**Quantitative analysis of aqueous phases of bio-oils resulting from
pyrolysis of different biomasses by two-dimensional comprehensive
liquid chromatography.**

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ABSTRACT

Agrifood by-products are perfect candidates to be further processed under the concept of circular economy, in order to produce their valorization. Although significant amounts of food-related wastes that are discarded are produced worldwide, these might still be rich in valuable compounds. A strategy to further valorize agrifood-related by-products is based on pyrolysis processes. The result of this process is a liquid product termed bio-oil which is composed of an organic phase and an aqueous phase. This bio-oil is rich on a variety of components and its analysis implies several challenges. In this work, quantitative on-line comprehensive two-dimensional liquid chromatography (LC×LC) is proposed for the first time to characterize several aqueous phases of different bio-oils. Rice husk, peanut shell, spent coffee grounds, peach core and Eucalyptus sawdust biomasses were analyzed. The developed quantitative LC×LC method presented very good linearity, precision, reproducibility, recovery and LODs and LOQs as low as 0.05 $\mu\text{g mL}^{-1}$ and 0.16 $\mu\text{g mL}^{-1}$, respectively. As much as 28 components were simultaneously separated and quantified in those samples. Our results found that the composition of these bio-oils was different but strongly related to the agrifood by-product submitted to pyrolysis. The developed methodology is foreseen as a valuable tool for the quantitative study of other bio-oils, considering the great complexity and high dimensionality of these samples.

Keywords: Bio-oil; by-products; pyrolysis; quantification; LC×LC

1. Introduction

Due to the increasing awareness on environmental protection, different strategies are constantly being developed in the frame of circular economy able to be implemented in biorefinery approaches. Agrifood by-products are perfect candidates to be further processed following strategies directed to produce their valorization. Significant amounts of wastes related to foods and crops production and processing are generated worldwide. However, these by-products are often still rich in valuable compounds that could be re-utilized. The selective extraction of high value compounds is a feasible alternative, even more interesting when advanced green extraction techniques are employed [1]. However, other processes can be designed in order to avoid the generation of wastes and their contribution to environmental pollution. Pyrolysis is an example of this latter kind of processes.

Pyrolysis is based on the application of high temperatures, typically around 500 °C in the absence of oxygen to produce the thermal decomposition of a particular biomass. In principle, any biomass composed of cellulose, hemicellulose and lignin is a good raw material for pyrolysis. Thus, agrifood wastes coming from vegetables may be used in this regard. As a result of pyrolysis, a liquid product termed bio-oil is produced. Bio-oils have been pointed out as starting materials for the production of biofuels as well as for high-added value chemicals. Deeper insight on the production of bio-oils by pyrolysis may be gained through different interesting review papers published elsewhere [2]. In general, the chemical composition of bio-oils is rather complex and it is composed by an organic phase (low-polarity components) and by an aqueous phase (high polarity compounds). Components belonging to different chemical families, including hydrocarbons, esters, ethers, ketones, acids, aldehydes, phenols or sugars, among others may be present.

As the use and application of a bio-oil will strongly depend on its particular chemical composition, which in turn depends on the composition of the initial biomass, the chemical characterization of bio-oils is an essential step. Gas chromatography coupled to mass spectrometry (GC-MS) and, most notably, comprehensive two-dimensional gas chromatography (GC×GC), have been widely used as analytical tools to characterize the organic phase of different bio-oils [3-8]. However, the analysis of the aqueous phase using GC-based techniques is more challenging since a previous extraction step (usually performed with dichloromethane), is necessary due to the unsuitability of water samples in GC. This step, can greatly affect the qualitative and quantitative composition of the samples, due to the different solvent affinity for each chemical class present. In this context, liquid chromatography-based techniques allow the direct analysis of the aqueous phase without any further extraction step [9]. Thus, it is expected that LC is complementary to GC for the whole comprehensive chemical characterization of bio-oils, since compounds with low volatility, high polarities and poor thermal stabilities are found in this sample [10]. In this regard, comprehensive two-dimensional liquid chromatography (LC×LC) could be a useful tool in order to characterize the typical complex profiles of aqueous phase from bio-oil.

Online LC×LC represents the most advanced two-dimensional liquid chromatography (2DLC) mode. It allows the combination of different separation mechanisms in each dimension through which the sample is analyzed. Typically, the sample is injected into the first dimension (¹D) where an initial separation is produced. Simultaneously, discrete fractions from the ¹D effluent are continuously collected and transferred to the second dimension (²D) with the aid of a modulator or interface. The most-commonly used interface is based on the use of, at least, a switching valve equipped with two sampling loops with identical inner volume that change function upon valve actuation. Using this

set-up, a fraction is collected in one of the sampling loops whereas the previous fraction is being injected using the other loop. This continuous procedure implies that slow separations are needed in the ¹D in order to produce effluent fractions at a relatively slow rate to allow enough time for each subsequent fast ²D separation.

By using on-line LC×LC, the separation potential and peak capacity are substantially improved compared to conventional one-dimensional separations. For this reason, comprehensive LC has already been demonstrated to be useful for the characterization of very complex food and agrifood-related samples [11,12], although the separation of bio-oils has only scarcely been explored, being limited to tree-related bio-oils [10]. Most of the developed approaches based on the use of on-line LC×LC in this field are focused on qualitative analyses. In fact, there are just few papers dealing with the quantification of, at least, part of the separated compounds [13-18]. Still, it is widely accepted that further developments are necessary to demonstrate the quantitative capabilities of LC×LC [11], even more if routine analysis is targeted.

Considering the analytical challenges related to the study of the aqueous phase of bio-oils derived from pyrolysis, the aim of the present work was to optimize a new quantitative LC×LC method for the separation and identification of compounds present in different bio-oils produced from important agrifood by-products, including rice husk, spent coffee grounds, peanut shell, peach core and Eucalyptus sawdust.

2. Materials and Methods

2.1. Reagents and Standards

All the solvents employed (acetonitrile, ethanol, methanol) were of HPLC-grade and acquired from VWR Chemicals (Barcelona, Spain). Ultrapure water was obtained from a

Milli-Q apparatus (Millipore system, Billerica, MA, USA) and used for the preparation of all solutions and mobile phases.

Reference standards used were at least 98% of purity and were purchased from Sigma–Aldrich (Saint. Louis, MO, USA). A stock solution (1000 mg L⁻¹) was prepared in water containing 21 reference standards: 3 ketones (3-methyl-1,2-cyclopentanedione, 4-hydroxyacetophenone, 4-hydroxy-3-methoxyacetophenone), 2 alcohols (homovanillyl alcohol, 3-p-hydroxyphenylpropanol), 9 phenols (1,2-benzenediol, phenol, 4-methyl-1,2-benzenediol, 2-methoxyphenol (guaiacol), 4-methylphenol, 2,6-dimethoxyphenol, 4-ethylphenol, 3,4-dimethylphenol, 2-acetyl-resorcinol), 6 aldehydes (5-hydroxymethyl furfural, furfural, 4-hydroxybenzaldehyde, 5-methylfurfural, vanillin, syringaldehyde) and an alkaloid (caffeine). The stock solutions were stored at 5 °C.

2.2. Biomass samples

Five biomass samples, which are agro-industrial wastes generated in large amounts per year in Brazil were investigated in this study. Rice husk was provided by Adib Peixoto Ltda (Pelotas, Brazil), peach core was supplied by Scharann Food Industry (Pelotas, Brazil), Eucalyptus sawdust was acquired from CMPC Cellulose Industry (Guaíba, Brazil), whereas peanut shell and spent coffee grounds were both obtained from a local market in Porto Alegre, Brazil.

All samples were milled to particle sizes between 40-60 mesh and dried in an oven at 105 °C, until constant weight, before bio-oil production.

2.3. Bio-oils production

Intermediate pyrolysis of biomass samples was performed in a homemade vertical furnace containing a tubular fixed-bed reactor of quartz glass. A schematic diagram of

the pyrolysis system is illustrated in Figure S1 and described elsewhere in detail [3]. The pyrolysis conditions were based on previous studies [4,19] and were as follows: 6.5 g of biomass (particle size 40-60 mesh) were used as starting material. N₂ flow of 100 mL min⁻¹, and a heating rate of 100 °C min⁻¹ up to a pyrolysis final temperature of 650 °C were established. The system was then maintained for 10 min at the final temperature till no more vapors were formed. The condensable products were cooled through a condenser using ethylene glycol and water (1:1) at -10 °C. The crude bio-oil produced (liquid product) from the pyrolysis of the different biomasses consisted of two immiscible phases: aqueous and organic phases. The phase separation was produced by decantation and the aqueous phase was collected without any further extraction or pretreatment. Aqueous phase samples were diluted in water, using a factor dilution of 1:30 v/v and 1:40 v/v according to the compounds concentration in the samples, and filtered through a 0.20 µm Chromafil PTFE membrane filter before qualitative and quantitative analysis by LC×LC/DAD-MS.

2.4. LC×LC instrumentation

The first dimension (¹D) was operated using an Agilent 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a diode array detector (DAD) which was connected at the exit of the second dimension. The second dimension (²D) was carried out using an additional LC pump (Agilent 1290 Infinity). An electronically-controlled 2-position 10-port switching valve (Rheodyne, Rohnert Park, CA, USA) was used to connect both dimensions. The interface between dimensions was based on two sampling loops of identical inner volume (30 µL). Separations were recorded at 280 nm although, in addition, UV-Vis spectra were collected from 200 to 400 nm at a sampling rate of 20 Hz in the DAD. The wavelength

(280 nm) chosen is suitable for the chemical families of compounds under investigation and has previously been used for analysis of aqueous phase samples by LC [5,9].

An Agilent 6320 ion trap mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used for the identification of the separated compounds interfaced by an ESI ion source. The flow eluting from second dimension was splitted via a T-piece in order to allow the introduction of a flow of ca. 600 $\mu\text{L min}^{-1}$ in the MS. The instrument was operated under negative and positive ESI modes using the following conditions: mass spectra recorded from m/z 50 to 200, according to the molecular weight of the compounds expected to be present in the bio-oils aqueous phase samples; capillary voltage: -3500 V; corona discharge: +4000 nA (positive)/-10000 nA (negative); nebulizer pressure, 60 psi; dry gas, 5 L min^{-1} , and; dry temperature, 350 $^{\circ}\text{C}$; vaporizer temperature, 400 $^{\circ}\text{C}$. The ion trap mass analyzer was controlled by the 6300 series trap control software (Bruker Daltonik GmbH, V. 6.2).

2.5. LC $\times$ LC separation conditions

After optimization of each dimension, the final selected separation method was based on the coupling of two RP separations, using the following conditions:

¹D separation: a X-Bridge amide (150 $\times$ 2.1 mm, 3.5 μm d.p., 141 $\text{\AA}$, Waters, Milford, MA, USA) column was employed in the ¹D, eluted using acidified water (0.1% formic acid, v/v, A) and acetonitrile (0.1% formic acid, v/v, B). Under optimum conditions, the linear gradient employed was: 0 min, 2% B; 3 min, 10% B; 50 min, 60% B; 61 min, 100% B. The flow rate was set at 25 $\mu\text{L min}^{-1}$, the column temperature at 30 $^{\circ}\text{C}$ and the injection volume was 10 μL .

²D separation: a short partially-porous column (Poroshell EC-C18 30 $\times$ 4.6 mm, 2.7 μm , Agilent, Santa Clara, CA, USA) was selected. Gradient, flow rate, temperature, type of

acid and solvents used were optimized separately. The optimum conditions were achieved using acidified water (0.1% formic acid, solvent A) and acetonitrile (0.1 % formic acid, solvent B) as mobile phases. The optimum linear gradient was as follow: 0 min, 10% B; 0.85 min, 90% B; 0.86 min, 100% B; 0.9 min, 100% B; 0.91 min, 10% B and re-equilibrium at starting conditions until 1 min. The flow rate was set at 2.5 mL min⁻¹. Once these parameters were optimized, the coupling between both dimensions was achieved employing 1 min repetitive second dimension separations; thus, the modulation time was 1 min. Other columns tested in both dimensions are described in Table S1.

2.6. Validation of the quantitative method

For the first time, an accurate quantitative analysis method using LC×LC has been developed and implemented on the analysis of the aqueous phase from bio-oils. Evaluation of method's performance was accomplished through the assessment of the main figures of merit, namely: linearity, dynamic range, determination coefficients (R^2), limit of detection (LOD), limit of quantification (LOQ), precision (intra and inter-day precision) and recovery. The validation protocol followed the guidelines established by ANVISA [20].

Calibration curves were constructed by the triplicate injection of eight different concentration levels of a standards mixture. Linearity was estimated in the concentration range between 1-250 mg L⁻¹ according the values of determination coefficients (R^2). LODs and LOQs were obtained based on the calibration curve parameters, being the standard deviation of the response at the lowest level of the intercept of the corresponding calibration curve divided by the average slope multiplied by a factor of 3 and 10, respectively. Precision was evaluated by intra and inter-day precision. Intra-day precision was expressed as the relative standard deviation (% RSD) of peak volumes obtained for

a 50 mg L⁻¹ standard solution (intermediate level) injected three times on the same day, while inter-day precision was calculated by repeating those injections on three consecutive days (n=9). System accuracy was evaluated by a spike recovery method, in which the aqueous phase from rice husk bio-oil was diluted by a factor 1:100 and quantified. Later on, this sample was spiked with a standard solution containing all the studied components at a final concentration of 100 mg L⁻¹ and analyzed in triplicate. Recoveries (%) for each compound were calculated using these quantitative data.

2.7. Other calculations

2.7.1. Peak capacity

Individual peak capacity for each dimension was calculated according to Eq. 1:

$$n_c = 1 + \frac{t_G}{\bar{w}} \quad (1)$$

where t_G is the gradient time and $\bar{w}$ is the average peak width (4σ). For ¹D individual ¹D and ²D peak capacity calculations, the average peak width was obtained from all the quantified peaks.

For each two-dimensional set-up, different peak capacity values were estimated, starting from the, theoretical peak capacity (^{2D} n_c). This value was calculated following the so-called product rule, using Eq. 2:

$$^{2D}n_c = {}^1n_c \times {}^2n_c \quad (2)$$

As eq. 2 does not take into consideration the deleterious effects due to the modulation process as well as possible ¹D undersampling, a more realistic peak capacity value was obtained from the equation proposed by Li et al. [21], denominated effective peak capacity (Eq. 3):

$$^{2D}n_{c, effective} = \frac{{}^1n_c \times {}^2n_c}{\sqrt{1 + 3.35 \times \left(\frac{{}^2t_c {}^1n_c}{{}^1t_G} \right)^2}} \quad (3)$$

being 2t_c , the 2D separation cycle time, which is equal to the modulation time. This latter equation includes the $\langle\beta\rangle$ parameter accounting for undersampling. Moreover, to more precisely compare among set-ups and in order to evaluate possible peak clusters along the 2D analysis and, thus, to estimate 2D space coverage, the orthogonality degree (A_0) was considered to offer the denominated 2D corrected peak capacity, as follows:

$${}^2Dn_{c,corrected} = {}^2Dn_{c,effective} \times A_0 \quad (4)$$

2.7.2. Orthogonality

System orthogonality (A_0) for each sample was calculated according to the method proposed by Camenzuli and Schoenmakers [22], taking into account the spread of each peak along the four imaginary lines that cross the 2D space forming an asterisk, that is Z_1 , Z_2 (vertical and horizontal lines) and Z_- , Z_+ (diagonal lines of the asterisk). Z parameters describe the use of the separation space with respect to the corresponding Z line, allowing to semi-quantitatively diagnose areas of the separation space where sample components are clustered, thus, reducing in practice orthogonality. For the determination of each Z parameter, the S_{Zx} value was calculated, as the measure of spreading around the Z_x line, using the retention times of all the separated peaks in each 2D analysis.

3. Results and discussion

3.1. LC×LC method optimization

Considering the aim of the present research directed towards the quantification of interesting compounds in different aqueous phases of bio-oils from several biomasses, the optimization of a LC×LC methodology able to provide with good separation capabilities is the first critical step.

The first approach consisted on the screening of several stationary phases under both HILIC and RP conditions to test the potential for the ¹D separation. Aqueous phase of rice husk bio-oil was selected as a model matrix to perform these experiments. Among the stationary phases studied, cyano, PEG, amino, ZIC-HILIC, diol and amide particles were included in combination with typical mobile phases. Table S1 summarizes the physical characteristics of the compared columns. In any case, HILIC conditions did not produce any appropriate distribution of the sample components, whereas RP conditions were demonstrated to be more suitable for some columns. Combining the information from this screening and keeping in mind the possible differential selectivity with the ²D, PEG and amide columns were selected as the most promising for the ¹D. Figure S2 shows RP separations attainable using these columns. As it can be observed, comparable results were obtained, although the amide column provided slightly more evenly separated peaks and, thus, it was selected for further optimization.

Once the column for the ¹D was selected, the separation conditions were re-optimized, adjusting a slower flow rate. In view of the column dimensions, 25 $\mu\text{L min}^{-1}$ was considered the target flow rate in order to produce discrete fractions for the ²D separations. Keeping this flow rate constant, the gradient was adapted to provide wide peaks that could be appropriately sampled to the ²D. Figure S3 shows the separations obtained for the 5 bio-oils studied in the present work under the ¹D optimum conditions selected. These conditions involved the use of an amide column in combination with water (0.1% formic acid) and acetonitrile (0.1% formic acid) as mobile phases eluting under RP conditions.

For the ²D optimization, rice husk bio-oil was directly injected in the candidate columns. In this case, a RP separation mode was straightforwardly selected, because RP has been repeatedly shown as the most appropriate separation mechanism to provide fast (<2 min)

and efficient separations with short column re-equilibration times [3]. These characteristics are strongly appreciated for ²D separations in LC×LC. In order to seek for a different selectivity compared to the ¹D, C18 (30 and 50 mm × 4.6 mm, 2.7 μm) and PFP (50 × 4.6 mm, 2.7 μm) short partially porous columns were tested in the ²D, establishing a target total time cycle of 1 min. This time would be the allotted time for each modulation and, thus, it should include the gradient and separation as well as the column re-equilibration. The optimization was performed trying to maintain a flow rate as low as possible to minimize sample dilution. In this sense, 2.5 μL min⁻¹ was the lowest possible flow rate that allowed proper separation and column reconditioning in 1 min. Figure S4 shows a comparison among the three tested columns under identical separation conditions (optimized gradient and flow rate). As it can be observed, albeit good separation of the sample components was possible using both stationary phases, C18 columns provided better peak shapes. Moreover, using the shorter alternative (Figure S4C), faster separations and more resolved peaks were attained. Thus, the 30 mm long column was selected for further LC×LC analyses. Subsequently, all the studied samples were analyzed using the previously optimized conditions without previous ¹D separation, confirming the potential of those experimental conditions to be used in the ²D (see Figure S5).

After individual optimization, the selected conditions were implemented in the new RPLC×RPLC method, setting 1 min as the modulation time. Two-dimensional plots (280 nm) of the five bio-oils analyzed (aqueous phase) using this method are shown in Figure 1. All the samples were characterized by the presence of a good number of components, which were distributed in the available separation space. Table 1 summarizes some of the figures of merit of the qualitative method for each sample studied.

As it can be observed, orthogonality degree ranged from 33% corresponding to spent coffee grounds to 50% for peanut shell and Eucalyptus sawdust. These orthogonality calculations are based on the use of the 2D plane as it is occupied by sample components [22]. Consequently, these values may be judged as good in spite of the use of the same separation mechanism in the two dimensions. This trend also corresponded to the corrected peak capacity reached, which considers the orthogonality to adapt $^{2D}n_c$ to provide more realistic figures. In any case, the obtained values were high, reaching peak capacities higher than 500 for Eucalyptus sawdust bio-oil, demonstrating the good separation capabilities of the developed method.

3.2. Development and validation of the quantitative method

Using the optimized LC×LC method, a wide group of 21 standard compounds that had been identified in other bio-oils were injected and analyzed. Ketones, alcohols, phenols, aldehydes and an alkaloid were included. Their chemical structures are found in Figure S6. As shown in Figure 1F, the separation obtained for this group of components was very good. Calibration curves for each compound were obtained by the triplicate injection and LC×LC analysis of eight different concentration levels after measuring their corresponding peak volume (Table 2). Determination coefficients (R^2) higher than 0.98 were obtained for all the tested compounds. Moreover, LODs obtained ranged from 0.05 $\mu\text{g mL}^{-1}$ for caffeine and 2.08 $\mu\text{g mL}^{-1}$ for phenol, whereas, LOQs values were lower than 6.29 $\mu\text{g mL}^{-1}$.

Besides, instrumental intraday and interday precision was assessed. For this purpose, a mixture (50 $\mu\text{g mL}^{-1}$ each) of the 21 quantified components was consecutively injected three times in the same day ($n = 3$) and also in three consecutive days ($n = 9$). RSD values obtained were in the range between 0.30 and 9.14 % for peak volumes in the same day.

Interday RSD values ranged between 0.30 and 14.56 % for peak volumes although were below 10% for 18 out of 21 quantified compounds. Accuracy was also estimated through analyte recovery (Table 3). Most of the standard compounds showed excellent accuracy (recovery range 90%-115%), except furfural and 2,6-dimethoxyphenol which show recovery of 85.2 % and 88.5 %, respectively. Since accuracy assays were performed using real samples, possible overlooked matrix effect-related issues were avoided. Regarding to the RSD% for accuracy data, all compounds analyzed showed values lower than 15%. Taken together, these values demonstrate the good capabilities of the quantitative LC×LC method developed, even more considering the analytical challenges commonly associated to this methodology such as: low response and low sensitivity, low reproducibility in the ¹D due to different cuts and, difficulties in the accurate determination of peak volumes [13,16,23].

Table 2 summarizes the information collected related to the calibration of the developed method, whereas Table 3 shows its validation figures of merit.

3.3. Analysis of bio-oils

The already validated LC×LC quantitative method was applied to the characterization of aqueous phase samples from 5 different bio-oils produced by pyrolysis from diverse agrifood by-products, namely, rice husk, peanut shell, spent coffee grounds, peach core and Eucalyptus sawdust. The potential of the utilization of these biomasses to obtain bio-oils, has been already studied in the literature. Many reports have shown the characterization of the organic phase of similar bio-oils, especially using GC×GC [3,5,8,24], identifying high-value compounds in the samples. In this line, LC×LC can be a good choice that can be considered complementary to GC×GC to obtain a

comprehensive characterization of these bio-oils, focused on the aqueous phases. However, the use of on-line LC×LC to this aim has been only scarcely explored [10]. Each sample was analyzed by LC×LC in triplicate and examined thanks to the use of DAD and MS detectors connected in series. This way, some tentative identifications for the separated compounds could be obtained, in addition to those for which commercial standards were available. Table 4 summarizes the information collected from the quantification of all the assigned peaks.

Twenty-eight compounds, including the major compounds, were identified and quantified in the aqueous phase samples. Twenty-one were positively identified by authentic standard injections, whereas seven were tentatively identified based on their UV and mass spectra. For the quantitative analysis, the concentration of analytes identified in the samples for which authentic standards were not available was estimated using the closest commercial standard from a chemical structure point of view. It was important to achieve good quantification of the most important compounds in the sample in order to define the potential use of the bio-oil. In a previous study [5], the quantification of aqueous phase from rice husk bio-oil using HPLC-DAD was reported; however, in that application, by means of conventional one-dimensional LC just 10 compounds could be identified and quantified, due to the great sample complexity. As demonstrated in the present contribution, LC×LC is able to offer enhanced analytical performance for the analysis aqueous phase samples from bio-oils, in terms of resolving power, sensitivity and selectivity.

LC×LC analyses evidenced the presence of polar compounds in bio-oils aqueous phases in high concentration. As a matter of fact, if GC techniques were used for the analysis of these samples, as commonly occurs, the most polar compounds would have not been totally removed from water in the extraction step. Consequently, their actual

concentration in the bio-oil would have been underestimated. Moreover, other compounds, such as 5-(hydroxymethyl)furfural, which cannot be detected by GC×GC without a derivatization step, as previously reported [6], were properly separated and quantified by LC×LC.

As can be observed in Table 4, 1,2-benzenediol is the main compound in all samples except for peanut shell where is the second highest, with concentration values ranging from 2.07 to 7.86 mg mL⁻¹, followed in absolute values by furfural (4.44 mg mL⁻¹ in peach core), 4-methyl benzenediol (3.61 mg mL⁻¹ in peach core), guaiacol (2.99 mg mL⁻¹ in peanut shell), 2-hydroxy-3-methyl-2-cyclopenten-1-one (1.98 mg mL⁻¹ in *Eucalyptus* sawdust) and caffeine (1.58 mg mL⁻¹ in spent coffee grounds).

Regarding chemical classes, Figure 2 shows the contribution of each group in terms of summed concentration values (mg mL⁻¹) in the 5 bio-oils (aqueous phase) analyzed. Phenols were majority in the samples, corresponding to the 65% of the quantified compounds in peach core and *Eucalyptus* sawdust. Among phenols, benzendiols were the most abundant class. Aldehydes, mainly furfurals, are also present as major components in the bio-oils from peach core (6.31 mg mL⁻¹), rice husk (4.66 mg mL⁻¹) and *Eucalyptus* sawdust (4.88 mg mL⁻¹). The high amount of benzenediols and furfurals found in the samples is in accordance with studies previously reported for other biomasses (coconut fibers, sugar cane straw and bagasse) representing a high proportion in terms of total peak volumes, although not quantitative information was included [9].

In the case of ketones, all identified components were cyclic ketones; the richest bio-oil on these components was *Eucalyptus* sawdust (3.75 mg mL⁻¹). Nitrogen compounds only appear in spent coffee grounds; this sample is characterized by high amount of caffeine representing 16% of the total quantified compounds. On the other hand, alcohols showed

overall concentrations between 0.11 and 1.67 mg mL⁻¹, being peanut shell bio-oil the sample with the lowest concentration while *Eucalyptus* sawdust bio-oil was the richest. The differences in the composition of bio-oil aqueous phases is totally related to the distinct chemical compositions of the biomasses processed by pyrolysis. Cellulose, hemicellulose and lignin, together with other components, appear in different proportions in the biomasses; this proportion influences the final composition of the obtained bio-oil by pyrolysis [25]. Thus, the predominant phenolic composition of peach core and *Eucalyptus* sawdust bio-oils is due to the high content of lignin in these biomasses. Lignin is composed of three phenylpropane units, which by depolymerization leads to a variety of phenols, such as benzenediols, methoxyphenols and alkylphenols [26]. Benzenediols, due their higher polarity, remain mainly in the aqueous phase while metoxyphenols and alkylphenols are commonly found in higher concentration in organic phase of the bio-oil. In contrast, the amount of aldehydes in aqueous phase samples is due to the cellulose content in the starting biomass. Aldehydes are derived from cellulose depolymerization, leading to a high amount of anhydro-saccharides and anhydro-oligosaccharides which formed aldehydes such as 5-(hydroxymethyl)furfural, 5-methylfurfural and furfural [26] by different reactions. These three compounds were identified in all aqueous phases analyzed. Regarding ketones, cyclopentenones and other cyclic ketones are derived mainly from hemicellulose and cellulose degradation [7]. Finally, the significant amount of caffeine in the aqueous phase from spent coffee grounds is due to its presence in high amounts in the raw biomass. Caffeine possibly does not suffer thermodegradation to a great extent during pyrolysis, which makes possible its determination in spent coffee grounds bio-oil [8].

Many of the separated and quantified compounds in the studied bio-oil aqueous phase samples, might be a viable alternative feedstocks within the chemical industry. As an

example, benzenediols have many important applications as antiviral and antioxidants with application in skin whitening products. Other phenols (alkyl and methoxy compounds) could be used as substitutes for phenols in phenolic resins [27,28]. On the other hand, furfurals have potential as liquid biofuels and in biopolymers production, whereas ketones can be applied in chemical synthesis [27].

4. Conclusions

Since pyrolysis has been confirmed as a viable alternative to valorize important industrial wastes, appropriate analytical methods to provide accurate elucidation of their products composition, are needed. This work reports the development of a new RPLC×RPLC-based methodology to quali- and quantitatively assess the composition of aqueous phases of five different bio-oils produced by pyrolysis from interesting agrifood by-products, namely, rice husk, peanut shell, spent coffee grounds, peach core and Eucalyptus sawdust. Albeit previous studies also explored the application of 2DLC the characterization of other different bio-oil aqueous phases, this is the first time where a quantitative methodology is implemented. The quantitative RPLC×RPLC developed method presented very good linearity, precision, reproducibility, recovery, and LODs and LOQs as low as $0.05 \mu\text{g mL}^{-1}$ and $0.16 \mu\text{g mL}^{-1}$, respectively. By using this procedure, 28 different components separated and detected in the aqueous phases studied were quantified. The composition of these bio-oils was different but strongly related to the composition of the agrifood by-product submitted to pyrolysis. The development of this methodology opens the door for the quantitative study of other bio-oils by LC×LC, as the complexity of these samples makes comprehensive LC an ideal alternative for their analysis.

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FIGURE LEGENDS

Figure 1. 2D contour plots (280 nm) obtained using the optimized LC×LC methodology for the analysis of the different bio-oil aqueous phases produced by pyrolysis from: rice husk (A), peanut shell (B), spent coffee grounds (C), peach core (D) and *Eucalyptus* sawdust (E). Panel F shows the separation obtained for the mixture of commercial standards analyzed ($50\text{ }\mu\text{g mL}^{-1}$ each). For peak identification see Table 4.

Figure 2. Quantitative contribution of the different compound families (phenols, ketones, aldehydes, alcohols and nitrogen compounds) in each of the analyzed aqueous phase samples by LC×LC.

Table 1. Peak capacity and orthogonality values calculated for the RPLC×RPLC analyses of the five bio-oils studied.

Parameter	Rice husk	Peanut shell	Spent coffee grounds	Peach core	<i>Eucalyptus</i> sawdust
¹ D peak capacity, ¹ <i>n_c</i>	33	37	32	37	36
² D Peak capacity, ² <i>n_c</i>	35	43	38	40	48
Theoretical peak capacity, ^{2D} <i>n_c</i>	1155	1591	1216	1480	1728
Effective peak capacity, ^{2D} <i>n'_c</i>	675	924	735	858	1013
Orthogonality, <i>A₀</i>	47%	50%	33%	44%	50%
Corrected peak capacity, ^{2D} <i>n_{c,corr}</i>	318	461	244	374	505

A₀, orthogonality; ^{2D}*n_c* = ¹*n_c* × ²*n_c*; ^{2D}*n'_c*: calculated according to [16]; ^{2D}*n_{c,corr}*: ^{2D}*n'_c* × *A₀*;

572 **Table 2.** Calibration data for the quantification of interesting compounds in the studied bio-oils using the new RPLC×RPLC method developed.

ID	Compound	t _R ¹ D (min)	t _R ² D (s)	Concentration range tested (µg/mL)	Slope (n = 8)	Intercept (n = 8)	R ²
1	5-(hydroxymethyl)furfural	24	16.40	1-250	879.84	558.4	1.0000
2	3-methyl-1,2-cyclopentanedione	28	21.50	5-150	85.885	359.99	0.9816
3	1,2-benzenediol	29	25.95	5-250	99.393	56.289	0.9985
4	furfural	30	21.90	1-250	916.53	4618.3	0.9937
5	homovanillyl alcohol	33	25.15	1-150	136.73	311.46	0.9957
6	caffeine	33	22.60	1-150	169.34	223.3	0.9976
7	phenol	33	36.85	25-250	53.425	128.14	0.9972
8	4-methyl-1,2-benzenediol	35	37.95	1-150	85.07	463.38	0.9909
9	3-p-hydroxyphenyl propanol	38	30.35	1-150	271.96	680.22	0.9989
10	4-hydroxybenzaldehyde	37	30.75	1-150	495.49	441.18	0.9936
11	5-methylfurfural	40	31.75	1-150	607.44	1147.4	0.9957
12	4-hydroxyacetophenone	43	32.85	1-150	516.58	41.262	0.9990
13	2-methoxyphenol (guaiacol)	43	40.55	1-150	71.189	89.862	0.9996
14	vanillin	43	33.00	1-150	696.8	2129.4	0.9972
15	4-methylphenol	45	48.15	1-150	64.696	71.253	0.9995
16	syringaldehyde	47	33.70	1-150	17.895	76.638	0.9855
17	2,6-dimethoxyphenol	47	39.60	5-150	130.84	481.87	0.9925
18	4-hydroxy-3-methoxyacetophenone	48	48.00	5-150	170.32	952.36	0.9948
19	2-acetyl-resorcinol	49	41.70	1-150	595.41	356.58	1.0000
20	4-ethylphenol	49	55.40	5-150	59.237	77.089	0.9997
21	3,4-dimethylphenol	49	58.20	5-150	69.468	252.35	0.9982

573

Table 3. Validation data of the quantitative RPLC×RPLC method developed for the characterization of aqueous phases from bio-oils.

ID	Compound	LOD (µg/mL)	LOQ (µg/mL)	Intraday precision, RSD (%) (n = 3)	Interday precision, RSD (%) (n = 9)	Recovery (%)
1	5-(hydroxymethyl)furfural	0.17	0.51	0.30	0.30	97.2
2	3-methyl-1,2-cyclopentanedione	1.30	3.92	7.93	8.18	103.0
3	1,2-benzenediol	1.19	3.62	1.48	9.93	99.2
4	Furfural	0.08	0.25	5.70	3.59	85.2
5	Homovanillyl alcohol	0.16	0.48	1.96	12.14	106.6
6	Caffeine	0.05	0.16	5.05	8.72	92.4
7	Phenol	2.08	6.29	1.71	5.09	93.8
8	4-methyl-1,2-benzenediol	0.10	0.30	2.73	1.72	93.7
9	3-p-hydroxyphenyl propanol	0.18	0.54	2.53	3.66	93.7
10	4-hydroxybenzaldehyde	0.05	0.15	5.38	10.27	92.7
11	5-methylfurfural	0.18	0.54	1.78	7.61	100.7
12	4-hydroxyacetophenone	0.16	0.49	9.14	3.82	90.5
13	2-methoxyphenol (guaiacol)	0.17	0.52	0.96	0.42	113.6
14	Vanillin	0.16	0.50	2.24	3.98	105.8
15	4-methylphenol	0.35	1.07	1.58	1.50	106.2
16	syringaldehyde	0.11	0.34	7.03	8.13	98.6
17	2,6-dimethoxyphenol	1.01	3.05	3.68	1.44	88.5
18	4-hydroxy-3-methoxyacetophenone	0.75	2.28	6.56	14.56	100.3
19	2-acetyl-resorcinol	0.20	0.62	0.77	0.81	104.3
20	4-ethylphenol	0.87	2.64	1.51	5.38	113.8
21	3,4-dimethylphenol	0.14	0.42	1.83	1.84	107.2

579 **Table 4.** Quantification of the identified compounds present in the aqueous phases of the studied bio-oils. Amounts provided as mg mL⁻¹ of aqueous
580 phase

ID	Compounds	Chemical family	Molecular ion detected ESI(+)/ESI(-)	Rice husk mg mL ⁻¹	Peanut Shell mg mL ⁻¹	Spent coffee grounds mg mL ⁻¹	Peach core mg mL ⁻¹	<i>Eucalyptus</i> sawdust mg mL ⁻¹
1	5-(hydroxymethyl)furfural ^a	Aldehyde	127 / 125	0.76	0.35	0.56	0.81	1.78
2	3-methyl-1,2-cyclopentanedione ^a	Ketone	114 / -	1.94	1.33	1.61	0.55	0.80
3	1,2-benzenediol ^a	Phenol	- / 109	3.55	2.68	2.07	7.86	7.41
4	Furfural ^a	Aldehyde	-	2.88	1.04	0.68	4.44	1.83
5	Homovanillyl alcohol ^a	Alcohol	151 / -	0.34	-	0.31	0.83	0.60
6	Caffeine ^a	Alkaloid	195 / -	-	-	1.58	-	-
7	Phenol ^a	Phenol	-	1.33	0.64	0.54	1.37	1.92
8	4-methyl-1,2-benzenediol ^a	Phenol	- / 123	1.09	0.99	0.41	3.61	3.22
9	3-p-hydroxyphenyl propanol ^a	Alcohol	153 / 151	0.24	0.11	0.25	0.17	0.47
10	4-hydroxybenzaldehyde ^a	Aldehyde	123 / 121	0.25	0.15	-	0.13	0.84
11	5-methylfurfural ^a	Aldehyde	111 / -	0.48	0.23	0.22	0.36	0.22
12	4-hydroxyacetophenone ^a	Ketone	137 / 135	-	0.13	-	-	0.29
13	2-methoxyphenol (guaiacol) ^a	Phenol	125 / 123	1.15	2.99	0.14	0.81	0.78
14	Vanillin ^a	Aldehyde	153 / 151	0.29	0.25	0.05	0.32	0.21
15	4-methylphenol ^a	Phenol	-	0.38	0.42	0.10	0.91	1.80
16	Syringaldehyde ^a	Aldehyde	- / 181	-	-	-	0.25	-
17	2,6-dimethoxyphenol ^a	Phenol	-	1.11	0.51	-	0.65	1.13
18	4-hydroxy-3-methoxyacetophenone ^a	Ketone	- / 149	0.33	0.25	-	-	-
20	4-ethylphenol ^a	Phenol	-	0.35	0.15	-	0.23	<LOQ
21	3,4-dimethylphenol ^a	Phenol	-	-	0.30	-	0.47	0.52
22	2-hydroxy-3-methyl-2-cyclopenten-1-one ^{b,c}	Ketone	114 / 111	1.00	0.34	0.66	1.96	1.98
23	3-ethyl-2-hydroxy-5-dimethylcyclopenten-2-en-1-one ^{b,c}	Ketone	- / 137	-	-	-	-	0.68
24	Homovanillyl alcohol isomer ^{b,d}	Alcohol	151 / -	0.24	-	-	0.33	0.60
25	Methyl-benzenediol isomer ^{b,e}	Phenol	- / 123	-	0.55	0.31	0.67	1.20
26	Hydroxyacetophenone isomer ^{b,f}	Ketone	137 / 135	0.06	0.10	0.09	0.09	-
27	Methylphenol isomer ^{b,g}	Phenol	-	0.50	0.48	0.15	1.67	0.96
28	Methylphenol isomer ^{b,g}	Phenol	-	0.17	0.13	0.05	0.42	0.45

581 (a) identified with commercial standards; (b) identified using mass spectra, Uv-Vis, and references; (c) quantified using 3-methyl-1,2-cyclopentenedione calibration curve; (d)
582 quantified using homovanillyl alcohol calibration curve; (e) quantified using 4-methyl-1,2-benzenediol calibration curve; (f) quantified using 4-hydroxyacetophenone calibration
583 curve; (g) quantified using 4-methylphenol calibration curve.