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RESEARCH PAPER

Development of an ultra-high-performance supercritical fluid chromatography method for the analysis of phenols in the pyrolysis aqueous fraction

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Abstract

The pyrolysis process consists of the thermal decomposition of biomass in an inert atmosphere, which produces a liquid (bio-oil) composed of a complex mixture of organic compounds, including an oil and water phase. The aqueous fraction can reach up to 45% w/w, and understanding its composition is of utmost importance in determining its intended destination, whether for the reuse of compounds in industrial applications or for treating the effluent for disposal. In this study, a fast, direct, and efficient method using ultra-high-performance supercritical fluid chromatography (UHPSFC) was developed and optimized for monitoring phenols in aqueous samples obtained from the pyrolysis processing of six different biomass sources. The following parameters were evaluated for method optimization: stationary phase type, mobile phase flow, organic modifier, sample diluent, temperature, pressure, and modifier gradient time. With a total analysis time of 26 min, out of the fourteen (14) investigated phenolic compounds, eleven (11) were successfully separated after method optimization, and among them, five (5) were quantified in all six aqueous fractions. The aqueous fractions of residue from cowpea pod (1.89 $mg.mL^{-1}$), sugar apple (3.09 $mg.mL^{-1}$), and acerola (4.79 $mg.mL^{-1}$) presented lower concentrations compared to grape (8.16 $mg.mL^{-1}$), pine nuts (6.68 $mg.mL^{-1}$), and guava (6.05 $mg.mL^{-1}$) fractions. However, even at lower concentrations, all biomasses showed promising results regarding the phenolic compound content, analytes that have high added value for the chemical industry.

Keywords Biomass · Pyrolysis · Aqueous fraction · UHPSFC-DAD · Phenols

Introduction

When compared to fossil fuels for obtaining fuels and chemical products, as a clean and sustainable raw material, biomass is a promising alternative [1]. Biomass can be obtained from plant residues or materials derived from plants, animal manure, and urban solids. It is a renewable resource that can be used in the production of chemicals or carbon–neutral and low-emission fuels [2, 3].

Among the different biomass conversion processes, gasification, combustion, and pyrolysis are among the most used. Pyrolysis consists of the thermal degradation of biomass at high temperatures in an inert atmosphere. As a result of this process, three products are obtained: a gas (biogas), a solid (biochar), and a liquid (bio-oil). The bio-oil consists of two phases: an oil and an aqueous phase [4, 5].

The aqueous fraction is formed from the moisture present in the biomass, and lignin dehydration reactions that occur during the pyrolysis process. Its composition may vary between 15 and 45% by mass of the total liquid generated during the process [6]. Nevertheless, most of the studies involving pyrolysis products in the literature are related to the oil fraction [6–9].

Both phases are complex mixtures of organic compounds consisting of carboxylic acids, alcohols, aldehydes, furans, hydrocarbons, and phenols, among others [10]. Due to its complex and highly toxic chemical composition (resulting from the presence of oxygenated compounds) [11], the aqueous fraction can be used as a source of inputs for various processes. Its components can be used in bio-refineries, applied in processes such as catalytic conversion of furans

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and phenols to hydrocarbons [12]; fermentation of levoglucosan to produce alcohols and diols [13]; production of hydrogen from organic acids through microbial electrolysis [14]; hydrocarbon production from catalytic hydrogenation of oxygenated compounds [15]; and biomethane production through anaerobic digestion [16].

Among the components present in the aqueous fraction, we can highlight the phenols. These are compounds that have broad applications in the industry, from the production of resins and adhesives [17] to their use in the food and pharmaceutical sectors [18]. Currently, the production of these compounds depends on fossil fuels; however, the aqueous fraction pyrolysis emerges as an alternative that can be investigated for obtaining these compounds.

So, knowing the chemical aqueous fraction pyrolysis composition is necessary to determine its potential applications, whether in energy production, fuels, or the chemical products industry [19]. The characterization of liquid product from pyrolysis (both phases) is commonly carried out through chromatographic techniques. Gas chromatography coupled to mass spectrometry (GC–MS) is the most widely used technique, and several studies have employed it to quantify phenols in these matrices [20–22]. However, when analyzing aqueous samples by gas chromatography, a sample preparation step involving organic solvent extraction and derivatization reactions is necessary. This step can affect potentially the qualitative and quantitative results [23]. As an alternative, liquid chromatography (LC) is a technique commonly employed, and its advantage lies in the possibility of direct insertion of this type of sample. Studies in the literature have used different LC modes, among them high-performance liquid chromatography (HPLC) [24], comprehensive two-dimensional liquid chromatography (LC × LC) [25], and online reversed-phase liquid chromatography × supercritical fluid chromatography (LCxSFC) [26].

Supercritical fluid chromatography (SFC) emerges as a separation technique that offers advantages over other well-established such as LC and GC. The mobile phase, composed of a supercritical fluid, has lower viscosity and higher diffusivity than that of LC, improving chromatographic kinetic performance. Additionally, there is a reduction in the consumption of organic solvents. SFC can analyze thermally labile compounds and high-molecular-weight compounds that are not compatible with GC [27]. When SFC is operated using stationary phases with sub-2-µm particles, the system is referred to as ultra-high-performance supercritical fluid chromatography (UHPSFC), which is used for very fast, even ultra-fast separations [28].

UHPSFC was considered a promising analytical tool for the analysis of complex samples. Studies have been reported in the literature using the technique for the organic compound characterization in oil phase samples from pyrolysis, but it has not been observed for aqueous samples nor has its quantification [29–31]. Thus, the present study aimed to develop and optimize a method using ultra-high-performance supercritical fluid chromatography with diode array detection (UHPSFC-DAD) to monitor and quantify phenols present in aqueous pyrolysis fractions.

Material and methods

Reagents and standards

The experiments were carried out using the solvents: dichloromethane (Dinâmica Química Contemporânea Ltda., Brazil), ethyl ethanoate (Vetec, Brazil), and methanol (MeOH) purchased from JT Baker (Xalostoc, Mexico) and isopropanol (i-PrOH) and HPLC grade acetonitrile (ACN) obtained from Panreac (Darmstadt, Germany). In this work, 14 phenols were analyzed by UHPSFC-DAD. The standards used for quantification were phenol, 2-methylphenol, 3-methylphenol, 4-methylphenol, 4-ethylphenol, 3,5-dimethylphenol, 2-methoxyphenol, 2,5-dimethylphenol, 4-methoxyphenol, benzene-1,2-diol, benzene-1,3-diol, 4-methylbenzene-1,2-diol, 2,6-dimethoxyphenol, and 2-methoxy-4-prop-2-enylphenol (Sigma-Aldrich, Brazil). In GC–MS analysis, pyridine (Pro Analisi) and the derivatizer trimethylsilyl 2,2,2-trifluoro-N-trimethylsilylethanimidate (BSTFA) (Sigma-Aldrich, Brazil) were used for derivatization.

Biomass: collection and preparation

The biomass sources used for pyrolysis in this work were dried at 60ºC for different time periods (from 5 to 24 h), to obtain a moisture content below 10%. Guava (*Psidium guajava* L.) and acerola fruit (*Malpighia emarginata*) seeds were collected from the fruit pulp processing industry (POMAR), while sugar apple (*Annona squamosa*) and cowpea pod (*Vigna unguiculata*) seeds were bought at the local fair, all in the city of Aracaju-Sergipe, Brazil. The pine nuts (*Araucaria angustifolia*) were purchased in the public market of Maringá-Paraná, Brazil; and the grape seeds (*Vitis vinifera* L.) were obtained from the Rio Sol winery, Lagoa Grande-Pernambuco.

Pyrolysis

To obtain the aqueous fraction, the pyrolysis experiments were performed in a laboratory-scale plant: a stainless steel fixed bed reactor (260 mm in length by 60 mm in diameter) at normal atmospheric pressure. The condensable vapors were collected in Teflon tubes and cooled in a thermostatic bath (MQBTC99-20, Microquímica Equipamentos LTDA brand) at a temperature of 10ºC. The pyrolysis time was 60 min (constant a heating rate of 30 °C•min^{-1}) and an N_2 flow of 5 mL•min^{-1}. The aqueous fraction was collected in the pre-cooling zone in the pyrolysis reactor spiral [32].

GC–MS instrumentation and chromatographic conditions

A gas chromatography system with a mass spectrometer (GC–MS), model QP2010 Plus, Shimadzu brand, was used. Helium (purity degree 99.995%) was the carrier gas at a flow of 1 mL•min^{-1}, and an injection volume 1 µL, and a split 1:20, on an SPB-5 column (60 m × 0.25 mm × 0.25 µm). The mass spectrometer operated with electronic ionization (70 eV) in scanning (SCAN) mode. The temperature program was set at 80 °C (5 min), 2 °C'min^{-1} to 105 °C (5 min), 2 °C'min^{-1} to 150ºC (2 min), and 10 °C'min^{-1} to 280 °C (10 min), with a 70-min analysis time. The identification of compounds was performed by comparing the spectra obtained with spectra presented in the (NIST and WILEY) libraries of the equipment's software, where only the peaks presenting an area above 0.10 and similarity greater than 80% were considered.

For the analysis, the organic compounds were extracted: the aqueous fractions were diluted in ultra-pure water (1:5) and went through a liquid–liquid extraction process with a vortex, using ethyl acetate as the extracting solvent. The extract went through a sodium sulfate column to remove water residues, and 200 µL were later derivatized with BSTFA according to the methodology of Santos et al. [32], being completed to 1 mL with dichloromethane.

UHPSFC-DAD instrumentation and chromatographic conditions

Analysis of the aqueous fractions using UHPSFC-DAD was performed at the Pharmaceutical Research and Chemometry (LabFarQui) Laboratory — Unicamp, using UltraPerformance Convergence Chromatography (UPC^2) — Waters Corporation (Milford, USA). The system consists of a binary pump, one dedicated exclusively to delivering liquid CO_2, while the other delivered an organic modifier. The injection was performed in automatic mode with a partial loop using MeOH and MeOH-i-PrOH (1:1) as respective strong and weak solvent washes. The system also contained a thermostated column oven, a convergence module with an automated back pressure regulator (ABPR), and a diode array detector (DAD) module.

The initial chromatographic conditions were a flow rate of 0.80 mL min^{-1}, temperature of 40ºC, and pressure at 10.3 MPa. The injected standard solution volume was 1.0 µL, with a linear modifier gradient ranging from 0 to 20% modifier over 10 min, maintaining the CO_2:MeOH ratio 80:20 for another 1 min, and returning to the initial percentage of CO_2 in 2 min, and the another 5 min (in this condition) to reach equilibrium before the next injection, totaling 18 min of analysis.

Method optimization

For method optimization, the following parameters were evaluated: stationary phase type, mobile phase flow, organic modifier, sample diluent, temperature, pressure, and modifier gradient time. These last three were evaluated according to a full factorial design (FFD) 2^3 (Table 1), using Fusion QbD software (S-Matrix, USA). All analysis and FFD were executed by Empower 2 Software (Waters Laboratory Informatics).

For the selection of the stationary phase, six columns from the Acquity UPC^2 line obtained from Waters Co. (Milford, USA) were investigated. Some characteristics are described in Table 2. All columns are composed of fully porous particles. More information can be found on the Waters website [33].

Quantification

For UHPSFC-DAD analyses, stock solutions of the 14 phenol standards were prepared using acetonitrile as the dilution solvent. The compound mixtures with 100 µg. mL^{-1} were initially prepared to optimize analysis parameters. The selectivity, range, linearity, precision, accuracy, and detection (LOD) and quantification (LOQ) limits were evaluated in this study. The accuracy was calculated as the proximity of the concentration obtained to the true value (by spiking with mixed standard solution) at two concentration levels ($n = 3$). On the other hand, precision was determined as the relative standard deviation among the repeated measurements ($n = 3$) at each concentration level. The protocol adhered to the guidelines set forth by ANVISA [34]. For analyses and quantification, the aqueous fraction samples were diluted in ultra-pure water at a ratio of 1:20, with the exception of the grape seed aqueous fraction (1:40).

Results and discussion

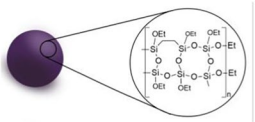
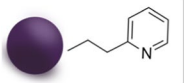
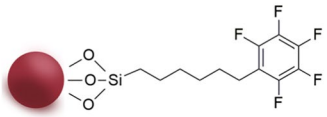
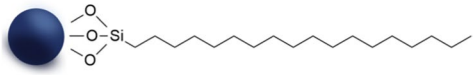
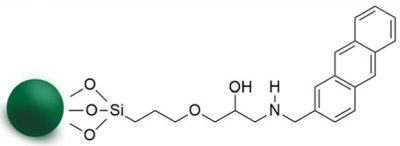
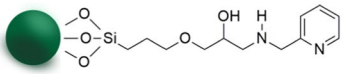
Characterization of aqueous fractions using GC–MS

The characterization of the aqueous fraction samples was carried out using analysis by GC–MS. The number of

Table 1 Factorial planning parameters 2^3

Parameters	Factorial points		Central point
Temperature (ºC)	+1 (50)	−1 (30)	0 (40)
Pressure (MPa)	+1 (15.5)	−1 (12.1)	0 (13.8)
Time gradient (min)	+1(18)	−1 (7.2)	0 (12.6)

Table 2 Column characteristics

Column	Stationary phase	Dimensions (mm)	Particle size (µm)	Pore size (Å)	Surface area (m^2/g)
Bridged-ethylene hybrid (BEH)		2.1 × 100.0	1.7	130	185
Bridged-ethylene hybrid 2-ethylpyridine (BEH 2-EP)		2.1 × 50.0	1.7	130	185
Charged-surface hybrid fluoro-pheny (CSH FPh)		2.1 × 50.0	1.7	130	185
High-strength silica C18 (HSS C18 SB)		2.1 × 50.0	1.8	100	230
Torus 1-amino anthracene (1-AA)		2.1 × 50.0	1.7	130	185
Torus 2-picolylamine (2-PIC)		2.1 × 50.0	1.7	130	185

peaks identified in the total ion current chromatography (TICC) was between 15 and 30% of the chromatographic peaks present, chromatograms in supplementary material (Fig. S1). The other peaks were not identified because of their low intensity or less than 80% similarity with peaks found in the mass spectrum library. In general, aqueous fractions are a mixture of compounds such as ketones, short-chain acids, phenols, nitrogenated, alcohols, and mixed-function compounds.

The six pyrolysis aqueous fraction samples studied presented phenols as the major compound class, a result of the presence of lignin in the biomass structure and of the interaction of lignin and cellulose [1]. The cowpea pod aqueous fraction presented the lowest percentage of phenol in its composition, at about 22%, while sugar apple, acerola fruit, guava, grape, and pine nuts waste presented between 35 and 50% (as show in Table 3).

The chief phenolic compound found in each aqueous fraction also varied. For cowpea pod, phenol presented the highest intensity, while for sugar apple, acerola fruit, guava, grape, and pine nut waste, benzene-1,2-diol was the main compound. Compounds belonging to the alcohols, acids, and ketones and mixed chain compounds, sugars, furans, and esters were found to a lesser degree.

It is important to make it clear that analysis by GC–MS was used in this study with the aim of characterizing the samples, particularly in terms of the content of phenolic compounds.

Optimization of the UHPSFC-DAD method

Optimization of the chromatographic parameters is essential in the development of analysis methods. For this purpose, a mixture of 14 phenol standards was used. The present work evaluated the factors that most influence retention time, analyte separation, and elsewhere, the stationary phase, organic modifier, pressure conditions, temperature, gradient time, and sample solvent.

Selection of stationary phase

The class of phenols is characterized by an aromatic ring linked to a hydroxyl group (OH), which gives acidic character to the compounds. Among the analytes evaluated in this work, there are those which in addition to phenol presented alkyl and methoxy substituents, and some presented two OH groups such as benzene-1,2-diol and derivatives. These are compounds that may interact with the stationary phases through hydrogen bonds and dipole–dipole interactions.

Interactions with the stationary phase can be evaluated by sorbent properties using Abraham's general solvation method. The equation used to describe the model (Eq. 1) is based on the theory that the total free energy related to the partition of a neutral solute between two phases can be

Table 3 Compound distribution by chemical class in the aqueous fractions of different biomasses

	Cowpea pod		Sugar apple		Acerola fruit		Guava		Grape		Pine nut	
	Area	Area (%)	Area	Area (%)	Area	Area (%)	Area	Area (%)	Area	Area (%)	Area	Area (%)
Phenols	13663422	22.61	14338796	35.33	20021581	42.09	40254705	51.76	29881684	56.40	20733954	43.71
Others*	13646979	22.58	7338385	18.08	8552574	17.98	16595180	21.34	6022851	11.37	8435146	17.78
Aldehyde	12407058	20.53	8980675	22.13	10233976	21.51	9295091	11.95	9805779	18.51	9149009	19.29
Alcohols	11647234	19.27	4473852	11.02	5492950	11.55	1862356	2.39	4656682	8.79	5958393	12.56
Acids	4804732	7.95	2535205	6.25	1799984	3.78	7523549	9.67	1472231	2.78	2136126	4.50
Ketones	4270575	7.07	2918528	7.19	1472583	3.10	2243736	2.88	1138792	2.15	1023981	2.16
Total	60440000	100.00	40585441	100	47573648	100	77774617	100	52978019	100	47436609	100

*Other classes of compounds that are present in smaller quantities, including hydrocarbons, mixed chain compounds, halides, and furans, among others

represented by the sum of the free energy contributions of the various molecular interactions [35, 36]:

$$\log k = c + eE + sS + aA + bB + vV \quad (1)$$

The solute descriptors are represented by uppercase letters (Eq. 1), where E is the molar excess refraction, providing a measure of the polarizable electrons in a molecule; S is the dipolarity/polarizability of the solute; A and B are acidity and basicity, respectively, of the solute's hydrogen bond; and V stands for the McGowan volume [36, 37].

The different intermolecular interactions during the solvation process of the solute by the stationary phase can be described by the coefficients of Eq. (1), where e refers to interactions involving non-bonding electrons and/or π electrons; s refers to interactions between dipoles; v refers to dispersive forces; and a and b refer hydrogen interactions, respectively, acidic and basic character of the stationary phase with respect to the formation of hydrogen interactions.

All these coefficients can be found using standard procedures for multiple linear regression analysis. The log k is the logarithm of the retention factor, and c is the intercept term of the model [35, 36]. The system constants enable the comparison of separation characteristics among different stationary phases, thereby aiding in identifying stationary phases with similar or distinct selectivity [38].

For the present study, six columns with different characteristics were investigated. The analysis conditions are described in the "Method optimization" section. Based on the study of West et al. [38], HSS C18 and CSH FPh columns show lower a values compared to the others. In this work, together with BEH, they demonstrated the poorest performance in the separation of phenolic compounds (Fig. 1). BEH, BEH 2-EP, Torus 1-AA, and Torus 2-PIC are classified as polar phase columns, displaying positive e, s, a, and b values, tending to interact better with these analytes. However, bare silica columns, such as BEH, have reduced a values and tend to better interact with bases.

BEH 2-EP, Torus 1-AA, and Torus 2-PIC demonstrated similar selectivity and better separation of phenolic compounds than the others; these columns presented numerically high a values, especially Torus 2-PIC, indicating a strong interaction of the analyte with these stationary phases [38]. Moreover, Torus 1-AA and Torus 2-PIC possess hydroxyl and amine groups in their stationary phase composition that favor interactions with analytes having a donor/acceptor character in hydrogen bonding; these were the columns with the best results. As the value of the a coefficient in Torus 2-PIC is numerically higher than in all the other columns, it presented better performance and managed to separate 12 out of the 14 compounds. Despite showing more asymmetric peaks, this can be resolved in the subsequent optimization steps. The coefficients of the

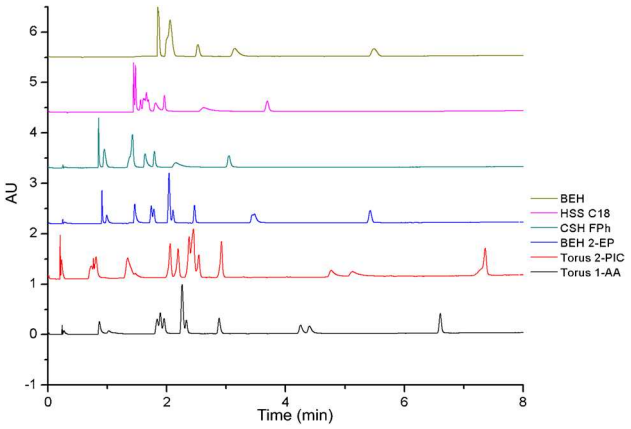


Fig. 1 Representative chromatograms for separation of the phenolic standards into different stationary phases. Chromatographic conditions: pressure of 10.3 MPa, temperature of 40 °C, flow rate at 0.8 mL min^{-1}, linear gradient elution from 0 to 20% of modifier in 10 min, injection volume of 1.0 µL, and detection wavelength set at 220 nm

Abraham descriptors can be found in the supplementary material (Table S1).

Flow (F)

Flow studies can reduce analysis time, but care is needed to avoid efficiency loss when working with extreme values. Furthermore, increasing the flow rate raises the inlet pressure, resulting in lower retentions and potential overlaps. In the present study, five different flow rates were evaluated: 0.60, 0.75, 0.80, 0.90, and 1.00 mL min^{-1}, using the 2-PIC column (chromatograms in the supplementary material, Fig. S2). Lower flows widened the base of the peaks (longitudinal diffusion), while higher flows can cause of the analytes of interest with samples interferents. For our analyses, a flow rate of 0.80 mL/min was chosen.

Mobile phase modifier

In SFC, using an organic modifier in the mobile phase brings improvement in peak shape, and analyte solubility, while avoiding sample precipitation in the column and reducing analyte interactions with residual silanol groups [39]. MeOH is a commonly used mobile phase modifier in analysis by SFC, and one of its advantages is its miscibility with CO_2 in various pressure and temperature ranges [40]. Being the most polar alcohol, it has a greater impact on increasing the elution strength of the mobile phase. Alcohols with longer chains, such as ethanol and propanol, may result in longer retention times and lower separation performance [41]. In the study by Woods et al. [41], changing the organic modifier from methanol to isopropanol significantly reduced the plate numbers. Additives are also commonly assessed in SFC separations, with organic acids being a frequent choice [42]. Studies mentioned in the literature show that the use of formic acid has resulted in improved peak capacity and reduced tailing effects [43, 44].

Therefore, MeOH was used as a modifier in this work, either in its pure form or in mixtures with water, ACN, and formic acid; chromatograms are represented in Fig. S3. The presence of formic acid distorted the baseline. The signal from the peaks of 4-methylbenzene-1,2-diol and benzene-1,2-diol increased proportionally with water but was most affected by the acid composition of the mobile phase, resulting in deformation. Furthermore, the use of ACN in the mobile phase did not lead to significant differences in the chromatogram profile. In terms of peak symmetry, the best results were obtained with the MeOH:H_2O (95:5) ratio and without using an acid additive.

Pressure, temperature, and gradient time modification

Pressure and temperature affect the density of the mobile phase and, consequently, compound retention. When the SFC mobile phase consists of pure CO_2, the density of the supercritical fluid defines the solubility and solute–solvent interaction of the compounds, thereby influencing changes in the retention factor [39]. However, the presence of an organic modifier in the composition can minimize the influence of pressure and temperature on the separation, depending on the amount [45]. The conditions, temperature, and pressure were investigated in this section. For this purpose, a FFD 2^3 was constructed, varying temperature, pressure, and modifier gradient time. Each test condition performed in this work can be seen in Table S2. The response was obtained in terms of the number of peaks, and based on these values, a contour surface (Fig. 2) was generated using the Fusion QbD software.

In the contour surface (Fig. 2), the white regions indicate the optimal conditions, where the highest number of peaks was obtained. The pressure of 15.5 MPa proved to be the most suitable for the proposed method, as no maximum response was obtained below this value in any region of the graph. Higher pressure values resulted in a greater number of peaks with a separation factor of ≥ 1.03. This effect of pressure on the separation factor has also been observed by Ovchinnikov et al. [45]. The authors noted similar behavior when analyzing resorcinol and pyrocatechol. A possible explanation for this behavior is that at higher pressures, sites with strong interactions become more available to the analytes, leading to enhanced analyte–stationary phase interactions.

Based on the work of Ovchinnikov et al. [45], phenolic compounds can be less susceptible to pressure changes. In some cases, the effects of pressure and temperature on

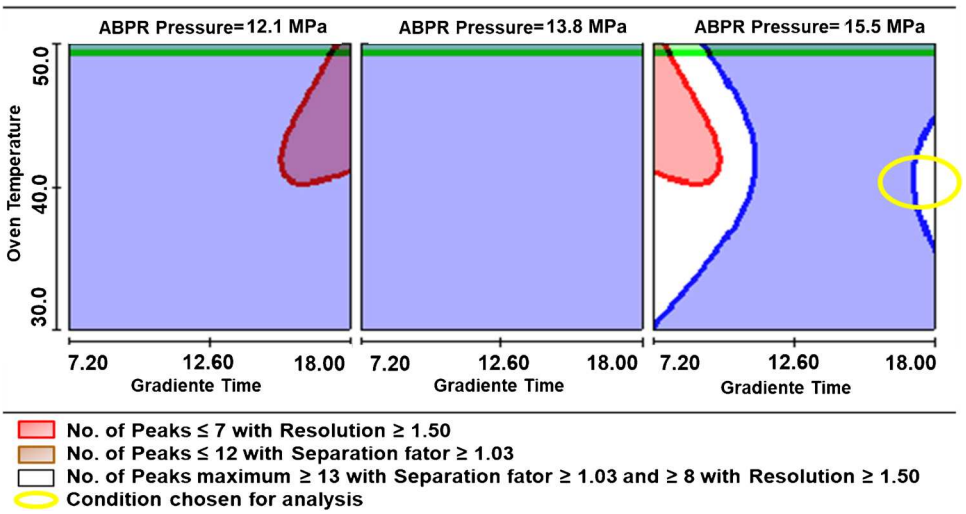


Fig. 2 Contour maps: gradient time vs temperature at the three pressures evaluated

separation can be small, but it can help when an organic modifier is present in small amounts in the mobile phase.

Thus, the selected analysis conditions are highlighted in yellow (Fig. 2). The pressure was set at 15.5 MPa, the temperature at 40ºC, and the gradient time at 18 min. These conditions were chosen to avoid potential co-elution of the target analytes with interferents present in the matrix, which may occur at 7.20 min.

Nature of the solvent

All phenolic compound standards used to optimize the described parameters were prepared in ACN and did not show any peak distortion or baseline noise. It is worth noting that no extraction steps were performed in the preparation of the aqueous fractions analyzed by UHPSFC in this study. The sample was directly diluted with the solvent at a ratio of 1:20 v/v, except for the grape seed aqueous fraction, which was diluted at 1:40 v/v. However, when the aqueous fraction samples were mixed with ACN, negative effects on the peaks of the target analytes were observed. To improve the chromatographic profile, other solvents and mixtures were evaluated: H_2O 100%, i-PrOH:ACN 1:3 (v/v), H_2O:ACN 1:3 (v/v), H_2O:MeOH 1:20 (v/v), and H_2O:MeOH 1:50 (v/v).

Of the solvent mixtures evaluated, water presented the best peak resolution, especially for 2-methoxyphenol. Compounds with higher retention were less likely to experience peak distortion. This behavior can be attributed to the difference in retention between the analyte and the diluent, allowing them to interact at different locations within the column without interfering with each other, as previously described in the literature [46].

Chromatograms representing the results are shown in Fig. 3. In the work of Fairchild et al. [47], highly polar solvents such as MeOH led to peak distortions (even with small injection volumes) due to the contrasting elution force between the sample solvent and eluent. Consequently, using water as the elution solvent could also be problematic. However, Sarrut et al. [31] observed that a higher proportion of acetonitrile in the injection distorted the shape of the 2-methylphenol peak more than water did. The authors attributed this behavior to the high affinity of analytes for water and the good affinity between water and the stationary phase. Other studies in the literature have also reported the positive effect of using water as the elution solvent, mainly at lower injection volumes ($\leq$2 µL) [46, 48]. Thus, water was used as the diluting solvent for the analysis of the aqueous fractions.

Quantitative analysis of aqueous fractions using UHPSFC-DAD

With chromatographic conditions optimized in this work (Table S3), eleven (11) of the fourteen (14) phenolic compounds (standards) were separated. Thus, for analysis of the aqueous fractions, the eleven compound were selected: 2-methoxyphenol, 2-methoxy-4-prop-2-enylphenol, 4-ethylphenol, 2,5-dimethylphenol, 2-methylphenol, 3,5-dimethylphenol, phenol, 4-methoxyphenol, 4-methylbenzene-1,2-diol, benzene-1,2-diol, and benzene-1,3-diol. They appeared in this same order of elution. The compounds 3-methylphenol, 4-methylphenol, and 2,6-dimethoxyphenol (peak 7) co-eluted with each other (Fig. 4A). The identification of these analytes in the aqueous fraction was done by means of retention time, which was confirmed by sample fortification and by comparing the UV spectra with the standards.

Five analytes were found to be present in the highest concentration among the six different aqueous fractions and could be quantified (Fig. 4). The remaining analytes co-eluted with other compounds or were not detected. Selectivity was investigated in terms of matrix effects by comparing

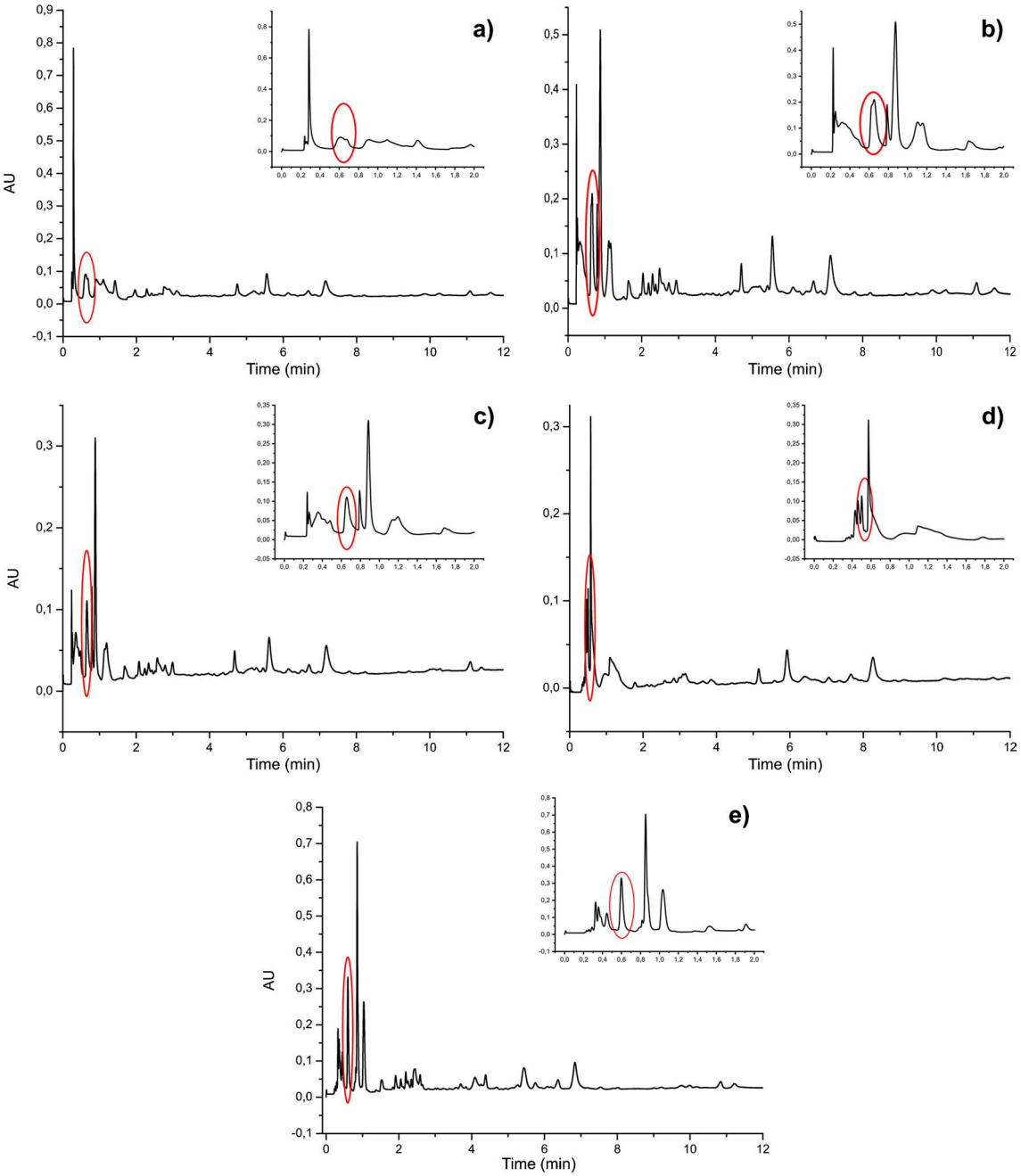


Fig. 3 UHPSFC chromatograms of aqueous fractions in different dilution solvents: **a** isopropanol:ACN (1:3); **b** H_2O:ACN (1:3); **c** H_2O:ACN (1:20); **d** H_2O:MeOH (1:50); **e** H_2O. Injection volume of 1 µL. 2-Methoxyphenol highlighted in red

two analytical curves (matrix and solvent). Quantification was performed using the matrix curve. The working range varied from 2 to 180 µg•mL^{-1}, depending on the analyte. The linear regression equations revealed a linearity greater than 0.99, and the LOD and LOQ, respectively, ranged from 0.92 to 8.41 µg•mL^{-1} and from 2.78 to 25.50 µg•mL^{-1}. The repeatability showed relative standard deviations (RSDs) of less than 5.0% for all compounds. Recovery tests fell within the range of 89 to 100%, except for two specific cases: phenol and benzene-1,2-diol. In general, the results were satisfactory and indicated good linearity, sensitivity, and precision. The results for each analyte can be found in supplementary material (Table S4 and S5).

Quantification of the compounds was performed using external standardization. The aqueous grape seed, guava, and pine nut fractions presented the highest phenolic compound concentrations (8163, 6049, and 6683 µg•mL^{-1}). The acerola fruit, sugar apple, and cowpea pod residues presented the lowest

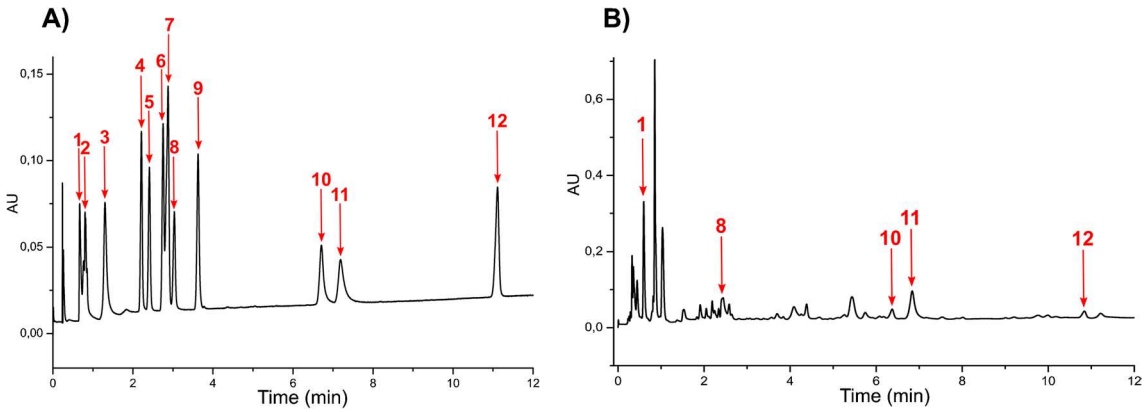


Fig. 4 Chromatograms of standards (**A**) and aqueous guava seed fraction (**B**). (1) 2-methoxyphenol, (2) 2-methoxy-4-prop-2-enylphenol, (3) 4-ethylphenol, (4) 2,5-dimethylphenol, (5) 2-methylphenol, (6) 3,5-dimethylphenol, (7) 3-methylphenol + 4-methylphenol + 2,6-dimethoxyphenol, (8) phenol, (9) 4-methoxyphenol, (10) 4-methylbenzene-1,2-diol, (11) benzene-1,2-diol, and (12) benzene-1,3-diol

concentrations (4789, 3097, and 1888 μg•mL^{-1}); the individual concentrations of the phenolic species are shown in Table 4.

With regard to the differences in concentrations among samples, they are related to the biomass lignocellulosic composition and its decomposition reactions. Lignin is considered the main source of phenolic compounds during pyrolysis [49] and the concentration of phenolic species in the bio-oil depends on their structural composition in each biomass, as well as the distribution proportion of the p-hydroxyphenyl, guaiacyl, and syringyl (Fig. S4) units that comprise it [3, 50]. The cowpea pod lignin content (7.31%) is found in smaller proportion than in the other biomasses [27], which explains the low concentration of phenolic compounds in its aqueous fraction. Thus, the high concentration values observed for guava and grape seed are a result of their lignin contents found in the literature, approximately 40% and 50%, respectively [51, 52].

When compared to another study by the authors using the aqueous fraction of guava seeds [53], the total concentration of phenols found (around 6000) was similar to that of the present study, even though Cerqueira et al. [35] quantified twelve (12) phenolic compounds using GC–MS, while this study quantified five (5). Techniques such as gas chromatography require extraction steps when dealing with an aqueous sample, which can lead to losses. Consequently, the quantification is done partially.

Although the use of GC and LC in the separation of compounds in bio-oil (oil and aqueous phase) is well-established in the literature [20–22, 24, 25], these techniques have their limitations, and their analysis times can reach up to 60 min or more. The possibility of direct sample analysis and shorter analysis times are significant advantages in a laboratory routine.

In the work of Lazzari et al. [24], using HPLC, 5 compounds were quantified in the aqueous pyrolysis fraction within an analysis time of 65 min. In recent studies using RPLCxSFC in the separation of the oil fraction [26], the analysis lasted 55 min, although the authors achieved good peak capacity. The present work, focusing on a specific class of compounds, quantified 5 target analytes in 26 min of analysis, but it also encountered limitations similar to other studies.

Despite the interferences in the samples making phenol quantification challenging, in future studies, coupling UHPSFC with a mass detector can assist in better performance in the identification and quantification of compounds, using, for example, the selected ion monitoring mode or deconvolution tools.

Table 4 Phenolic compound concentrations in aqueous fractions analyzed by UHPSFC-DAD

Concentration (μg.mL^{-1})*						
	Cowpea pod	Pine cone seed	Acerola seed	Grape seed	Pinion seed bark	Guava seed
Phenol	303 ± 42	982 ± 12	555 ± 24	< LOQ	1787 ± 35	285 ± 15
4-Methylbenzene-1,2-diol	< LOQ	290 ± 16	674 ± 3	1808 ± 6	1177 ± 33	418 ± 15
Benzene-1,2-diol	836 ± 56	1378 ± 4	1647 ± 3	5828 ± 4	3276 ± 81	1835 ± 6
Benzene-1,3-diol	749 ± 11	448 ± 8	1913 ± 8	362 ± 9	265 ± 10	374 ± 5
2-Methoxyphenol	< LOQ	< LOQ	< LOQ	165 ± 3	179 ± 2	3137 ± 59
Total	1888 ± 109	3098 ± 40	4789 ± 38	8163 ± 22	6684 ± 161	6049 ± 100

*Concentration of phenols with dilution correction

Conclusions

The sample with the highest phenol concentration was the aqueous fraction obtained from grape seeds, while the cowpea pod sample presented the lowest concentration. However, even at the lowest concentrations, the aqueous fractions exhibit a high content of phenolic compounds, which have high added value for the industry. Therefore, these samples are potential sources for obtaining these inputs that are originally derived from fossil fuels.

The present method used for analysis of phenols in pyrolysis aqueous fractions was optimized using the UHPSFC-DAD technique and a Torus 2-PIC polar column, with organic modifier in an 18-min gradient (MeOH:H2O; 95:5) to 18%, a flow rate of 0.80 mL•min^{-1}, pressure at 15.5 MPa, and an oven temperature of 40ºC. The samples were prepared in water.

The UHPSFC method developed proved to be adequate for analysis of aqueous pyrolysis samples and can be used to monitor phenolic compound concentration in complex matrices from aqueous fractions originating in pyrolysis processes. It is worth emphasizing that, since we are working with a highly complex sample, in order to enhance the method's precision in determining the five (5) quantified analytes and resolving the issue of co-elution with interferents, future studies can be conducted to investigate methods of clean-up for this type of sample. Moreover, other detectors can be explored to assist in the identification and, consequently, characterization of these compounds.

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Declarations

Competing interests The authors declare no competing interests.

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