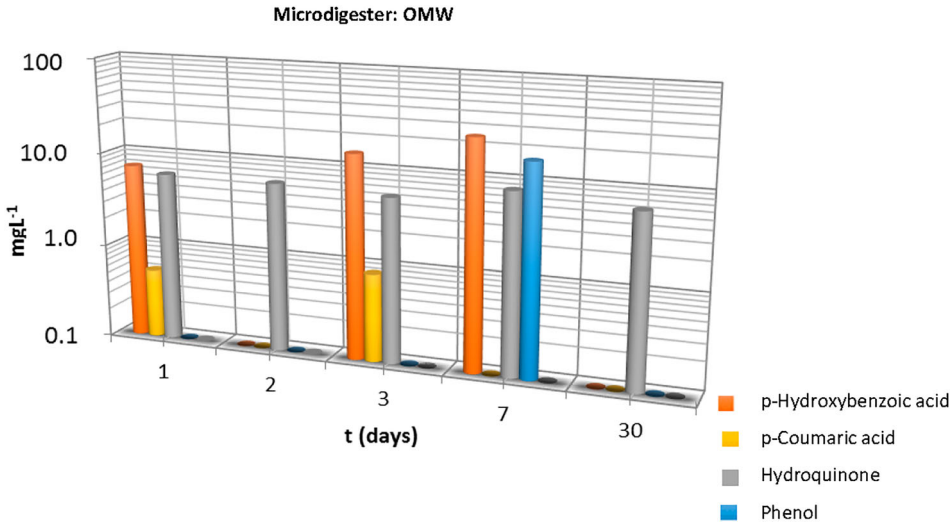

Figure 4. Results of the OMW microdigester HPLC analysis.

(ranging from 4.7 to 7.4 mg L^{-1}) in every reactor. *p*-hydroxybenzoic acid was the compound with the highest concentration in this period (from 10 to 57 mg L^{-1}), signaling an intermediate stage in the pCA degradation process.

Within a week, all pCA derivatives by degradation had been detected and their concentrations slowly decreased, with hydroquinone and *p*-hydroxybenzoic acid still delivering significant concentrations. Phenol was the compound with the highest concentration after 7 days.

Finally, after a month, pCA concentration was <1 mg L^{-1} and the compound with the highest concentration in all reactors was hydroquinone.

3.1.5.4. OMW spill. A set of analysis was carried out in the Alcarayón Stream with the following results: upstream, DO, pH, EC and COD values indicated a fine environmental condition. Nonetheless, the situation was diametrically opposed in the spillage zone, where OD approached near-zero values and EC and COD increased dramatically – common symptoms of an industrial spill (Table 9).

The RP-HPLC-UV chromatographic analysis revealed the presence of pCA and pCA derivatives (Figure 7).

4. Discussion

Our assays were intended to simulate the different conditions of the shallow and deep waters of a watercourse. These two mediums differ in oxidation state, causing pCA to degrade following different routes in each one. The different degradation routines for pCA in several oxidation states have been determined by tracking pCA and its derivatives via the HPLC-RP-UV as described earlier in this paper.

The HPLC-RP-UV is a technique that relies on simple, inexpensive and easy-to-carry equipment yet delivers the same resolution as orthodox HPLC equipment and outruns them as far as response times and specificity are concerned. That is to say, the HPLC-RP-UV is the ideal tool for field use – it enables *in situ* tests in strategic points of a suspectedly polluted watercourse, making it possible to determine the presence and quantity of pCA and pCA derivatives and thus approximately date a spillage in relation to the concentration of each compound.

Table 7. Results of the pCA microdigester HPLC analysis.

Microdigester 5: pCA					
	Time (days)				
Compound (mg L^{-1})	1	2	3	7	30
p-hydroxybenzoic acid	12.40 ± 0.80	–	57.0 ± 4.0	12.70 ± 0.60	–
p-hydroxybenzaldehyde	–	–	–	0.0154 ± 0.018	–
pCA	1.090 ± 0.050	–	–	1.350 ± 0.070	0.52 ± 0.03
Hydroquinone	14.1 ± 1.8	5.90 ± 0.80	7.4 ± 1.0	6.90 ± 0.90	6.80 ± 0.90
Phenol	2.00 ± 0.11	23.4 ± 1.2	–	19.4 ± 1.2	–

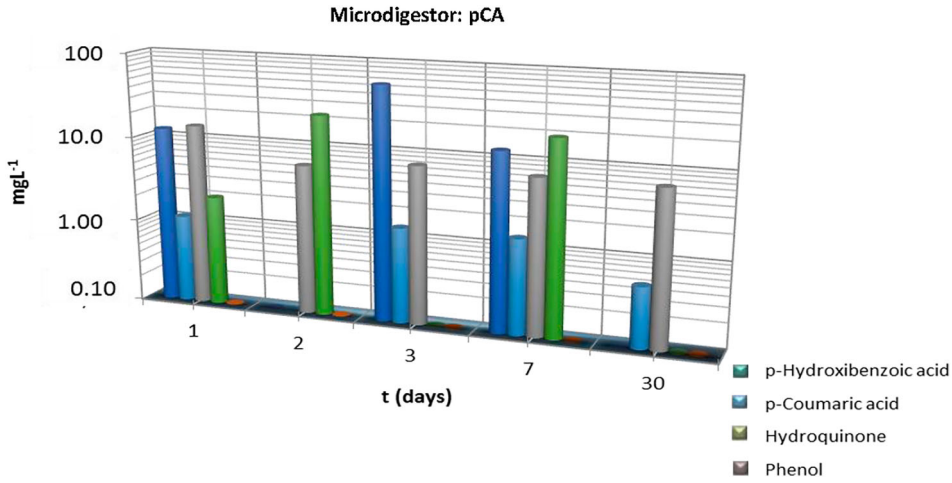

Figure 5. Results of the pCA microdigester HPLC analysis.

Table 8. Results of the HPLC analysis of the equal mixture of standards microdigester.

	Microdigester 7: equal mixture of standards				
	Time (days)				
Compound (mg L^{-1})	1	2	3	7	30
p-hydroxybenzoic acid	11.70 ± 0.80	3.41 ± 0.23	33.3 ± 2.3	–	–
pCA	0.940 ± 0.050	–	–	1.520 ± 0.070	0.424 ± 0.020
Hydroquinone	19.1 ± 2.5	4.30 ± 0.60	4.70 ± 0.60	11.8 ± 1.6	6.10 ± 0.80
Phenol	4.90 ± 0.30	61.0 ± 3.0	–	53.0 ± 3.0	–

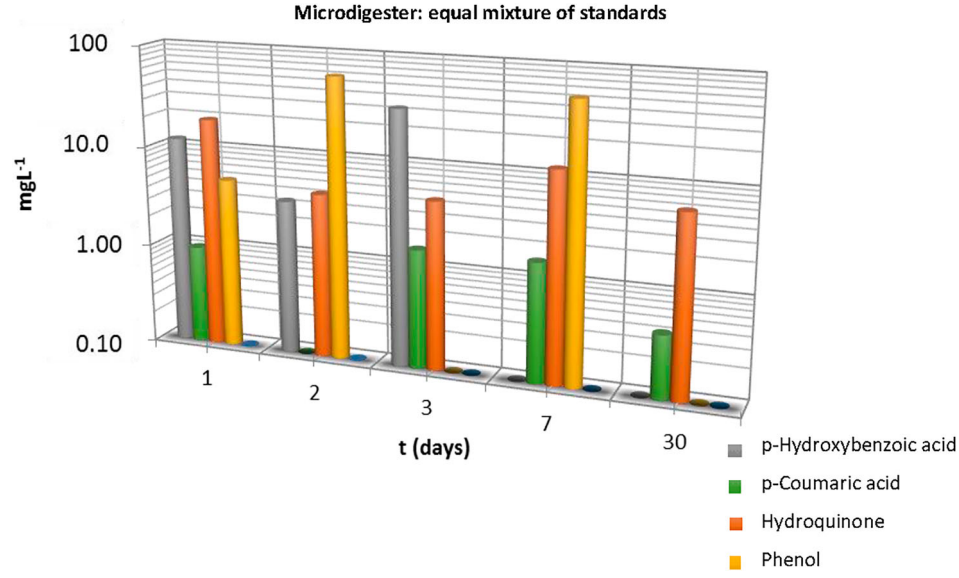

Figure 6. Results of the equal mixture of standards microdigester HPLC analysis.

In greater detail, our study covered a total time span of 30 days, matching specific concentrations of each OMW-related chemical to specific periods of time. pCA will be detected for the whole 30-day period although its concentration will decay steadily over time. Hydroxybenzoic acid being the major chemical present in the water indicates that the degradation process has already begun but the spill did not occur more than 15 days ago. If phenol is found to be the main pCA derivative diluted in the water, the spill took place between 15 and 20 days ago. Finally, when hydroquinone is the major pCA derivative to be found, the spill can be assessed to have an approximate age of 30 days.

Table 9 . Results of the sets of analysis at the Alcarayón Stream.[a]

Samples	Parameters				
	pH	Temperature (°C)	EC (mS cm^{-1})	OD (mg L^{-1})	COD (mg L^{-1})
	September–October				
US (Upstream)	7.80	28.1	1.409	10.05	8.2
S1	7.30	33.9	7.88	0.04	12.1
DS (DownStream)	7.64	29.9	1.869	3.85	11.9
	November–December				
US (Upstream)	7.86	20.9	3.49	6.25	7.9
S1	9.14	22.3	7.14	0.05	12.5
DS (DownStream)	7.90	22.1	2.80	0.15	11.4
	January–February				
US (Upstream)	7.00	16.5	1.25	4.65	8.7
S1	8.35	18.3	3.98	0.11	11.7
DS (DownStream)	7.79	16.3	1.20	1.20	11.5

[a]The values presented are an average of those obtained throughout the 6-month sets of analysis.

The procedure presents a wide variety of applications.

- Determination of the geographic point where the spillage took place, unmistakably pointing to the spilling facility. This is a useful tool in situations where more than one factory is placed near a watercourse and the source of OMW is unclear.
- Qualitative and quantitative assessment of the potential damage to the different sections of the watercourse according to their distance to where the spillage point.
- Establishing a concrete and certain date to a spillage – a basic data when it comes to legal action against spilling facilities.
- This detailed study of the several pCA degradation routines allows a reliable prediction of the chemical agents that will appear over time, buying a critical time to take action against their effects in the water.

Assessing the ecological state of a river, where oxidation potential plays a major role, enables one to expect a certain degradation routine for OMW agents.

A separate analysis of the different depths of a watercourse is as important as that of its geographic sections. At a certain location, a watercourse may have different oxidation states at different depths. That is to say, pCA will follow divergent degradation routines and different polyphenols will arise in shallow and deep waters, as analysis will clearly indicate. Observing and contrasting parallel, often aerobic vs. anaerobic degradation processes will confer a much greater accuracy to dating and locating the spill.

In short, this spillage control protocol delivers a thorough insight of the spillage footprint in a river, providing much more consistent evidence than that of organic matter variations, allowing legal ecologic sanctions to be imposed.

In the real-life test carried out in the Alcarayón stream, the RP-HPLC-UV protocol allowed pCA, *p*-hydroxybenzoic acid, hydroquinone and phenol to be identified and quantified. Furthermore, it became apparent that the Alcarayón stream contained these OMW-originated chemical agents only after crossing the olive mill industrial zones — agents that are only originated as byproducts of the olive industry. For this reason, we can positively claim that the RP-HPLC-UV protocol was proved successful in the process of identifying an OMW spill in a river and assessing its severity.

5. Conclusions

We have developed a tool that enables the determination of pCA and its degradation intermediates through the RP-HPLC-UV analysis, and verified its suitability for the control of spills from the olive industry.

The sample treatment this analysis protocol requires is minimal, making the global process easily reproduced. The composition of the mobile phase is simple yet it ensures high resolution and a good separation of the compounds. These features, together with the short overall running time of the process (between 7 and 10 min) prove it optimal for analysis.

The calibrated linear intervals ranged from 10 to 500 mg L^{-1} obtaining good linear adjustments. Detection limits L_D ($S/N = 3$) and quantification limits L_Q ($S/N = 4$) were calculated from their calibration lines in the range of 0–500 mg L^{-1}.

pCA was chosen as a representative molecule of OMW discharges. Degradation compounds of pCA by chemical oxidation and anaerobic biodegradation have been identified and quantified. Both scenarios (oxidant and anaerobic) were studied in order to emulate the behavior of an actual polluted watercourse, which has oxidant surface conditions and clearly anaerobic conditions in the deep zones. Organic charge, as well as the evolution of the different derivatives by degradation, has been monitored, enabling not only the detection of OMW spills but also their dating by observing what compounds are present in the water.

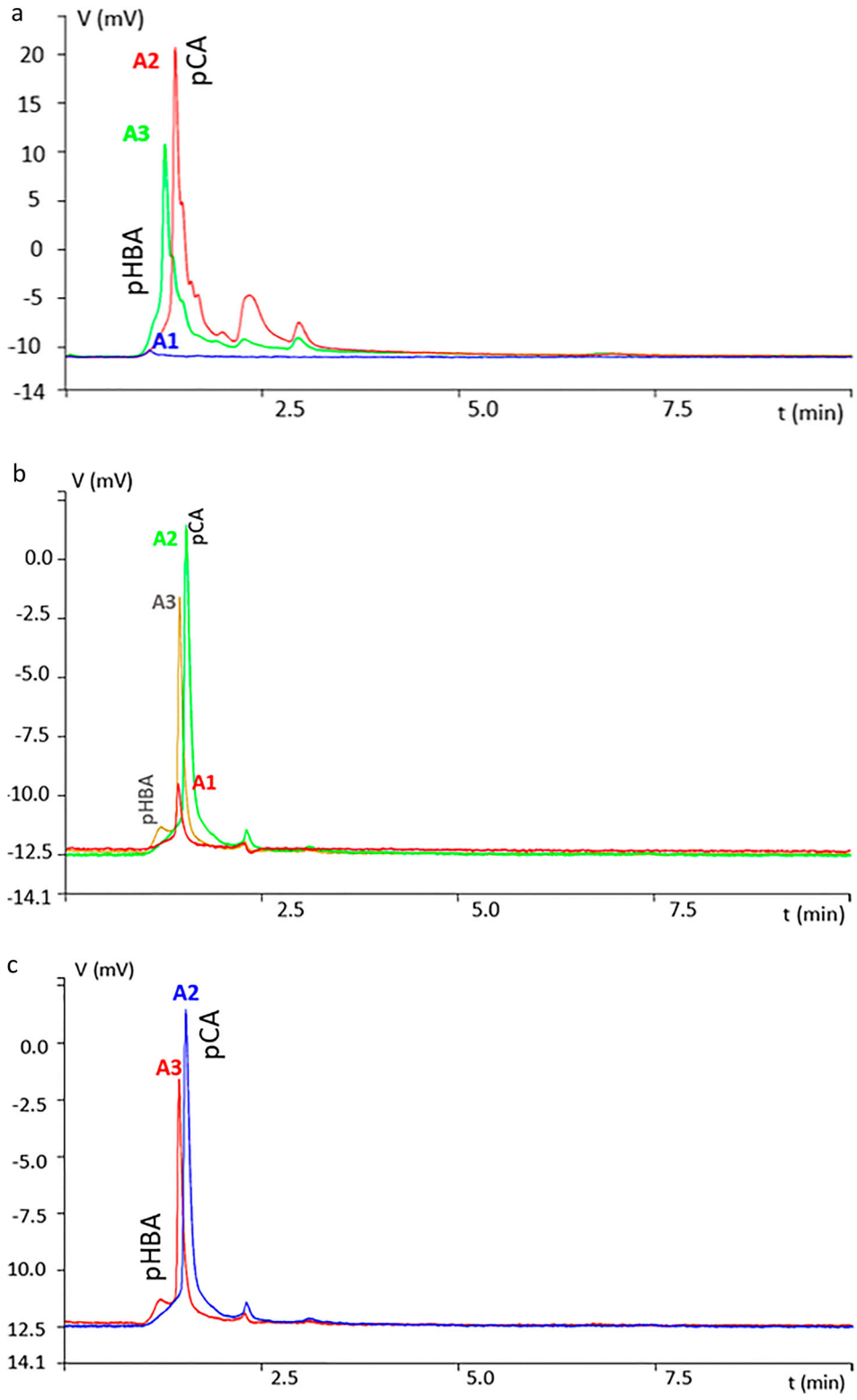

Figure 7. Chromatograms of water samples of the Alcarayón stream: point S1, nearest to the olive industry (a. September–October, b. November–December, c. January–February).

Regarding oxidant conditions, corresponding to the shallow zones of a watercourse, a relation between the increase of $KMnO_4$ and the decrease of TOC was observed (phenol proved the most degradation resistant compound). The RP-HPLC-UV analysis confirmed that pCA oxidation occurs in two different paths. One consists of oxygen attacking its double exocyclic ring and originating *p*-hydroxybenzaldehyde, whose oxidation by

decarboxylation gives rise to hydroquinone and phenol. The other, which occurs simultaneously, consists of the decarboxylation of pCA, which forms *p*-hydroxybenzoic acid and phenol.

As far as anaerobic biodegradation is concerned (emulating the conditions of the deep zones of a river), a COD decrease with good r^2 was observed over time for pCA, *p*-hydroxybenzoic acid and phenol. Hydroquinone and hydroxybenzaldehyde did not register good adjustments.

The RP-HPLC-UV analysis revealed how during the first 7 days pCA concentration diminishes while significant amounts of hydroquinone and *p*-hydroxybenzoic acid appear. Also, phenol reaches relevant concentrations after a week. Given a month, hydroquinone was the only compound to be detected in significant amounts.

This tool presents much smaller implementation costs and quicker response times than other mass HPLC methods, yet with the same reliability and with a specificity superior to that of the colorimetric methods. The great simplicity of the equipment makes it ideal as an *in situ* field tool. A viable, reliable tool for OMW spillage control is, therefore, introduced.

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