**Valuable chemicals identified from *Flourensia* species using vacuum and analytical pyrolysis**

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**ABSTRACT**

The genus *Flourensia*, present throughout most of the Americas has been identified as a source of renewable chemicals. Here, we characterised the aerial biomass and matrix polysaccharides of *Flourensia campestris*, *F. oolepis* and *F. riparia*, and explored their potential as a source of chemicals through the study of two techniques: analytical pyrolysis (PY-GC/MS) and vacuum pyrolysis (VP). In all species, hemicellulose was more abundant than cellulose and lignin, with galacturonic acid being the most abundant monosaccharide. Bio-oils from VP exhibited a complex combination of phenols, terpenes, furans and nitrogen-containing compounds which have a wide range of industrial applications. Differences between the composition of VP pyrolytic oils derived from the mixture of leaves and stems or from individual organs suggest possible interactions between biopolymers. Combining PY-GC/MS and principal component analysis(PCA) we identified fingerprints for leaves and stems of the three *Flourensia* species.

**Keywords:** Py-GC/MS. Metabolite fingerprints. *F. campestris*. *F. oolepis*. *F. riparia*.

**1. Introduction**

New sustainable feedstocks and bio-based chemicals are essential to replace fossil fuels and decarbonise heavily oil dependent industrial sectors. Industrial biorefineries in which total biomass is converted into value-added products, chemicals and biofuels are a key component for the creation of bio-based industries. Pyrolysis is considered one of the most promising technologies for biorefineries, and many countries are developing industrial-scale plants [1], some of them mobile to improve the process logistics [2,3]. Thermochemical conversion of plant biomass allows for the complete utilization of the biomass, yielding bio-oil and valuable by-products (bio-syngas and biochar). In fast pyrolysis biomass is rapidly heated at a rate >1000°C/s in the total absence of oxidizing agents, and the process is carried out at moderate temperatures (400–600°C) [1]. Particularly in vacuum pyrolysis, high yields of bio-oils can be obtained due to the short residence time of the organic vapour generated in the reactor, which reduces the occurrence and intensity of secondary reactions [4].

Depending on its composition, bio-oils have different applications as bio-fuels [5],[6]. Bio-oil can also be used to produce specialty chemicals such as resin precursors, additives in fertilizers, pharmaceuticals, flavouring agents, etc. [7,8] .

Although the characterization of pyrolytic bio-oils can be performed using different techniques, gas chromatography coupled to mass spectrometry (GC-MS) has been the most widely used method [9]. In turn, analytical pyrolysis coupled with gas chromatography/mass spectrometry (PY-GC/MS) is an efficient, precise, and simple methodology for the characterization of plant biomass. The resulting pyrograms can be used to analyze the fingerprint patterns of complex plant-derived materials by means of multivariate statistical techniques as principal component analysis (PCA) and hierarchical cluster analysis (HCA) [10].

In the quest for new feedstocks that can be transformed into bio-oils, gas, biochar and valuable sub-products with industrial applications, researchers are continuously testing new plant species and crops, and investing in new and more efficient processing technologies.

The genus *Flourensia* (Family Asteraceae; Sub-family Asteroideae; Tribe Heliantheae; Subtribe Enceliinae) emerges as an interesting alternative crop for semi-arid and arid areas of Southern USA and Latin America. The 25 species within the genus are long-lived perennating shrubs, typically resinous and widely distributed along a steep latitudinal and altitudinal range. A sound body of research on the genus highlight the potential of a number of species as prospective candidates for sustainable exploitation and as new sources of bio-renewables [11-15].

In a previous work, our research group explored the potential of *Flourensia oolepis* to produce bio-oil and biochar using fast pyrolysis -under vacuum- of aerial biomass at different temperatures [16]. Overall, results showed that vacuum pyrolysis of aerial biomass of *F. oolepis* could be a source of terpenes and phenolic compounds with a wide range of applications.

From these results and the capacity of these species to grow in arid and semi-arid environments, we carried out an in-depth evaluation of the potential of *Flourensia* spp. as alternative sources of bio-products. In this study we characterised the lignocellulosic aerial biomass of three species of *Flourensia* (*F. campestris*, *F. oolepis* and *F. riparia)*, using analytical (PY-GC/MS) and vacuum pyrolysis as complementary techniques in order to explore their potential as sources of valuable by-products for industries. We studied the raw biomass and cell wall constituents and explored the suitability of PY-GC/MS (at 450 °C) and PCA as a fingerprinting technique for species/organs discrimination. Preparative vacuum pyrolysis was carried out between 280-450 °C, the product yields were determined, and an exhaustive characterisation of the bio-oils was performed in order to establish which liquid might have a future application.

**2. Materials and methods**

*2.1.* *Materials*

Plant material from the evergreen shrubs *Flourensia campestris* Griseb (FC) and *Flourensia oolepis* S.F. Blake (FO) was collected in natural areas corresponding to “El Cuadrado” (FC, S 31°06′18″ W 64°27′36″, 900-1000 MASL), Punilla Valley, Córdoba province, Argentina. A voucher of both specimens (BAA 26.498) was deposited at the herbarium “Gaspar Xuárez” of the Facultad de Agronomía, Universidad de Buenos Aires, Argentina. *F. riparia* Griseb (FR) was collected in natural areas corresponding to Chicoana (FR, S 30°56′04″ W 64°29′28″, 900-1000 MASL), Salta province, Argentina. A voucher specimen (SI043385) was deposited at the herbarium of the Instituto de Botánica Darwinion, Buenos Aires, Argentina.

Shoots from at least 15 specimens of FC, FO and FR were collected in early summer (January) of 2018. Harvested shoots were air dried in the shade, and separated in two fractions: leaves and stems, milled and stored at ca. 20 °C.

All organic solvents used in this study were analytical grade and used without further purification. Trifluoroacetic acid (TFA), isopropanol (IPA), polytetrafluoroethylene (PFTE), acetone (ace), sulfuric acid (H2SO4) and ethanol (EtOH) were purchased from Honeywell Fluka and VWR Chemical. Oxygen-free dry nitrogen 5.0 used in pyrolysis experiments was supplied by Linde. Arabinose (Ara), fucose (Fuc), galactose (Gal), galacturonic acid (GalA), glucuronic acid (GlcA), glucose (Glc), mannose (Man), rhamnose (Rha) and xylose (Xyl) were purchased from SIGMA (purity > 99%).

*2.2.* *Sample preparation and characterisation*

Elemental analysis for carbon (C), hydrogen (H) and nitrogen (N) was carried out using a Perkin Elmer 2400 Series II analyzer. Carbon, hydrogen and nitrogen content (wt% on dry basis) were analysed in duplicate and average values taken. Oxygen (O) was calculated by mass difference.

*2.3. Chemical composition of leaves and stems of FC, FO and FR*

*2.3.1.* *Polysaccharide matrix* *analysis*

The monosaccharide composition analysis of the air-dried aerial parts of FC, FO and FR was performed using high-performance anion exchange chromatography (HPAEC) (Dionex, Camberley, UK) according to the methodology previously described [17]. A calibration curve containing a mixture of nine monosaccharides (Fuc, Ara, Rha, Gal, Glc,Xyl, Man, GalA, GlcA) was used for determination and quantification.

*2.3.2. Crystalline cellulose content*

The content of cellulose was determined by measuring the glucose formed by acid hydrolysis (Saeman hydrolysis). The glucose content was assayed using the colorimetric Anthrone assay [18].

*2.3.3. Extractives, volatiles, Klason lignin and ash content*

One gram of each milled sample was washed with 96% EtOH at 100 °C for 30 min and the supernatant was recovered (repeated four times). Extractives free residue, and the supernatant were dried at 45 °C for 3 d. Supernatants were weighed to determine the extractive fraction. Klason lignin was determined as the insoluble residue after hydrolysis of the extractive free residue with 72% sulfuric acid [19].

Simultaneously, 1 g of plant material was placed in weighed crucibles and kept in an incineration oven (550 °C) for 24 h in order to obtain a direct measure of ash content.

*2.4. Analytical pyrolysis-gas chromatography-Mass spectrometry (Py-GC/MS)*

Py-GC/MS results were obtained using a CDS 5250-T Trapping Pyrolysis Autosampler, Agilent Technologies 7890B GC System as gas chromatography unit and Agilent Technologies 5977A MSD as mass spectrum unit. The samples pyrolysed at 450 oC for 15 s, under trapping mode. The volatile materials released were carried into the GC/MS unit, via a heated transfer line (340°C), in a helium flow for GC/MS analysis. The following GC/MS parameters were applied: GC inlet temperature at 350oC, the initial temperature at 40 oC for 2 min, ramp rate at 10 °C/min until 300 oC, holding at 300 oC for 12 min, split ratio with 50:1. Helium carrier gas flow was 1ml/min and the separation performed using an Agilent HP-5ms Ultra Inert (30m x 0.25mm x 0.25µm). The separation was performed using an Agilent HP-5ms Ultra Inert (30 m x 0.25 mm x 0.25 µm). Volatile compounds were identified by comparing the mass spectra with NIST Lab database (Match ≥ 85%).

Three spectra were collected for each sample, and the triplicate-averaged spectrum was used for principal component analysis (PCA). PCA was carried out using The Unscrambler X 10.5.1 software (CAMO). Peak identification was initially done manually using MassHunter Qualitative Analysis B.07.00 and NIST 14 library.

Before PCA analysis, the acquired data were processed as follows [20]: Agilent.D files were converted to zlib and compressed to 32 bit precision .mzML files using ProteoWizard MSConvert version 3.0.19172 using the following filters: peakPicking vendor, zeroSamples removeExtra. Using custom scripts in R 3.0.0 operating in a Linux 64 bit environment, .mzML files were further processed to bin, smooth, and baseline to correct the input data. Briefly, spectra were extracted from input files using the mzR package and subsetted to a retention time range between 50 and 2000 s, and a m/z range between 35 and 550. Across scans, masses were then binned at 1 m/z intervals using the binYonX function from the xcms package, with a base intensity value of 1, and then smoothed over a moving window (n = 3), using the rumean function from the caTools package. Baseline was calculated and subtracted from the running minimum value of the smoothed traces (n = 50). Binned, smoothed, and baseline subtracted data were then saved into a new .mzML file using the copyWriteMSData function from mzR.Peak detection was then performed using successive iterations of the findPeaks.centWave function from xcms (pe akwidth windows spanning 1 to 60 s) to maximize coverage, with duplicates ≤2 s apart removed, and filtered to retain peaks with gaussian shapes. Gaussian peaks with a signal/noise ratio > 3 were used for linear retention time correction across all samples using the XCMS retcor function, grouped using group.density with bw = 2 and minsamp= 3, and the fillPeaks function used to calculate missing areas. The CAMERA package was used to identify isotopes and correlated m/z values likely to originate from a single compound. For each cluster of retention time and m/z values identified, the most intense was kept as a representative for the underlying compound, and additionally any m/z retention time pairs manually identified as likely pyrolysis products by reference to the literature. This process produced a matrix of baseline corrected peak areas for samples x unique masstags (m/z and retention time), for further analysis.

*2.5. Vacuum pyrolysis experiments*

Pyrolysis reactions were conducted in a tubular reactor under inert atmosphere and vacuum as described in previous work [16]. Crashed leaves and chopped stems samples of FC, FO and FR (1.00 g) were used for the experiments at 280, 350 or 450 °C. The conversion of the starting material took place in 20 min, and due to the vacuum system, contact times of the generated products were very short (~ 1s). The liquid pyrolysate was extracted with acetone, after evaporation of this solvent, the bio-oil was weighed. The carbonaceous solid was removed from the sample holder and weighed, and the gas yield was calculated by difference. All yields are expressed as the average of at least three experiments. The first set of pyrolysis experiments were performed at 280, 350, and 450 °C with a mix of leaves + stems (50:50 w/w; named as LSmix) of each species. The second set of pyrolysis experiments were performed at 450 °C using either the leaves or stems of each species.

The components of the bio-oil from the pyrolysis reactions of FC, FO and FR were analysed by gas chromatography coupled to mass spectrometry (GC-MS). These analyses were performed with a Shimadzu GC–MS-QP 5050 spectrometer equipped with a VF column (30 mm x 0.25 mm x 5 m) by using helium as eluent at a flow rate of 1.1 mL/min. The injector and ion source temperature was 300 °C, the oven heating ramp was 10°C/min from 150 up to 280°C, and the interface temperature was 280 °C. The pressure in the MS instrument was 1.3 mPa and MS recordings were made in the electron impact mode (EI) at ionization energy of 70 eV. Compounds were tentatively identified based on the comparison between mass spectra experimentally obtained with those from the National Institute of Standards and Technology (NIST 14) library (Match > 85%).

**3. Results and discussion**

*3.1. Feedstock characterisation*

Plant biomass consists of several major polymers: cellulose, hemicellulose, pectins, lignin, and minor components such as organic acids, proteins, tannins as well as secondary metabolites of intrinsic value. It is well known that the relative fractions of the different components may vary among plant species, organs, developmental stages, and growth conditions [21].

In our study, biopolymer differences were detected both, within species (leaves vs. stems) and among species. Figure 1 and Table 1 show the content of cellulose, hemicellulose, and lignin of the two biomass samples of *Flourensia campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) used in this study: leaves and stems. Elemental analyses, O:C and H:C molar ratios are also shown.

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**Fig. 1**. Composition of leaves and stems of *Flourensia campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR).

**Table 1**

Elemental composition of leaves and stems of FC, FO and FR (on dry basis).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **FCa** | | **FOb** | | **FRc** | |
| **Leaves** | **Stems** | **Leaves** | **Stems** | **Leaves** | **Stems** |
| C (wt. %) | 50.56 | 55.08 | 45.44 | 45.67 | 36.94 | 40.85 |
| H (wt. %) | 3.68 | 5.79 | 5.71 | 3.26 | 2.59 | 1.87 |
| N (wt. %) | 2.17 | 3.04 | 2.47 | 1.56 | 12.62 | 13.91 |
| O (wt. %)d | 41.14 | 35.00 | 44.01 | 47.37 | 38.87 | 40.37 |
| Ash (wt. %)e | 2.45 | 1.09 | 2.37 | 2.14 | 8.98 | 3.00 |
| O:C molar ratio | 0.61 | 0.48 | 0.73 | 0.78 | 0.79 | 0.74 |
| H:C molar ratio | 0.87 | 1.26 | 1.51 | 0.86 | 0.76 | 0.55 |

a*Flourensia campestris*, b*Flourensia oolepis*, c*Flourensia riparia,* dCalculated by difference, eCalculated after one gram of dried sample was placed in weighed crucibles in an incineration oven at 550 °C for 24h.

In general terms, the cell wall composition of the three species falls within the broad spectrum found in lignocellulosic biomasses [8,22,23]. Extractives and volatiles together accounted for ca. 35% of the leaves weight, but only 22.5% of the dry biomass of stems (Fig. 1). Volatiles ranged from ca. 7 to 10%, with slightly higher values in leaves relative to stems. The major differences between organs were related to the extractive component, in which leaves showed significantly higher amounts (40-50%) compared to stems.

The high percentage of extractives found in *Flourensia* is most probably related to the high amount of resins present in these species, which would be extractable with ethanol. Previous results showed that crude resins in *Flourensia* accounted for 20-40% of leaves dry weight [24], being similar to other resinous shrub species such as *Larrea* spp. [25], *Grindelia chiloensis* [26] and *Haplopappus* spp. [27].In addition, the thick layer of cuticular waxes in surface deposits could also contribute to the amount of extractives found in these species. GC-MS analysis of surface deposited resins collected from stems of FC and FO before spring regrowth revealed substantial differences in composition [11].

Cellulose and matrix polysaccharides represented 40-58% of the biomass in leaves and 49-65% in stems, from which matrix polysaccharides contributed between 32-40% of the total dry matter of leaves and stems. The percentages of cellulose found in *Flourensia* species are in the lower range of those reported for other lignocellulosic materials [21,28,29]. Cellulose was significantly higher in stems relative to leaves in the three species, however while FC and FO showed similar percentages, FR exhibited almost 50% less cellulose in both organs. In contrast, matrix polysaccharides values were similar in both, leaves and stems (Fig. 1). These percentages were close to those found in herbaceous plants which contain between 30-40% of hemicellulose [30], and significantly higher than other reported values for softwoods and hardwoods [31].

The lignin content varied between 10 to 23%, depending on species and plant organ (Fig. 1), and were within the expected range for dicotyledonous species and some grasses [28]. Stems showed higher values relative to leaves in all species tested (14-23% stems vs. 10-16% leaves), where lignin content in FR ˃ FC ˃ FO.

The chemical composition of the lignocellulosic biomass depends not only on the genetic background of the species, but also on environmental factors and the interaction between them. Even within the same species, environmental stress (abiotic and biotic) trigger adaptive responses that modulate cell wall composition and wood components to increase plant resistance [32,33].

Matrix polysaccharides include hemicelluloses and pectins. Xylans, mainly as glucuronoxylans (GX) and glucuronoarabinoxylan (GAX), constitute the main components of secondary cell walls of hardwoods and herbaceous plants (20–30%, respectively), while glucomannans (GM) and galactoglucomannans (GGM), are the major components of softwoods and are seldom present in hardwoods [21,35,36].

As mentioned before, the matrix polysaccharides fraction represents the largest component of the total biomass of the *Flourensia* species investigated (Fig. 1), hence their composition and the contribution of the different monosaccharides becomes relevant for their potential applications, and for the analysis and interpretation of the pyrolysis experiments.

In the three *Flourensia* species, the fraction of GalA was by far the most abundant in both leaves and stems, accounting for 30-40% of all polysaccharides (Fig. 2).

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**Fig. 2**. Fractions of hemicellulose-derived monosaccharides present in the leaves and stems of *Flourensia campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR).

The relative abundance of the other groups depended on the organ considered. Typically, the second most important monosaccharide was arabinose in leaves, and xylose in stems. The leaves of FC and FO exhibited very similar patterns of polysaccharides with GalA˃˃Ara≥Glc≥Gal˃Xyl˃Rha, while FR showed GalA˃Ara≈Gal˃Xyl˃Glc≈Rha (Fig. 2). In stems instead, patterns were very similar for FC and FR with much larger contributions of xylose (GalA˃˃Xylose˃˃Ara˃Gal/Glc˃Rha˃Man), relative to that found in FO (GalA˃˃Xylose ˃Ara˃Glc=˃Gal˃Man˃Rha) (Fig. 2).

Although analysing the structures of the monosaccharides present in *Flourensia* was beyond the scope of this study, it is interesting to note the predominant contribution of arabinose and galacturonic acid in the leaves of *Flourensia* spp., since they may have important applications. These monosaccharides are general components of pectins, suggesting the presence of rhamnogalacturonan and homogalacturonan in this fraction. The high content of arabinose suggests the presence of arabinogalactans (AGs, [37]).

The elemental composition of the species (Table 1) indicates that the C, O and H contents are in the range of values obtained for other lignocellulosic biomasses [6]. However, it should be noted that the N content is markedly higher in *F. riparia*. Another characteristic of this species was that the ash content in leaves was significantly higher than that obtained for the rest of the biomasses evaluated.

***3.2. Analytical Pyrolysis-Gas Chromatography-Mass Spectrometry (PY-GC/MS) of leaves and stems of FC, FO and FR.***

Analytical pyrolysis experiments were carried out as a first screening to characterise the main families of compounds present in the biomasses of the three species, and to identify metabolic fingerprints for species and organs. The temperature chosen for analysis was 450 °C, a temperature typically used for studying the behavior of lignocellulosic biomass [38]. During analytical pyrolysis, the organic molecules present in the biomass are thermally decomposed to small volatile fractions which are analysed by a GC-MS interfaced with the pyrolyser, allowing for the structural identification and quantification of the pyrolysis products. The lack of information on the pyrolytic behavior of specific compounds and/or the mixture of the complex composition of secondary compounds of *Flourensia* spp. would preclude the elucidation of all the metabolites. However, as shown by Tianniam, et al. [39] for *Angelica acutiloba* roots, a complete metabolite identification is not necessary for the metabolite fingerprinting approach we selected to discriminate species and organs.

In the pyrograms of the leaves and stems of the three species (Fig. S1 and Fig. S2, supporting information), between 128 -200 peaks were initially detected (Table S1, supporting information). All the volatile products identified in the samples were classified into six classes of compounds: aromatic hydrocarbons (Ar), furans (Fur), organic nitrogen-containing compounds (Nit), other oxygenated compounds (Oxy), cyclic hydrocarbons (CyH) and other minor products + unknown (Others) (Fig. 3). Results show that Oxy and Ar compounds were the major classes present in all samples, contributing between 65 to 93% to the total of volatile compounds. Oxygenated compounds were ca. 25% higher in the stems of FC and FR compared to the leaves, while no difference was detected in FO between organs. Nit represented 4-29% of the identified compounds, where the larger value corresponded to FR leaves. Leaves of FC and FR had higher values of Nit than stems, while percentages were almost identical in both organs for FO. The percentage contribution of the other classes was ≤ 4% for each class. The general pattern for the relative contribution of the different classes may be described as Oxy > Ar > Nit > Others > Fur = CyH.

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**Fig. 3**. Volatile products distribution of Py GC-MS from leaves and stems of FC, FO and FR at 450 °C.

The PCA of the samples analysed by PY-GC/MS are shown in Figures 4 and 5. In the PCA scores plot (Fig. 4), each point represents the average spectrum obtained for each species and organ, and their spatial position provides information on how samples are related to each other. The scores plot shows that an overall 86% of the total variation is explained by the two first components. PC1 (68%) is responsible for discriminating leaves from stems (right and left quadrants, respectively), showing that regardless of species, samples of either leaves or stems showed closer metabolic composition. In turn, PC2 (18%) discriminated between species (upper and lower quadrants). The clustering of the leaves of FC and FO in the scores plot suggests that these species share similar metabolite profiles compared to the more distant and dissimilar leaves of FR. The same is true for the cluster of FO and FR stems, compared to FC stems. Since FR leaves and FC stems shows the larger distances from the plot origin it would be expected that both have a strong impact on the model. A difference in the relative contribution of the same metabolites, a different composition of metabolites and/or a few distinctive metabolites could explain why FR leaves and FC stems do not cluster together with the other two species.

Gráfico, Gráfico de dispersión

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**Fig. 4.** PCA scores plot of the PY-GC/MS data of leaves and stems of *F.campestris* (FCLe, FCSt), *F. oolepis* (FOLe, FOSt) and *F. riparia* (FRLe, FRSt).

![Gráfico, Diagrama, Gráfico de dispersión

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**Fig. 5.** PCA correlation loadings plot of PY-GC/MS data of leaves and stems of *Flourensia campestris*, *F. oolepis* and *F. riparia*.

In the PC loadings plot (Fig. 5) the compounds that are found within the 95% confidence limit, and particularly those positioned near the outer edge of the ellipse, are those that are more important for differentiating leaves (right quadrants) from stems (left quadrants) and species (upper and lower quadrants). In addition, PC loadings plot displays the relationships between the detected compounds. Compounds grouped together at the right or left quadrants in Fig. 5, have similar contributions and are positively correlated. The differences between leaves and stems were determined by 18 and 15 compounds, respectively (Table 2), with important variations in the percentage in which each class of compounds was represented. Leaves are characterised by a preponderance of Oxy (39%), and a similar contribution of Ar (22%) and Nit (17%) compounds. In contrast, stems are characterised by a majoritarian contribution of Ar (53%) followed by Oxy (33%), and a total absence of Nit and CyH compounds.

In addition, five compounds seem to be the most relevant in differentiating the leaves of FR from those of FC and FO, namely three Oxy species: 2-methyl propanal (**5**), 2-oxo-propanal (**7**) and *n*-hexadecanoic acid (**6**), and all the nitrogenated compounds listed in Table 2, while 2,3-dihydro-benzofuran (**1**) seems to characterise FC and FO leaves. In the case of stems, two Oxy compounds: acetoin (**8**) and 2-hexanone (**9**) appear to be involved in differentiating FC from FO and FR, while another oxygenated compound, 3-methyl-3-buten-1-ol acetate (**10**) seems to characterise FC and FO.

The structure of main compounds that differentiate the species are shown in Figure 6.

**Table 2**

List of compounds that are more important in differentiating the clusters depicted in the PC scores plot, classified according to the six classes: aromatics (Ar), furans (Fur), nitrogen derivatives (Nit) oxygen derivatives (Oxy), cyclic hydrocarbons (CyH) and others.

|  |  |  |
| --- | --- | --- |
| **Organs** | | |
| **Type of compounds** | **Leaves** | **Stems** |
| Ar | \*\*\* 1,2,3-trimethoxy-5-methyl-benzene  \*\* toluene  \*\* phenol  \* *p*-cresol | \*\*\* 4-ethyl guaiacol  \*\* 4-vinyl guaiacol  \*\* syringol  \*\* 3',5'-dimethoxyacetophenone  \*\* 5-methyl-*m*-cresol  \*\* creosol  \* 5-methyl-guaiacol  \* guaiacol |
| Fur | \*\* **2,3-dihydro-benzofuran** (1) | \* 2-furanmethanol |
| Nit | \*\*\* **glycine betaine** (2)  \*\*\* ***N*,*N*-dimethyl-methylamine** (3)  \*\* **13Z-docosenamide** (4) | nd |
| Oxy | \*\*\* 1-ethoxy propene  \*\* **2-methyl-propanal** (5)  \*\* **n-hexadecanoic acid** (6)  \*\* **1-methyl glyoxal** (7)  \*\* (methoxymethyl)-cyclopropane  \*\* acetone  \* 3-butenyl propyl ether | \*\*\* **acetoin** (8)  \*\*\* **2-hexanone** (9)  \*\*\* **3-methyl-3-buten-1-ol, acetate** (10)  \*\* acetic acid, methyl ester  \* 2-butanone |
| CyH | \*\*\* nerolidol | nd |
| Others | \*\* 2-hexyl-1-decanol  \*\* succindialdehyde | \* 2,6-dimethyl-2,4-heptadiene |

The number of asterisks denote the relative importance of each compound in differentiating leaves and stems. Compounds in bold indicate those that are key in differentiating species. The numbers in brackets correlate with the structures of the Figure 6. Nd: not detected.

The high level of nitrogenated compounds found in FR corresponds with the high N levels found in FR leaves, and with previous results which revealed large contents of glycine betaine (**2**) in FR leaves [40]. Some authors have reported the interconversion between *N*,*N*-dimethyl glycine methyl ester and glycine betaine as an example of the transformations that alkylated amino acids undergo by heating [41]. This interconversion process may be responsible for the appearance of this metabolite and for being recognised as one of the markers for FR.



**Fig. 6**. Structure of key compounds that differentiate *Flourensia campestris*, *F. oolepis* and *F. riparia*.

***3.3 Vacuum pyrolysis of FO, FC and FR***

Based on 1) the results from the analytical pyrolysis (at 450 °C) showing that thermal degradation of the biomasses yielded a whole range of compounds and families of compounds of industrial interest, and 2) our previous results using vacuum pyrolysis in FO [16], we decided to use vacuum pyrolysis in order to investigate product yields and to characterise the resulting bio-oils using either the whole aerial biomass or separate organs of *Flourensia spp*.

The first set of pyrolysis experiments were performed at 280, 350, and 450 °C with a mix of leaves + stems (50:50 w/w; named as LSmix) of each species. Results with LSmix show that biomass decomposition for the three species started at 280 °C, and that the increase in temperature produced an increase in the gaseous fraction at the expense of a decrease in the solid fraction (Fig. 7).

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**Fig. 7**. Liquid, solid and gas yields from pyrolysis of a mixture of leaves + stems (LSmix) of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at different temperatures.

Biochar yields were 43-49% at 280 °C and dropped to 22-11% at 450 °C, whereas non-condensable gases yielded similar values at 280 °C (40-46%) but peaked up to 74% at 450 °C (Fig. 3). Bio-oil yields increased with temperature and attained maximum values at 450 °C for FO and FR (36% and 15%, respectively), while in FC the higher yield was obtained at 350 °C (25%). In FO, the increase in the liquid fraction was mirrored by a decrease in the solid fraction (Fig. 7).

Although temperatures for maximizing bio-oil yield in fast pyrolysis (50-70%) are typically documented in the range 400-500 °C [42], ours results suggest that at least for these *Flourensia* species, the highest bio-oil yields are obtained at temperatures ≤ 450 °C. It is plausible that the observed response may be explained by the interactions between the different biopolymers present in each species [21] and the heavy loads of resins (compatible with the high levels of extractives and volatiles shown previously, Fig. 1). In fact, a fast pyrolysis study in *Euphorbia rigida*, a resinous species with a chemical composition comparable to *Flourensia* spp., also showed low bio-oil yields that could be increased using alumina and zeolite catalysts [43].

The temperature selected for the second set of pyrolysis experiments (450 °C) in which we used either the leaves or stems of each species, was decided based on the temperature that produced the maximum bio-oil yield in two (FO and FR) out of the three species investigated in the first set of experiments.

Results show that the pyrolysis of the individual organs was different from that of LSmix at 450 °C in all species (Fig. 8). FC leaves produced a similar amount of biochar (24%) to that obtained from LSmix, while stems produced ca. 40% less. The production of bio-oil was higher when each organ was pyrolysed separately (25% leaves, 19% stems), representing a 2x increase over that obtained in LSmix (11%). The gaseous yields did not exhibit major differences compared to LSmix and followed the changes in the other two fractions.

FR showed some similarities with FC regarding the yield response of the liquid fraction. Bio-oil yields of the pyrolysed organs were 40% (leaves) to 73% (stems) higher than that obtained for the LSmix. Biochar yields were equivalent to that produced by LSmix, and differences in the gaseous yields reflected the changes in the bio-oil fraction.

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**Fig. 8.** Liquid, solid and gas yields from pyrolysis of leaves and stems of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at 450 °C.

In contrast, FO leaves and stems showed a distinctive behaviour relative to the other two species when compared to LSmix yields. As previously shown, FO LSmix achieved the highest bio-oil yield of the three species (36%, Fig. 7) but interestingly, the yields of individual leaves and stems decrease two-fold relative to LSmix. The solid fraction also showed a significant reduction in both organs relative to LSmix. The decrease observed in the liquid and solid fractions in leaves and stems were mirrored by a corresponding increase in the gaseous fraction.

In the case of FO, the higher yields obtained in LSmix compared to that of individual organs suggest a possible synergistic effect of the biopolymers and resins present in the biomass during the pyrolytic reactions. Conversely, in FC and FR, the possible antagonist effects of the constitutive biopolymers and resins observed in LSmix (at 450 °C) could have been removed when the pyrolysis was performed separately on leaves and stems. Although the three species of *Flourensia* are highly resinous, resins composition as well as the density of the glandular trichomes and secretory ducts, responsible for their production, may vary between organs and species [11,12,24]. In fact, results from previous studies on FC and FO show that the density of glandular trichomes (gt, number per mm2) exhibit 12 to 33x variation between organs (flowers, leaves and stems), and between species for a specific organ. The higher densities were detected in young stems where FO showed between 2 to ca. 4x more glandular trichomes relative to FC (344-656 vs 162-170 gt mm2, respectively). The same stands for the density of secretory ducts found inside leaves and stems, although the range of variation was smaller [12,24]. The differences in the amount, exposure, and specific composition of resins would influence the pyrolytic behaviour of each biomass, which may contribute to explain the contrasting results in product yields observed in our pyrolysis experiments.

The importance of feedstock composition in determining pyrolysis yields has been shown previously. For instance, Hassan et al. studied the pyrolytic behaviour of four different fractions of loblolly pine and cotton-wood and found that bio-oil yields and the remainder fractions were significantly affected by the type of biomass [44]. Differences were detected not only between species for the same biomass fraction but also among fractions of the same species. Authors suggested that differences in the levels of volatiles and biopolymers composition (homocellulose and lignin) were involved in the observed results.

Altogether, these results show that the use of either the whole aerial biomass of a species or specific organs can target the composition of the final product. This experimental data can contribute to the scale up of industrial projects.

***3.3. Bio-oil characterisation from vacuum pyrolysis experiments***

The resulting bio-oil was extensively analysed by GC–MS technique, where the peak area percentage of the detected products depends on the response factor of the mass spectrometer detector, making it difficult to provide an accurate quantification of products. Bearing this in mind, the peak area of an individual compound was directly proportional to the concentration of such compound in the liquid pyrolysate. The peak area percentage of any compound was used for comparison purposes to assess its variability among the experimental conditions. Based on the ion chromatograms of the bio-oils obtained for the three species at different temperatures, we identified a total of 146 compounds (by NIST, match ≥ 85% - Table S2, supporting information-).

These compounds were classified into the same six groups defined in the analytical pyrolysis: aromatic hydrocarbons (Ar), furans (Fur), organic nitrogen-containing compounds (Nit), oxygenated compounds (Oxy), cyclic hydrocarbons (CyH) and other minority products + unknown (Others) (Fig. 9 and 10).

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**Fig. 9.** Product distribution in the bio-oil from pyrolysis of a mixture of leaves + stems (LSmix) of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at different temperatures.

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**Fig. 10.** Product distribution in the bio-oil from pyrolysis of leaves and stems of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at 450 °C.

Results from the first set of pyrolysis experiments (at 280, 350, and 450 °C) show that although most of the groups were present in all LSmix bio-oils, their relative contribution differed between species and temperatures tested (Fig. 9). FC bio-oils were characterised by a predominant contribution of Ar (ca. 50%) followed by oxygenated compounds (15-21%), a small percentage of CyH and only trace amounts of Nit compounds. The general pattern for all temperatures can be described as Ar > Oxy > Fur > Others > CyH. In contrast, FO and FR bio-oils were characterised by CyH and Ar as major classes. In FO values ranged from 26-47% for CyH and 10-36% for Ar; the higher percentages were obtained at 350 °C. In FR contributions ranged from 10-49% for CyH and 11-34% for Ar. Two additional distinct features characterised FR bio-oils: a significant presence of Nit (8-36%) and a small contribution of oxygenated compounds (7-17%).

The main compounds identified by GC-MS in the Ar class were phenol; hydroquinone; 2-methoxy-phenol; 2-methoxy-4-vinylphenol; 2'-acetonaphthone; and 2-acetonaphtone. Syringol and guaiacol derivatives were also present but in small proportions. These compounds have been identified as products during the main conversion step of lignin that occurs from 200-450 °C, with the higher decomposition rate taking place between 360 and 400 °C [35,45]. At lower temperatures, the breaking of bonds between monomer units can also produce the release of guaiacol derivatives [8]. Syringols are usually detected within 275-350 °C [8,46], and the maximum rate of production of phenols occur between 360–400 °C [35].

Furans contribution ranged from 3 to 19%, being present in all species except in FR at 450 °C. The higher values were detected in FC at 350 °C and FR at the lower temperature tested (280 °C). These results would also agree with the range of temperatures at which cellulose and matrix polysaccharides decompose [8]. The most important compounds identified by GC-MS in LSmix bio-oils were benzofuran derivatives for FC and FO, and naphthofuran derivatives for FR.

Organic-nitrogen containing compounds were present in FO (0-13%) and FR (4-36%), being negligible in FC bio-oils. The highest percentage of Nit in FO was found at 280 °C, while in FR the higher values were obtained at 350 °C (36%) and 450 °C (24%). The main compounds were aliphatic amines of low molecular weight and polinitrogenates as diaza-derivatives. The importance of this class of compounds in FR is consistent with the higher content of nitrogen measured in the leaves and stems of this species (13-14%, respectively-Table 1) compared to FC and FO. These results are also in line with a previous study in which we investigated the abundance of quaternary ammonium species in the leaves of eight South-American species of *Flourensia*. Results showed that FR was the species exhibiting the highest content of glycine betaine with as much as 90 ± 9 μmol g−1 DW [40].

Oxygenated compounds were present in all species and temperatures. Oxy typically include a complex combination of the products derived from the individual pyrolysis of cellulose, hemicellulose, lignin and extractives.In the case of *Flourensia*, this mixture was mainly composed of ketones and long chain acids.

Cyclic hydrocarbons were mainly represented by terpenes, such as limonene, caryophyllene oxide, spathulenol and hydrogenated naphthalenes, and their relative contribution changed according to species and temperature. For instance, while limonene was obtained in all reactions, its contribution increased with temperature accounting for as much as 49% of FR bio-oil at 450 °C. These results agree with our previous results from FO pyrolytic bio-oils, in which we found that CyH consisted of a mixture of limonene, bisabolene and spathulenol [16]. Figure 10 shows the results of the pyrolysis experiments performed separately on the leaves and stems of each species at 450 °C. The relative contribution of the different classes of compounds differed between organs in each species, between species, and with LSMix in each species (cf. Fig. 9 and 10). However, similarities in the pattern of classes and relative contribution were found between LSmix bio-oils and individual organs for two species: FCmix and FC stems, and FOmix and FO leaves. In contrast, no similarities were observed for FR.

Regarding the leaves, the bio-oils from FC and FO showed almost identical patterns of classes (Fig. 10). CyH and Ar represented 67% of the total bio-oil blend with similar contributions of CyH and Ar. Oxy compounds showed a significant contribution in FO (17%), while Others accounted for 16% and 9% in FC and FO, respectively. The relative abundance of the latter reflects a complex matrix of compounds with more diverse chemical groups. A completely different response was observed in FR leaves, where Nit contributed ca. 72% of the total compounds and CyH and Ar represented only 17%.

In contrast, the pyrolytic bio-oils of stems were very similar in the three species investigated where Ar>Oxy. Ar and Oxy contributed between 67 to 84%, and Ar was ca. 2 to 3x higher than Oxy. The reminder classes varied slightly in order of importance among species, with individual contributions ≤ 10% for each class.

These patterns are very different to those described for the analytical pyrolysis experiments (450 °C) of leaves and stems of the three species, which were characterised by a predominance of Oxy and Ar compounds, and a negligible contribution of CyH (cf. Fig. 3 and Fig. 9). Compared to pyrolytic bio-oils, Oxy species were ≥ 3-26x larger than those found in leaves (the higher value corresponds to FR), and 2.3-2.8x larger than in the stems of the three species. These apparently controversial results strongly suggest that a large proportion of Oxy compounds are not trapped in the bio-oil during fast pyrolysis and would be present in the gaseous fraction. In this sense, it is important to highlight that the large load of resins in these species may have contributed to the high levels of low molecular weight Oxy compounds (>58-102 g/mol) found in the volatiles, as a result of the decomposition and fragmentation during the analytical pyrolysis process.

The number of compounds identified by GC-MS within each class, as well as their relative contribution was found to vary according to species and organ. For this reason, we will only mention those which contributed ≥ 5% of the total, and that were either present in most bio-oils or showed a distinctive feature (i.e.: highly represented; exclusively present in one species/organ).

Compounds in Ar class corresponded to phenol derivatives such as: phenol, hydroquinone, 2-acetyl-3,6-dimethyl-1,8-naphthalenediol, guaiacol, 4-vinyl-guaiacol, syringol (only in stems), 2',4'-dihydroxychalcone (in FO leaves), 3-ethoxy-phenol (in FC stems), 3,5,6,7,8,8a-hexahydro-8a-(4-hydroxyphenoxy)-2*H*-1-benzopyran-2-one (in FR stems). The relative contribution of this class to the bio-oil of each species and organ does not seem to correlate with the measured values for lignin (cf. Figs. 10 and 1). For instance, FR exhibited 30-66% more lignin in leaves and 2x higher levels in stems compared to FC and FO. However, the contribution of Ar to the mix of compounds in bio-oils was similar for stems and 6x times smaller for the leaves.

CyH were the major contributors of the bio-oils obtained from FO and FC leaves, with terpenoids accounting for ca. 70 to 100%, respectively. The major compounds identified were bisabolol in FC (18%) and bisabolene epoxide in FO (16 %), while spathulenol contributed between 5-7%. The higher content of extractives found in the leaves compared to stems (≥ 96 % higher, Fig. 1) could explain the large contribution of CyH in these bio-oils. The composition of CyH agrees with previous studies on FC, FO and other *Flourensia* species in which volatile and non-volatile resins were found to be primarily constituted by terpenoids (mono and sesquiterpenes) and whose composition may vary according to species and plant organs [24]. As in present results, Piazza et al. [11] have also shown the majoritarian contribution of bisabolene derived compounds in glandular trichomes (53%) and leaf epidermis (33%).

Oxygenated compounds were represented by a diverse number of compounds with percentages 5%, of which 2-butenoic acid, 2-methyl- and 3-methyl- cyclopentane-1,2-dione were present in most samples. Other Oxy were found exclusively in one organ and/or species: 1-(acetyloxy)-2-propanone in stems; levoglucosan in FO stems and leaves; 10,11-dihydroxy-3,7,11-trimethyl-2,6-dodecadienyl acetate in FO leaves; 3-methoxy-3,7-dimethylocta-1,6-diene in FC leaves. The contribution of Oxy to each bio-oil can be correlated with the content of hemicellulose, which is higher in stems relative to leaves (cf. Figs. 10 and 1). Within this group, anhydrosugars did not represent a significant fraction of products, probably due to the low cellulose content (≤ 25 wt.%) in the leaves and stems of *Flourensia* species. Additionally, it has been shown that the interaction of hemicellulose and cellulose during pyrolysis inhibits the formation of some anhydrosugars, reducing their yields [47].

Nit compounds were mainly present in FR bio-oil from leaves, in which 80% of this class was accounted for just two compounds: 1-methyl-5-piperidin-1-yl-imidazolidin-2,4-dione (19%), 4-amino-4-methyl-2-pentanone (22%). These results also agree with the high Nit values obtained in FR LSmix bio-oil at 450 °C (Fig. 9), although with a different blend of compounds.

Furans were poorly represented in these bio-oils, except for FO stems (10%), for which ca. 80% corresponded to furanmethanol and benzofuran derivatives. The class Others included a heterogeneous group of ca. 30 compounds distributed among species, typically with low percentage contribution (< 2%).

Differences in the quantity and the quality of resins may play a crucial role in the way each biomass is pyrolysed and may explain the contrasting results in product yields observed in the pyrolysis experiments described.

Results from vacuum and analytical pyrolysis cannot be readily comparable due to the differences in the degradation process that biomass undergo under each technique as well as the type of products that can be trapped for further analysis. However, it is interesting to note that several of the fundamental units typically derived from lignin and furans degradation, and that characterise these species, were present in both types of experiments.

In summary, bio-oils from FC, FO and FR are a complex mixture of phenols, terpenes, furan derivatives, nitrogen-containing compounds and other oxygenated compounds including aldehydes, ketones, carboxylic acids and anhydrosugars. The pyrolysis of leaves and stems of FC, FO and FR could represent a promissory source of phenolic, terpenoid and nitrogen compounds with a wide range of applications, which could be separated and valorised. The high content of phenolic compounds in the bio-oil (particularly in the organic phase) can be a feedstock for synthesizing phenol-formaldehyde (PF) resins or used directly in boilers, engines and furnaces for electricity generation.

**4. Conclusion**

The analysis of the biomass composition of three *Flourensia species* (FC, FO and FR) highlights a large proportion of matrix polysaccharides compared to cellulose and lignins. In all species, GalA is the most abundant monosaccharide in both leaves and stems, followed by Ara in leaves, and Xyl in stems.

PY-GC/MS is a valuable technique to characterise *Flourensia* spp. biomass undergoing thermal degradation. The combination of PY-GC/MS and PCA allowed to identify between 18 and 15 compounds as fingerprints for leaves and stems of the three *Flourensia* species investigated, and specific markers for each species. These results could be useful in chemotaxonomic studies and for the bioprospection of new species within the genus.

Bio-oils from vacuum pyrolysis of leaves and stems (mixed or separately) of FC, FO and FR are a complex combination of phenols, terpenes, furans, nitrogen-containing compounds and other oxygenated compounds. Our results show that bio-oils obtained from pyrolysis of a mixture of leaves and stems have different characteristics compared to those obtained from pyrolysis of individual organs. The main differences were observed in the relative contribution of Ar and CyH.

Overall, this study highlights the importance of investigating native species that are already adapted to grow and survive in semi-arid and arid environments, many of which may arise as new crops for the exploitation of natural populations. This is particularly relevant in the scenarios in which sustainable biorefineries will be oriented in maximizing the use of regional resources. This report, the first of this kind for the genus *Flourensia*, shows the potential of these native species to be used as sources of high-value chemicals for a variety of industrial platforms.

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**Author statement**

Mariana P. Silva: Conceptualization, Methodology, Writing, Software, Validation, Investigation, Formal analysis. Leonardo D. Gomez: Review & editing, Resources. Tony R. Larson: Methodology, Software analysis of metabolomics. E. Laura Moyano: Conceptualization, Design, Review, Financial Support. Ana L. Scopel: Conceptualization, Writing, Review and Editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Figure Captions**

**Fig. 1**. Composition of leaves and stems of *Flourensia campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR). Data are expressed as means of three independent experiments ± SE. Numbers indicate the mean percent contribution of each product; decimals were rounded to the nearest integer. For clarity, percentages ≤ 3% are not shown.

**Fig. 2.** Fractions of hemicellulose-derived monosaccharides present in the leaves and stems of *Flourensia campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR). Data are expressed as means of three independent experiments ± SE. Decimals were rounded to the nearest integer; for clarity, values ≤ 3% are not shown.

**Fig. 3**. Volatile products distribution of Py GC-MS from leaves and stems of FC, FO and FR at 450 °C. Products were classified as: aromatics (Ar), furans (Fur), nitrogenated compounds (Nit), oxygenated compounds (Oxy), cyclic hydrocarbons (CyH) and others. Data are expressed as means of three independent experiments ± SE. Numbers indicate the mean percent contribution of each product. Decimals were rounded to the nearest integer; for clarity, values ≤ 4% are not shown.

**Fig. 4.** PCA scores plot of the PY-GC/MS data of leaves and stems of *F.campestris* (FCLe, FCSt), *F. oolepis* (FOLe, FOSt) and *F. riparia* (FRLe, FRSt). The scores represent the means of three independent experiments.

**Fig. 5.** PCA correlation loadings plot of PY-GC/MS data of leaves and stems of *Flourensia campestris*, *F. oolepis* and *F. riparia*.

**Fig. 6**. Liquid, solid and gas yields from pyrolysis of a mixture of leaves + stems (LSmix) of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at different temperatures. Data are expressed as means of three independent experiments ± SE. Numbers indicate the mean percent contribution of each product; decimals were rounded to the nearest integer.

**Fig. 7.** Liquid, solid and gas yields from pyrolysis of leaves and stems of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at 450 °C. Data are expressed as means of three independent experiments ± SE. Numbers indicate the mean percent contribution of each product; decimals were rounded to the nearest integer.

**Fig. 8.** Product distribution in the bio-oil from pyrolysis of a mixture of leaves + stems (LSmix) of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at different temperatures. Products were classified as: aromatics (Ar), furans (Fur), organic-nitrogenated (Nit), oxygenated (Oxy), cyclic hydrocarbons (CyH) and minority products (Others). Data are expressed as means of three independent experiments ± SE. Numbers indicate the mean percent contribution of each product. Decimals were rounded to the nearest integer; for clarity, values ≤ 5% are not shown.

**Fig. 9.** Product distribution in the bio-oil from pyrolysis of leaves and stems of *F. campestris* (FC), *F. oolepis* (FO) and *F. riparia* (FR) performed at 450 °C. Products were classified as: aromatics (Ar), furans (Fur), organic-nitrogenated (Nit), oxygenated (Oxy), cyclic hydrocarbons (CyH) and others (Others). Data are expressed as means of three independent experiments ± SE. Numbers indicate the mean percent contribution of each product. Decimals were rounded to the nearest integer; for clarity, values ≤ 5% are not shown.