Development of a supercritical fluid chromatography method with ultraviolet and mass spectrometry detection for the characterization of biomass fast pyrolysis bio oils

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Abstract

The characterization of complex mixtures is a challenging issue for the development of innovative processes dedicated to biofuels and bio-products production. The huge number of compounds present in biomass fast pyrolysis oils combined with the large diversity of chemical functions represent a bottleneck as regards analytical technique development. For the extensive characterization of complex samples, supercritical fluid chromatography (SFC) can be alternative to usual separation techniques such as gas (GC) or liquid chromatography (LC). In this study, an approach is proposed to define the best conditions for the SFC separation of a fast pyrolysis bio-oil. This approach was based on SFC data obtained directly from the bio-oil itself instead of selecting model compounds as usually done. SFC conditions were optimized by using three specific, easy-to-use and quantitative criteria aiming at maximizing the separation power. Polar stationary phases (ethylpyridine bonded silica) associated to a mix of acetonitrile and water as polarity modifier provided the best results, with more than 120 peaks detected in SFC-UV.

Keywords: Supercritical fluid chromatography; Mass spectrometry; Bio-oil; Fast pyrolysis; Complex sample

1 Introduction

Because of the necessity to develop new sources of energy for the future, the production of second generation (2G) biofuels from lignocellulosic biomass seems to be a promising option, implying different ways of conversion [1]. Fast pyrolysis is a thermochemical process operated within 400–450 °C range, enabling biomass liquefaction. The resulting product, also called pyrolysis oil or bio-oil, is very rich in oxygen compounds, corrosive and thermally unstable. It therefore needs to be upgraded to be used as biofuels [1]. To characterize bio-oils chemical composition at a molecular level, several analytical techniques were investigated [2], in particular chromatographic techniques. Gas chromatography (GC) permits to characterize a part of bio-oil composition (estimated at about 40%) [3–5]. However GC is currently unsuitable for compounds with high polarity and/or low volatility and/or poor thermal stability. In those cases where GC cannot be applied, liquid chromatography (LC) can be an alternative for the characterization of bio-oils [6,7]. On-line comprehensive two-dimensional liquid chromatography (LCxLC) was successfully applied to the aqueous fraction of bio-oils [6–8] and compared more recently to on-line LC × SFC [8]. SFC may be a promising approach to analyze bio-oils as it combines the advantages of GC (low fluid viscosity and high diffusivity of solutes) with those of LC including (i) the possibility of separating polar and/or low volatile compounds and (ii) the availability of a large panel of stationary phases providing very different selectivities. Furthermore, SFC conditions are expected to be soft with a usual temperature range between 30 and 60 °C, compatible with most of the compounds present in bio-oils.

Over the last decade, a new generation of SFC devices has been commercialized and the interest for SFC has grown in various fields [9] (pharmaceuticals, bioanalysis, agrochemicals, food products). The addition of an active backpressure regulator associated with a new generation of pumps, able to generate low flow-rates in a reproducible way, leads currently to robust and reliable analyses. However, in view of published results, it seems that SFC has

been mainly used for the separation of relatively simple samples. As regards bio-oils, a major issue is to find out well representative compounds able to mimic their great chemical complexity in terms of functional groups and molecular weights. To take into account the matrix complexity in the early stages of method development, we chose in this study to investigate SFC conditions by directly analyzing a biomass fast pyrolysis oil. The ultimate objective of our work was to achieve a detailed molecular description of such a bio-oil. To do so, several steps were required, starting from the optimization of SFC-UV conditions (stationary phase, mobile phase, temperature, etc…) and going to an in-depth analysis based on hyphenation between SFC and high resolution mass spectrometry. The present work corresponds to the first step of our methodology and aims to get a better understanding on how SFC retention is influenced by key experimental parameters such as temperature, pressure or mobile phase composition. As a result, the purpose of this paper was not to provide bio-oil molecular data but rather to propose a relevant methodology, based on quantitative and objective criteria, allowing to select the best chromatographic conditions in order to separate the highest number of compounds that are present in complex matrices such as biomass fast pyrolysis oils. Accordingly, a novel approach was proposed with three optimization criteria directed towards maximizing peak capacity. As an outlook, preliminary results based on the resulting optimized SFC separation and hyphenation to mass spectrometry detection are illustrated in order to point out the potential of this technique for bio-oil analysis.

2 Materials and methods

2.1 Chemicals and samples preparation

Solvents (acetonitrile, methanol, water) were MS grade from Sigma Aldrich (Steinheim, Germany). Carbon dioxide SFC grade (99,97%) was purchased from Air Liquide (B50 bottle under pressure). Tetrahydrofuran (THF) was purchased from VWR (Fontenay sous bois, France). The fast pyrolysis bio-oil was obtained from conifer sawdust, provided by IFP Energies nouvelles. The sample was diluted in THF (1/5 w/w) before analysis.

2.2 Instrumentation and chromatographic conditions

All experiments were performed on an Acquity UPC^2 instrument (Waters, Milford, MA, USA). Key parameters (stationary and mobile phases, back pressure, column temperature and gradient conditions) were optimized according to a procedure developed in the ‘Results and discussion’ section. The type of stationary phase was optimized according to the procedure described in the next section. The studied columns and their characteristics are reported in Table 1. The mobile phase flow-rate was 1.4 mL/min for columns #1, 2, 3, 5 and 7, except for column #4 and 6 whose geometry characteristics were taken into account by using a flow-rate at 3.5 mL/min.

Table 1 Characteristics of the columns investigated in this study.

alt-text: Table 1

Column number	Column name	Manufacturer	Stationary phase chemistry	d_c[a] (mm)	L_c[b] (cm)	d_p[c] (µm)	L_c/d_p
1	Acquity UPC² BEH	Waters	Unbonded silica	3.0	10	1.7	5.88
2	Acquity UPC² HSS C_{18} SB	Waters	C_{18}	3.0	10	1.8	5.55
3	Acquity UPC² BEH 2-EP	Waters	Ethyl Pyridine	3.0	10	1.7	5.88
4	Luna Cyano	Phenomenex	Cyanopropyl	4.6	15	3.0	5.00
5	Acquity UPC² BEH RP18 Shield	Waters	Polar embedded C_{18}	3.0	10	1.7	5.88
6	Nucleodur Polartech	Macherey-Nagel	Polar embedded C_{18}	3.0	10	2.5	4.00
7	Acquity UPC² CSH Fluorophényl	Waters	Fluorophenyl	3.0	10	1.7	5.88

[a] Column internal diameter.

[b] Column length.

[c] Particle diameter.

The injection volume was 1 µL for all experiments. The injector needle was washed with 600 µL of methanol after each injection. The variance due to extra column band broadening was measured under liquid chromatographic conditions by the method of statistical second moment [10] and estimated at 85 µL^2. The measured dwell volume, corresponding to the instrument volume between the meeting point of the solvents and the column inlet, was 425 µL. This value is higher than UHPLC ones (typically about 100 µL) which is related to the large mixing chamber (approximately 300 µL) used in this study to avoid any demixing phenomenon. The column outlet was connected to a photo-

diode array detector (PDA) equipped with a high pressure UV cell (400 bar) with a volume of 8 µL and a path length of 10 mm. The detection wavelengths varied between 210 and 400 nm with a bandwidth of 1.2 nm. The sampling rate was set at 40 Hz. The instrument was controlled by Empower 3 software (Waters).

Mass spectra were obtained using a LCMS 2020 instrument (Shimadzu, Kyoto, Japan) equipped with either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources both working in negative and positive modes. A simple quadrupole ensured the mass measurement in the range 80–800 *m/z*. MS parameters were optimized to favor the detection of pseudo-molecular ions. Such an optimization was based on model compounds detection. Nebulizing gas flow and dry gas flow were 0.5 and 10 L/min respectively. The interface temperature was set at 400 °C and the desolvation line temperature was 250 °C. For ESI source, the interface voltage was +3.5 kV and −5 kV in positive and negative ion modes respectively. For APCI source, the corona current in negative mode and positive modes were respectively 80 µA and 70 µA.

3 Results and discussion

3.1 Methodology for optimizing key SFC parameters

This work aims at developing an efficient method for the SFC separation of fast pyrolysis bio-oils. The lack of extensive knowledge on the components of such very complex mixtures prevented us from considering model compounds to develop the separation method as it was done in the past for closely related samples [6]. The development of the separation method was therefore carried out on the bio-oil. In this way, a strategy was implemented to optimize key parameters from a few preliminary experiments. Key parameters for any SFC separation, include the type of stationary phase, the type of co-solvent, the gradient conditions, the column geometry, the column temperature and the backpressure of the SFC system. The flow-rate was selected so that the maximum pressure and hence the minimum analysis time were attained. The choice of optimization criteria was based on sample peak capacity. This latter is known to be a powerful descriptor to assess the separation power of a given chromatographic system for a given sample under given gradient conditions and furthermore to compare the ability of different chromatographic systems to separate the components of this sample. The sample peak capacity, n_{grad}, in gradient elution was defined by Dolan et al. [11] as

$$n_{grad} = \frac{t_n - t_1}{w} \quad \textbf{(1)}$$

where t_n and t_1 are the retention times of the most and the least retained solutes under gradient conditions and w, the average 4σ peak width. Eq. (1) can be useful to compare different chromatographic systems provided that the number of compounds in the sample is limited. However, gradient separations of very complex samples such as bio-oils lead to multiple peak coelutions which make it impossible to determine the average peak width and hence the sample peak capacity from Eq. (1). Furthermore, an additional peak capacity, due to the isocratic initial step, has to be taken into account for the determination of the total sample peak capacity. Under isocratic conditions (corresponding to the initial composition), the sample peak capacity, n_{iso}, is given by Eq. (2) defined as [11]

$$n_{iso} = \frac{\sqrt{N}}{4} \times \ln \frac{1 + k_{n,iso}}{1 + k_{1,iso}} \quad \textbf{(2)}$$

where N is the column plate number, $k_{1,iso}$ and $k_{n,iso}$ are the retention factors of the least and most retained compounds under initial isocratic step. N is related to the column length, L_c and the particle diameter, d_p, by Eq. (3):

$$N = \frac{L_c}{h \times d_p} \quad \textbf{(3)}$$

where h is the reduced column plate height.

35 years ago, Snyder and co-workers developed the semi-empirical Linear Solvent Strength Theory (LSST) based on a linear relationship between the logarithm of the solute retention factor and the time:

$$\log(k) = \log(k_i) - b\frac{(t - t_{delay})}{t_0} \quad \textbf{(4)}$$

k_i is the retention factor in initial gradient conditions; t_0 is the column dead time; t_{delay} is the delay time composed of the dwell time, t_D, and of a possible programmed initial isocratic hold, t_{ini} ($t_{delay} = t_D + t_{ini}$); b is the gradient steepness given by

$$b = S \times s \quad \textbf{(5)}$$

s is the normalized gradient slope ($s = \frac{\Delta\varphi}{t_G} \times t_0$, $\Delta\varphi$ being the gradient range expressed in volume fraction of the strong solvent and t_G, the gradient time) and S is the slope of the relationship between the logarithm of the solute retention factor and the volume fraction of the strong solvent:

$$\log(k) = \log(k_0) - S\varphi \quad \textbf{(6)}$$

k_0 being the solute retention factor in the weak solvent and φ, the volume fraction of the strong solvent, B.

According to LSST and considering (i) an average S value and (ii) high k_i values for all solutes, the following equation can be derived from Eq. (1) [11]:

$$n_{grad} = 2.303S\left(\varphi_{n,grad} - \varphi_{1,grad}\right) \times \frac{1}{1+2.303b} \times \frac{\sqrt{N}}{4} \quad \textbf{(7)}$$

where $\varphi_{n,grad}$ and $\varphi_{1,grad}$ are the composition at elution of the most and least retained compounds under gradient conditions.

In case of very complex samples, it can be assumed that the least retained compound is eluted in the void volume ($k_{1,iso}$=0) while the last eluted peak in the initial isocratic step is eluted at the beginning of the gradient which means that $\varphi_{1,grad}$ corresponds to the initial composition φ_{ini} and that

$$k_{n,iso} = \frac{t_{delay}}{t_0} \quad \textbf{(8)}$$

From Eqs. (2), (3), (8) and (9), the resulting equation for the total sample peak capacity can be expressed as

$$n = \frac{1}{4}\sqrt{\frac{L_c}{hd_p}}\left(\ln\left(1+\frac{t_{delay}}{t_0}\right) + 2.303S\left(\varphi_{n,grad} - \varphi_{ini}\right) \times \frac{1}{1+2.303b}\right) \quad \textbf{(9)}$$

It was first necessary to verify that Eq. (7) was valid, namely that LSST could be applied to linear gradients in SFC as can be done in RPLC (reversed phase liquid chromatography). LSS parameters, k_0 and S (Eq. (6)) can be calculated from the retention data of two preliminary gradient runs [12,13] which allows then the accurate prediction of any gradient retention times provided that LSST is valid. Experimentally we performed, on the Acquity BEH-2EP column, two linear gradient runs, running from 1% to 40% ACN and using two different normalized gradient slopes, (0.01 and 0.03) so that k_0 and S could be calculatedby OSIRIS V4.1 (Datalys, Grenoble, France). This was done for 6 peaks selected in such a way that (i) they could cover a large retention range and (2) they could be easily tracked from the two preliminary experimental chromatograms. A third linear gradient with a different normalized gradient slope of 0.005 $\frac{\Delta\varphi}{t_G} \times t_0$ was performed experimentally. In order to demonstrate whether LSST can be applied to linear gradients in SFC, a simulated chromatogram was obtained from OSIRIS for the 6 peaks (Fig. 1a) and compared to the experimental chromatogram of the bio-oil. (Fig. 1b). As shown the simulated and experimental chromatograms are in very good agreement, thereby validating LSST for linear gradients in SFC. Resulting S and log(k_0) values are shown in supplementary material. It is interesting to notice that, unlike RPLC separations with methanol or THF as strong solvent [14], the dependence of log(k_0) vs S is decreasing, suggesting that a slightly convex gradient should be more suitable in SFC than a linear one. However, considering the low variation of S (values between 4 and 7 with an average value of about 5), linear gradients remains the most suitable for the analysis of bio-oils. Only linear gradients were therefore evaluated in the rest of this study. A similar trend (not shown) was observed with chromatographic systems which were studied in this work.

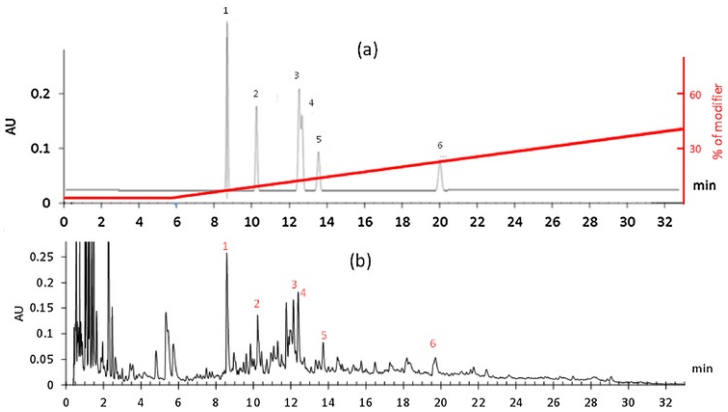

Fig 1 Comparison of (a) predicted and (b) experimental separation of a fast pyrolysis bio- oil with a normalized gradient slope of 0.005 (stationary phase: Acquity BEH 2-EP, modifier: ACN/H_2O (98/2), temperature: 30 °C, BPR pressure: 150 bar). Applied gradient in red.

alt-text: Fig 1

At first, some key parameters of Eq. (9) were fixed. Those include φ_i (5%), t_{delay}/t_0 (15) and the gradient steepness, b (s value of 0.01, see Eq. (5). These parameters were optimized in the second step once the chromatographic system (mobile and stationary phases) had been selected thanks to a screening of seven columns (see Table 1) and three co-solvents (acetonitrile, methanol and a mix of acetonitrile and methanol (50/50 v/v)). For sake of consistency, the

ratio L_c/d_p was approximately the same for all studied columns (see Table 1). Both column temperature (30 °C) and backpressure (150 bar) were fixed in the first step and further considered in the second step.

Since it was not possible to directly determine the sample peak capacity from chromatograms composed of more than one hundred peaks, the concept of peak capacity was evaluated through three criteria. The first one (Criterion A) represents the total number of observed peaks. The choice of this criterion is based on the assumption that peaks are randomly spaced on the chromatogram in case of very complex samples. In this case, the statistical theory of peak overlap, developed by Giddings and Davis [15] can be applied. According to this theoretical approach, for a given sample (i.e. a given number of compounds), the peak capacity is expected to be related to the number of observed peaks in the chromatogram. The second criterion (Criterion B) measures the size of the elution window. It corresponds to the composition at elution of the most retained compound, $\varphi_{n,grad}$, which is a key factor in Eq. (9). The elution windows of SFC-UV chromatograms (detection at 210 nm) were divided into two equal parts. The third criterion (Criterion C) is the number of observed peaks in the second part of the chromatogram. This criterion aims at limiting the occurrence of peak clusters, especially in the first part of the separation, and hence to promote a good ~~repartition~~distribution of the peaks. The count of peaks was carried out by means of a home-made software INDIGO based on a robust detection algorithm from mathematical morphology. This approach can detect all local maxima with a minimum intensity which was set at a level equal to 3 times the signal to noise ratio without calculation of derivatives. To use efficiently the three criteria we propose, each of them has been normalized by the maximum and the minimum values encountered for all the experiments. The relevance of the three chosen criteria is highlighted in Fig. 2, showing the dependence of Criterion A vs Criterion B (Fig. 2a) and of Criterion B vs Criterion C (Fig. 2b) for the different studied systems. It is clear that there is a poor correlation between the three criteria, thereby underlining their complementarity. Finally a response function, corresponding to the geometrical mean of the three criteria, was calculated to assess the performance of the different chromatographic systems. The different steps of our methodology are illustrated in Fig. 3.

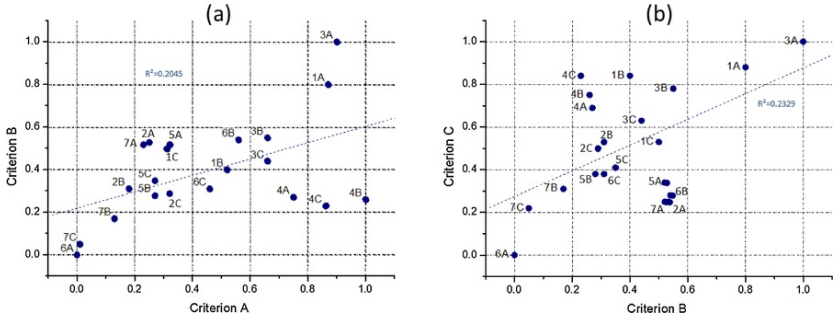

Fig. 2 Dependence of (a) criterion B vs criterion A and (b) criterion C vs criterion B for the studied chromatographic systems identified by combination of number (see Table 1) and letter (A: ACN, B: mix ACN/MeOH 50/50, C: MeOH).

alt-text: Fig. 2

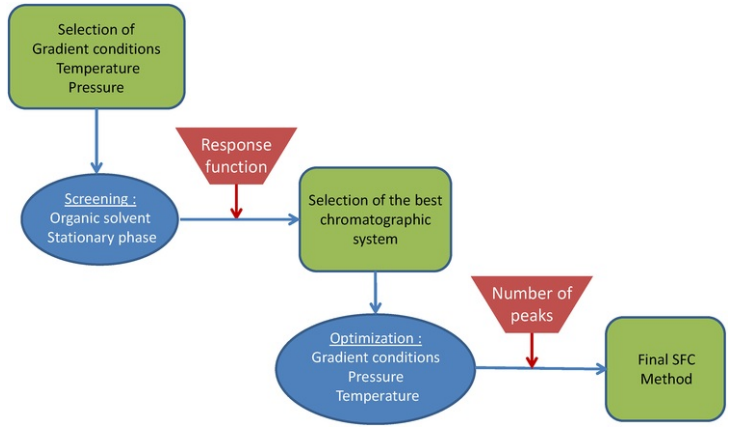

Fig. 3 Methodology for optimizing SFC key parameters.

alt-text: Fig. 3

3.2 Screening of mobile and stationary phases

The first step to determine the best conditions consisted in a screening of both stationary and mobile phases. Because SFC mobile phases are composed of a pressurized mixture of non-polar CO_2 and polar organic modifier, there is no restriction in the choice of stationary phases. According to West et al. [16,17], it is possible to cover almost all interaction possibilities from a set of five stationary phases involving dipole–dipole interactions, interactions due to n and π electrons, acid-base interactions and steric interactions [17]. In our work, we therefore investigated five silica-based stationary phases, including an unbounded one and four bonded ones (C_{18}, C_{18} with polar embedded groups, ethylpyridine and cyanopropyl). Typical SFC columns dimensions (3 mm x 100 mm) and typical size particles (1.7 or 1.8 µm) for UHPSFC were tested as listed in Table 1.Two additional stationary phases were chosen to complete this study: Luna CN (#4 in Table 1) and Nucleodur Polartech (#6 in Table 1). According to the classification of West et al. [9] their characteristics are close to BEH-2EP (#3 in Table 1) and BEH RP18 (#5 in Table 1) respectively. This was done in order to highlight any difference of performance within a given group of stationary phases. At the same time, several mobile phases were investigated. SFC mobile phases being mixtures of CO_2 and an organic modifier, their polarity and hence their eluent strength are dependent on the nature of the modifier [18]. As a result, different strong solvents (B) were investigated in this study, including methanol (MeOH), mostly used in SFC due to its high polarity, acetonitrile (ACN) and a mix (50/50 v/v) of both. Fig. 4 shows the response function value for each chromatographic system. As can be seen, the best stationary phases are either unbonded silica (#1 in Table 1) or polar bonded silica (Ethyl pyridine and Cyanopropyl, #3 and #4 respectively). Those promote dipole–dipole and H donor-acceptor interactions according to LSER (Linear Solvation Energy Relationship) classification [19]. Bio-oils are composed of a large number of polar compounds (alcohols, phenolics, organic acids, furans, ketones) [20]. The presence of such oxygen functional groups seems to be in accordance with observed interactions.

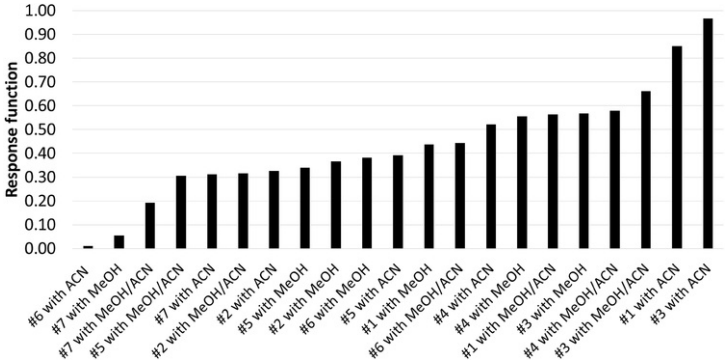

Fig. 4 Response function values for the different studied chromatographic systems. The studied columns are numbered as in Table 1.

alt-text: Fig. 4

The BEH 2EP column (#3) appears to be the most attractive. However it is interesting to note that MeOH as solvent B leads to a lower value for the response function compared to MeOH/ACN (50/50 v/v) or, even better, to pure ACN. This can be easily explained by a larger elution window (criterion B) in case of ACN due to its lower eluent strength as also shown in Fig. 5. Although ACN allows to enlarge the elution window resulting in an increase in peak capacity, this solvent is not prone to hydrogen-bonding interaction, yet of great interest with oxygenated compounds. Furthermore, this interaction also exists between the compounds and the silanols of the stationary phase and may have a negative effect on peak shapes. To address these issues, the use of 2% water in ACN was also investigated. The molecules of water can strongly interact with the silanols thereby leading to a competitive adsorption on these sites. As shown in Fig. 5, the effect of water is not significant as regards Criteria B and C while Criterion A (number of peak detected) is slightly increased. Because criterion C remains the same with and without water, we can suppose that addition of water affects more significantly compounds that are poorly retained. As a conclusion, addition of water is beneficial since it increases the peak capacity in the isocratic part of the chromatogram by reducing peak broadening.

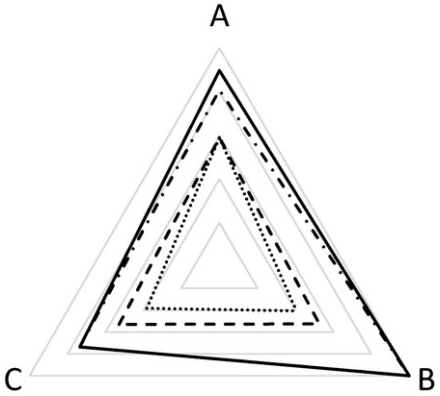

Fig. 5 Evolution of Criteria A, B and C using the Acquity BEH-2EP column (#3 in Table 1) depending on the strong solvent B: Acetonitrile (▬ · ▬), Methanol (·····), Acetonitrile / Methanol (50/50; v/v) (▬ ▬ ·), Acetonitrile/Water (98/2; v/v) (▬▬).

alt-text: Fig. 5

Similarly, others additives were tested (i.e. trifluoroacetic acid, ammonium acetate, diethanolamine and formic acid) but none of them had a significant impact on the number of peaks detected.

Finally, the best chromatographic system was composed of an Acquity UPC^2 BEH 2EP column (3 × 100 mm, 1.7 µm) and a mix of acetonitrile and water (98/2 v/v) as strong solvent B. This combination was selected to optimize the other key parameters.

3.3 Optimization of mobile phase composition

The variation of mobile phase composition is splitted into two parts: isocratic and gradient parts. For the isocratic part, the initial composition (φ_{ini}) was set at 5% in the preceding screening, according to the usual value reported in the literature for SFC separations. However a lower initial composition was expected to provide a higher peak capacity according to Eq. (9) ($\varphi_{n,grad}$-φ_{ini} larger). Consequently, φ_{ini} was varied from 5% to 0% while maintaining the same ratio t_{delay}/t_0 and changing the gradient time so that the normalized gradient slope was kept constant. Criteria B and C were expected to remain unchanged regardless of the initial composition. We therefore focused on the number of peaks detected (Criterion A). As shown in Fig. 6a, the initial composition had little impact on the number of peaks (less than 10% variation) during the isocratic part, suggesting that the peak capacity was maintained constant as could be expected from Eq. (9) provided that the reduced plate height, h, was not affected. Conversely, there is a significant increase (up to 30%) in the number of peaks detected during the gradient part when the initial composition is decreased. That is related to the increase in the composition range. The best initial composition should be 0% (106 peaks) or 1% (104 peaks). However, with an initial composition of 0%, a small pressure bump occurred at the gradient start resulting in baseline disruption. We therefore preferred to select an initial composition of 1% for the rest of this study. Retention time reproducibility might be an issue with such a low solvent content due both to the need for longer equilibration times and to the fact that Pump B must be able to deliver low flow-rates in a reliable way. To assess retention time reproducibility in these conditions, three different runs were performed under the same conditions over three different days. The relative standard deviation (RSD) was calculated from the three retention times for five reference peaks which were well distributed along the entire chromatogram and could be easily recognized from run to run (Table 2). As shown in Table 2, RSD was always below 0.6% thereby demonstrating that 1% was suitable as initial composition for gradient runs with the selected chromatographic system.

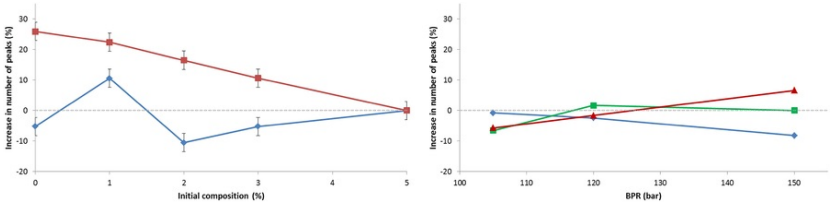

Fig. 6 Increase in the number of peaks detected at 210 nm as a function of (a) the initial composition (reference 5%) for isocratic part (♦) and gradient part (■) and (b) BPR pressure for 3 column temperatures: 20 °C (♦), 30 °C (■) and 60 °C (▲) (Reference 30 °C and 150 bar). Other conditions: Acquity BEH 2-EP (3 × 100 mm; 1.7 µm); A: CO_2; B: ACN/H_2O (98/2 v/v); 1.4 mL/min.

alt-text: Fig. 6

Table 2 RSD of retention times for reference peaks depending on the initial composition of the gradient elution (30 °C and 150 bar).

alt-text: Table 2

Initial composition (%)	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5	
	RSD (%)	Tr (min)	RSD (%)	Tr (min)	RSD (%)	Tr (min)	RSD (%)	Tr (min)	RSD (%)	Tr (min)
5	0.27	0.70	0.16	0.92	0.48	3.08	0.41	5.61	0.53	9.47
1	0.29	1.08	0.52	2.57	0.19	5.52	0.27	7.89	0.15	12.48

As regards the gradient part, as discussed in the first section (see Section 3.1), a linear gradient was expected to be suitable for the separation of bio-oils (small variation of S with log (k_0)). With a view to optimize the normalized gradient slope, we compared the number of peaks and the peak intensity of a reference peak both as a function of the gradient time. As shown in Fig. 7, the number of peaks increases with the gradient time. However this is correlated to a decrease in sensitivity. A good trade-off between these two opposite effects consisted in selecting a gradient time in the range 10–20 min so that the slope of the curve giving the variation of the number of peaks with the gradient time was not too low. Accordingly, a normalized gradient slope of 0.01 (i.e. a gradient time of 14 min) was selected.

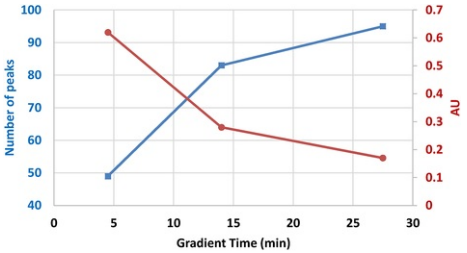

Fig. 7 Number of peaks (blue) and peak intensity of a reference peak (red) observed at 210 nm as a function of the gradient time. Conditions: Acquity BEH 2-EP (3 × 100 mm; 1.7 µm), A: CO_2; B: ACN/H_2O (98/2 v/v); from 99:1 (A:B) to 60:40 (A:B); 30 °C; BPR pressure: 150 bar; 1.4 mL/min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

alt-text: Fig. 7

3.4 Optimization of the column temperature and BPR pressure

Both pressure and temperature have a potential effect on mobile phase density and hence on solute retention. For this reason, we optimized BPR pressure (in the range 100–150 bar) and the column temperature (in the range 30 °C to 60 °C) simultaneously. Usual conditions (30 °C and 150 bar) were defined as reference in Fig. 6b, showing the increase in the number of peaks detected as a function of BPR pressure for 3 column temperatures.

Results reported in Fig. 6b show that, within the studied range, the effect of temperature and BPR pressure on the number of peaks detected is not as significant as the effect of the initial composition (Fig. 6a). The increase in the number of peaks does not exceed 10% except for 150 bar at 60 °C. However 60 °C is the maximum recommended temperature for this stationary phase. In order to prevent any thermal degradation, this configuration was not selected. Considering the small increase in the number of peaks under alternative conditions, we preferred maintain the original conditions (*i.e.* 150 bar and 30 °C). The small variation of peak capacity with temperature and pressure can be explained by the complexity of the sample. Several studies [21–23] reported the effect of temperature and/or pressure on retention, but the related samples contained a few components only, which did not give rise to many coelutions. In case of complex mixtures, Davis and Giddings [24] demonstrated that only 18% of the theoretical peak capacity could correspond to a single-component peak. In our case, it is obvious that even under the best conditions, numerous co-elutions occur. Although the impact of pressure and temperature is different depending on the component, the number of peaks detected should remain nearly the same. To conclude, usual SFC conditions (*i.e.* 30 °C and 150 bar) seem to be suitable for the analysis of the fast pyrolysis bio-oil.

3.5 Final SFC-UV method

By following the different steps presented in Fig. 3, we selected the conditions allowing to detect a large number of peaks (*i.e.* 122) and hence to provide a large sample peak capacity for a bio-oil within a reasonable analysis time of 22 min. The relevance of this approach can be assessed by comparing in Fig. 8 two separations of bio-oil. The first one (Fig. 8a) was not optimized. In particular, both stationary and mobile phases were not suitable for bio-oil compounds. In these inappropriate conditions, only 15 peaks were detected. The second separation (Fig. 8b) was obtained under the above optimized conditions. Using our methodology, we determined that the best set of conditions

involves polar stationary phases (BEH-2EP) associated to a mix of acetonitrile and water as polarity modifier. With a further adjustment of the conditions, namely a low initial composition (1%), a normalized gradient slope of 1%, a temperature of 30 °C and BPR pressure of 150 bar, the number of peaks detected in UV (210 nm) was increased to about 120.

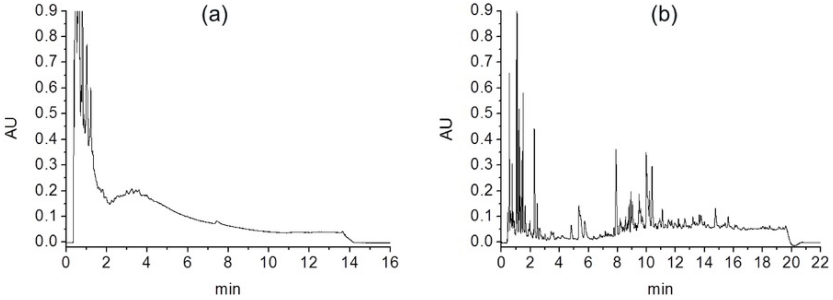

Fig. 8 SFC-UV chromatogram of the fast pyrolysis oil using (a) bad conditions (stationary phase: Acquity Fluorophenyl, modifier: MeOH, temperature: 30 °C, BPR pressure: 150 bar) and (b) optimized conditions (stationary phase: Acquity BEH 2-EP, modifier: ACN/H_2O (98/2), temperature: 30 °C, BPR pressure: 150 bar).

alt-text: Fig. 8

3.6 Hyphenation with MS detection

To enhance information about fast pyrolysis bio-oil composition, hyphenation of our SFC-UV device with mass spectrometry (MS) was carried out. As the mobile phase coming out from SFC column was pressurized (i.e. 150 bar), a decompression could occur at the MS-source inlet, thereby preventing sufficient vacuum to be ensured. That means that only MS sources working at atmospheric pressure can be used. In this work, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources were chosen to cover a large range of polarity and molecular weight.

To take into account the specificity of a CO_2-based mobile phase, MS parameters had to be optimized. The source temperature needed to be higher than that in usual conditions to prevent from any freezing during the decompression of CO_2 and to limit the formation of clusters due to the cold spray. The decompression of CO_2 requires the use of an additional solvent (make-up flow) before the entry in the ionization source. Without that, solutes may precipitate in the transfer capillary. The addition of methanol (protic solvent) as make-up solvent, ensured a suitable inlet flow and enhanced the ionization process by charge exchange with analytes. CO_2 decompression has also an impact on the nebulizing flow. This latter had to be as low as possible to avoid a too wide spray and hence a loss of information. The Fig. 9 illustrates the interface used in both ESI and APCI modes.

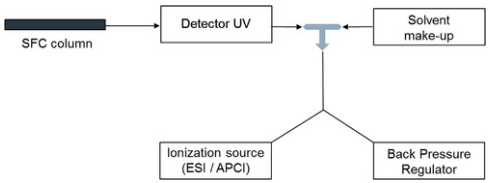

Fig. 9 Schema of interface between SFC-UV and Ionization source of MS.

alt-text: Fig. 9

In optimized conditions, positive-ion and negative-ion ESI and APCI chromatograms of the bio-oil are presented in Fig. 10. The different ionization modes enabled a successful ionization and the detection of compounds contained in the bio-oil. It is interesting to notice that the four obtained chromatograms are quite different. As expected, our results demonstrate that these different modes of detection are complementary and thus can provide an extensive information about the chemical composition of bio-oils. 36 peaks, 46 peaks, 61 peaks and 59 peaks were detected in positive-ion and negative-ion ESI and positive-ion and negative-ion APCI chromatograms respectively. The number of peaks detected is lower than with UV detection (i.e. 122) which is consistent with the fact that (i) MS detection is more specific and (ii) additional peak tailing due to dispersion phenomena in MS reduces peak separation and sensitivity (iii) the 210 nm selected as detection wavelength in the UV allows a much 'broader' detection of compounds.

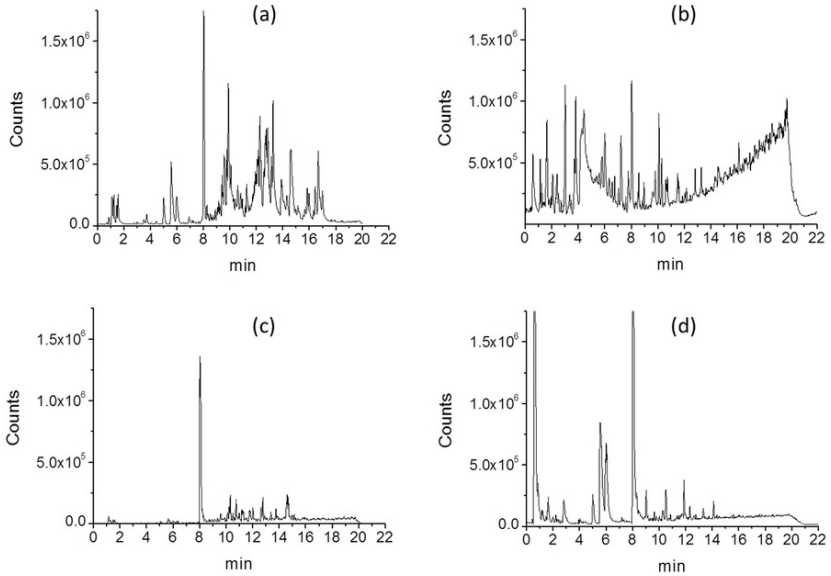

Fig. 10 SFC/MS chromatograms of a fast pyrolysis oil with different ionization sources: (a) ESI negative, (b) ESI positive, (c) APCI negative, (d) APCI positive. SFC methods: Stationary phase: Acquity BEH 2-EP, Modifier: ACN/H_2O (98/2), T°: 30 °C, BPR pressure 150 bar.

alt-text: Fig. 10

The use of positive-ion modes (APCI and ESI) seems to be relevant to enhance information on less retained compounds. ESI negative mode seems to be the best one to analyze the last part of the chromatogram. It can also be expected that negative-ion mode will be more appropriate for acids, phenolic compounds, whereas positive-ion mode will be preferred for carbohydrates, ketones and furans. A suitable combination of these different detection modes will be required to get an exhaustive information about a large number of~~on all~~ compounds present in bio-oils.

4 Conclusion

Despite SFC analysis has gained increasing interest for several years now, a better knowledge is required on how key experimental parameters such as stationary phase, temperature, pressure, mobile phase composition affect the retention of oxygenated polyfunctional compounds present in complex products. In this work, an approach for the selection of suitable SFC conditions for separating a biomass fast pyrolysis oil is proposed based on three quantitative, objective and easy-to-use criteria. The novelty of this methodology stems from the fact that there was no need for model compounds since method development was directly performed with the bio-oil. Stationary and mobile phases, initial mobile phase composition, and gradient slope were optimized in order to achieve a maximum separation power.

We found that a polar stationary phase (i.e. ethylpyridine bonded silica) associated with a mix of 98% acetonitrile and 2% water, as strong solvent led to the best chromatographic system. Under optimized conditions, the number of peaks detected by UV spectroscopy could reach more than 120 in 22 min. Some first preliminary results using a simple quadripole mass spectrometer were shown to illustrate the potential interest of such a hyphenated system. To go further on, we analyzed biomass fast pyrolysis oils using a high resolution mass spectrometer (SFC-IT-Tof/MS). Results should be soon submitted in a dedicated paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.06.003.

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Appendix A. Supplementary data

The following are Supplementary data to this article:

Multimedia Component 1

Highlights

- A novel SFC-UV/MS method for the analysis of a biomass fast pyrolysis oil.
- Optimization based on a complex industrial sample using three specific criteria.
- Separation of more than 120 peaks by SFC-UV.
- Complementary information obtained with APCI+/− and ESI+/− modes.

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