Journal of Chromatography B 1253 (2025) 124442

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Analysis of aliphatic and phenolic compounds present in industrial black liquors using HPLC-DAD and IC-MS/MS methods

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ARTICLE INFO

Keywords:
Black liquor
Carboxylic acids
Phenolic compounds
Liquid chromatography
Ionic chromatography
Mass spectrometry

ABSTRACT

Carboxylic acids and aromatic compounds are essential building blocks and starting materials for the production of a wide range of fine chemicals and materials. Their recovery from kraft black liquor, an industrial effluent from pulp and paper mills, is a promising way to produce alternative bio-based chemicals. Reliable methods are needed to identify and quantify the molecules of interest in complex mixtures such as black liquors. First, an HPLC-DAD-based method was developed for the determination of aliphatic acids and phenolic compounds. It allowed the separation of 31 aliphatic and phenolic compounds. The method was applied to the identification of aliphatic and phenolic compounds in industrial black liquors. Then, an IC-MS/MS method was developed to confirm the identification and quantification of organic compounds in black liquor samples. 22 compounds were detected and identified by MS/MS detection.

According to both methods, the major aliphatic acids in softwood kraft black liquor are formic acid (9.8 g/L), acetic acid (7.1 g/L), lactic acid (5.2 g/L), glycolic acid (4.7 g/L), 2-hydroxybutyric acid (2.3 g/L), and oxalic acid (1.3 g/L). Phenolic compounds were detected at very low levels (total concentration 1.4 g/L). This study demonstrates the value of a multi-technique strategy for the identification and quantification of organic compounds in complex matrices such as black liquor.

1. Introduction

Carboxylic acids and aromatic compounds are essential building blocks and starting materials for the production of fine chemicals, flavors, and polymers; yet their manufacture is highly reliant on petrochemical derivatives or processes that demand a vast amount of energy and generate CO_2 emissions. To guarantee long-term sustainable production, multiple renewable feedstock choices have been investigated. One of these alternatives is to recover the carboxylic acids found in Kraft black liquor (BL) [1]. BL is formed during the pulping process as a byproduct of paper pulp produced from wood chips. The non–cellulosic biomass compounds (*i.e.* hemicellulose and lignin) degrade during Kraft cooking and yield in a large fraction of carboxylic acids (≈ 30 % dry matter) and small amount of aromatic molecules (< 1 %) [2]. At industrial level, the Kraft process is the dominant pulping technology, it employs a solution of Na_2S and NaOH added during the cooking stage to breakdown the biomass, easing the pulp recovery [3].

The origin of biomass as well as the recipe and proportions of inorganic compounds may slightly modify the carboxylic/aromatic composition but overall, the impact is minimal [2]. In the carboxylic acid fraction of BL, more than 20 compounds have been identified and in exhaustive lists this value may be higher [2]. Within this fraction, acetic, formic, lactic, glycolic and isosaccharinic (gluco- and xylo-) acids stand out as the most relevant compounds in quantity [4,5]. In the aromatic fraction, catechols, cyclopentenones, and thiophenes were reported [6]. Particularly, vanillin and its derivatives (vanillic acid, acetovanillone), syringaldehyde and its derivative (syringic acid), 4-hydroxybenzoic acid, 4-hydroxyacetophenone, guaiacol were detected in black liquors, as well as di-aromatic compounds such as stilbene [7–9].

The carboxylic acid fraction it is most commonly analyzed by high-performance liquid chromatography (HPLC) [10–16] or capillary electrophoresis [1,17,18]. Nonetheless, other chromatography techniques

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https://doi.org/10.1016/j.jchromb.2024.124442
Received 28 October 2024; Received in revised form 17 December 2024; Accepted 24 December 2024
Available online 6 January 2025

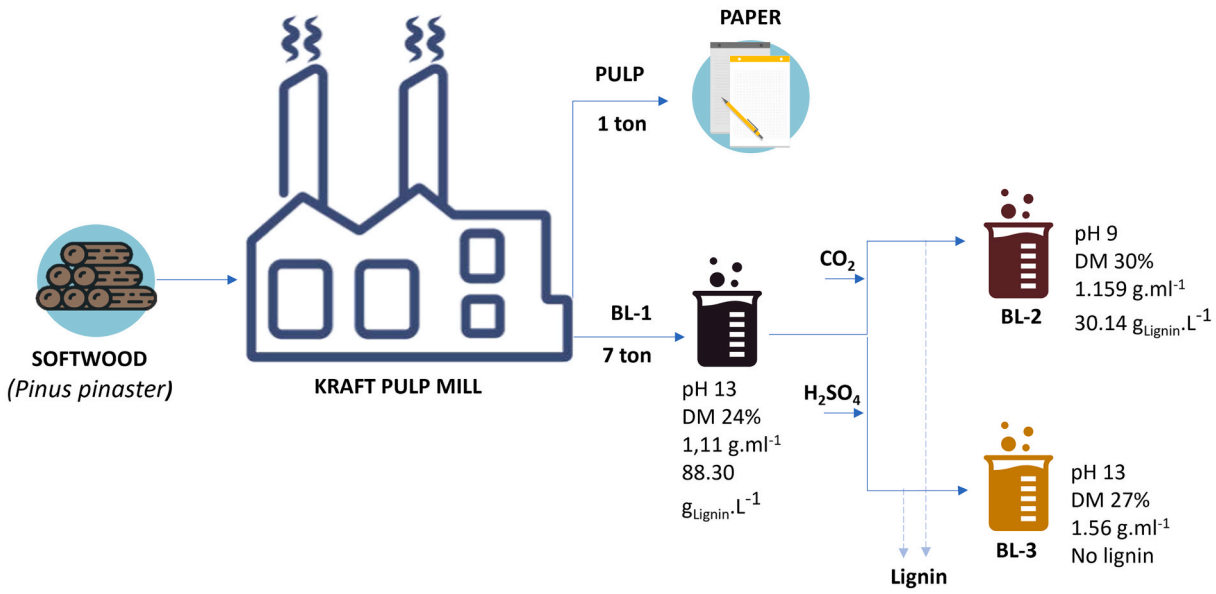

Fig. 1. Black liquors and their pretreatments. DM: dry matter.

such as GLC [19,20], GC-FID [4,21], GC–MS [1], HPLC-APCI-MS [22] and IC-CD [23] have been employed and reported for the quantification of these types of molecules present in BL. Some of the analysis techniques can also be applied to the aromatic section such as the HPLC.

The identification of aromatic compounds in BL was less investigated. Faustino et al used mass spectrometry to identify some phenolic compounds in hardwood BL [7]. Electrophoresis methods were also applied for detection of aromatic species [8]. In addition, gas chromatography allowed to identify guaiacol and stilbene derivatives in liquor extracts [9].

Ion chromatography (IC) is suitable to analyze ionizable molecules in paper process samples [24]. This technique is based on analytes separation with a cation or anion exchange resin and depending their total charge, their size and the pH of the eluent [25]. To characterize process liquors, ion chromatography was generally combined with suppressed conductivity or with electrochemical detector [24,26,27] or with pulsed amperometry detector [28,29]. In 2008, for the first time, a study performed ion chromatography with suppressed conductivity detector (IC-CD) and also suppressed mass spectrometry (IC-MS) to determine organic acids in black liquors [23]. Samples came from conventional laboratory scale kraft cooks of industrial wood chips from birch, aspen, and softwoods consisting of 75 % Scots pine and 25 % Norway spruce. The suppressor, set up before the detector, aims to remove the inorganic salts and neutralize the eluent in water. Thus, it allows not only to decrease the background noise in conductivity but also to prevent salt contamination in MS [30]. IC-CD was chosen to quantify simultaneously inorganic anions (chloride, sulfate, thiosulfate) and low-molecular-mass aliphatic carboxylic acids as their carboxylate anions (*i.e* lactate, 2-hydroxybutanoate, formate and oxalate) in 27 min run time [23]. Moreover, complementarity method with IC-MS was developed to realize a qualitative analysis of black liquor. The IC-MS achieved a scan between 50 and 200 Da to get mass-to-charge ratio (m/z) of unknown compounds as the simple quadrupole detector does not provide fragmentation information. The identification used these values, model compounds and black liquor analyses results obtained by gas chromatography coupled with mass spectrometry detector and previously published by the same research team. This article pointed out that this method was also able to separate other acids and inorganic anions, but it was decided to focus only on compounds known to be present in significant amounts in black liquors.

Accurately quantifying and identifying the organic species present in BL is a rather complex task. This is due to the heterogeneity of compounds present as well as the casuistic and dilute nature of the solution. In addition, generally several techniques are required for identification and quantification of several compounds, and even when a single analytical technique is employed, the accuracy of the method can be arguable. Since the correct quantification of the species is the cornerstone to determine the performance of the processes applied to the BL valorization, it is necessary to develop reliable, accurate and less time-consuming analytical techniques.

In this study, we focused on the development of two complementary chromatographic techniques applied to the analysis of the BL aromatic and carboxylic fractions. Both HPLC with DAD detector and IC combined with conductivity and tandem mass spectrometry were used to characterize black liquors. The goal was to identify as many compounds known to be found in black liquors as possible (including compounds present in low concentration), with a minimum of sample preparation, and to compare the results obtained with these two analytical methods. Moreover, for unknown peaks, the triple quadrupole detector allows fragmentation data in addition to the m/z of the deprotonated compounds.

2. Materials and methods

2.1. Black liquor

Kraft BL-1, -2 and -3 were kindly provided by CTP (Centre Technique du Papier) and FCBA Institute (Grenoble, France). They come from industrial Kraft plants in France processing maritime pine (*Pinus pinaster*) as feedstock. The solutions are considered as weak BLs (dry matter < 60 %wt). BL-1 solution refers to a crude liquor whereas BL-2 and BL-3 were semi-industrially treated. Those fractions correspond to the liquid phase collected after lignin precipitation by carbonation (CO_2) at 70 °C, and acidification (H_2SO_4) at 70 °C, of crude BL, respectively (Fig. 1). The main physicochemical properties of the different BLs employed in this study are available in ESI (Table S1).

2.2. HPLC analysis

2.2.1. Commercial compounds

For the HPLC samples preparation, HCl 37 % was purchased from VWR chemicals. For the formulation of the HPLC mobile phases, phosphoric acid (H_3PO_4) 85 % was purchased from Laurylab, monosodium phosphate (NaH_2PO_4) from Acros Organics and HPLC grade acetonitrile

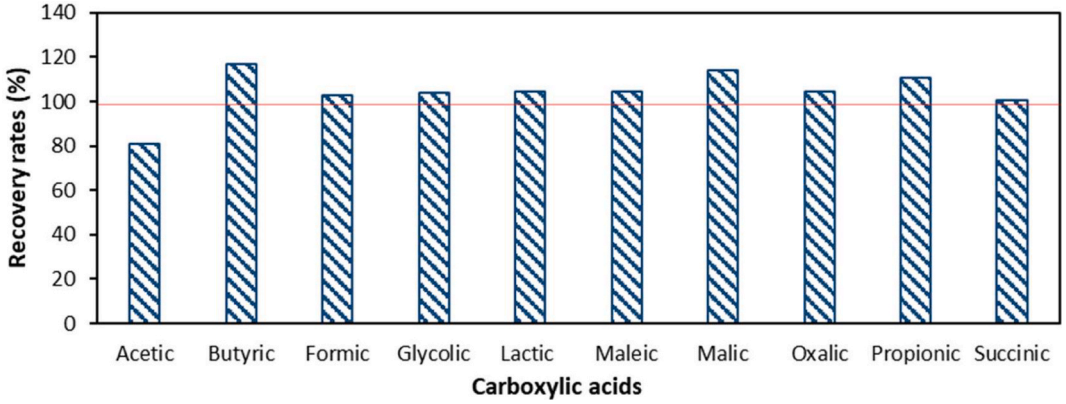

Fig. 2. Recovery rates of 10 carboxylic acids in BL-1.

(ACN) from Sigma Aldrich. Commercial compounds used as standard were purchased from various suppliers (see Table S3 in ESI for details).

2.2.2. BL sample preparation

Each liquor sample was diluted by mass (1 g sample/10 g H_2O) and acidified with concentrated HCl (37 % wt.) until pH 2–3. Precipitation of lignin was observed (brown precipitate). Then it was filtered with PTFE syringe filters (0.45 μm) and kept in the freezer until analysis.

2.2.3. HPLC equipment

The analysis of the carboxylic acids and aromatic compounds were carried out on a Shimadzu apparatus composed of a DGU-20ASR degasser, a LC 20AD quaternary pump, and a SIL-20A HT autosampler with a 100 μL injection loop, a CTO-10AS VP column oven. It is equipped with SPD-M10A VP DAD detector. The parameters of DAD detector were: acquisition frequency 12.5 Hz, time constant 0.24 s, range of wavelength 190–400 nm (D_2 lamp). The RID signal was not used for this study. The software Shimadzu LabSolutions was used for the monitoring of HPLC analysis and the post-treatment of HPLC data, including calibration curves and standard error calculations.

The HPLC column was a Phenomenex Synergi Hydro-RP column (250 × 4.6 mm, particle diameter 4 μm) with a Phenomenex SecurityGuard cartridge AQ-C18. Between two sequences of analysis, the column was rinsed with a mixture 50/50 pure deionized water/ACN and was stored at room temperature, sealed by plugs.

2.2.4. Mobile phases

Two mobile phases were prepared for the analysis. An aqueous mobile phase buffered at pH 2.6 was prepared with orthophosphoric acid (H_3PO_4 85 %, 340 μL for 1 L of pure deionised water) and sodium dihydrogenophosphate dihydrate (NaH_2PO_4 99 %, 780 mg for 1 L of pure deionised water) and filtered under vacuum on 0.2 μm pore membranes. HPLC grade commercial acetonitrile (ACN) was used as the second mobile phase without further purification.

2.2.5. HPLC analysis – Operating conditions

During a typical analysis run, the HPLC column was kept at 30 °C and the total liquid flowrate was set at 0.7 mL min^{-1}, corresponding to a fluid velocity of 0.098 cm s^{-1} and a theoretical plate height of 12.4 μm. For a standard test a 10 μL aliquot of sample was injected at 0.5 min running time. Within the analysis course a gradient of solvent was applied from 100 % aqueous mobile phase to 22 % ACN mobile phase (details of the gradient in Table S2 in ESI).

In total, the analysis method lasts 90 min per sample. The gradient led to a drift of baseline at low wavelength (<230 nm) which is attributed to the increasing amount of acetonitrile in the mobile phase. Each sequence, from 10 to 20 samples, was preceded by a blank run (run without sample injection) and followed by a run with a standard solution as control.

2.2.6. Determination of aliphatic and phenolic compounds in liquors

Aliphatic and phenolic compounds were profiled by comparing retention times with those of standards. The list of compounds employed for the standard solutions, as well as their supplier and purity, are available in Table S3 in ESI. Their identification was confirmed by UV–Vis spectral analysis between 190 and 400 nm.

2.2.7. HPLC method validation

The following parameters were used for method validation: linearity, sensitivity, reproducibility, accuracy, limit of detection, limit of quantification. The accuracy was measured following the analysis of standard solutions in triplicates, standard error was calculated on the areas of peaks corresponding to each standard compounds. The calibration curves of each standard were plotted against its concentration using least squares regression analysis. The limit of detection (LOD) and limit of quantification (LOQ) were estimated as 3.3 σ/slope of the calibration curve and 10 σ/slope of the calibration curve, respectively. σ is the standard deviation of the response.

2.3. IC-MS/MS analysis

2.3.1. Commercial compounds

Acetic acid (Sigma Aldrich solution 96 %), formic acid (Sigma Aldrich solution 98 %), oxalic acid (Sigma Aldrich ≥99 %), glycolic acid (Sigma Aldrich 99 %), malic acid (Sigma Aldrich ≥99 %), maleic acid (Sigma Aldrich ≥99 %), propionic acid (Acros Organics solution 99 %), succinic acid (Sigma Aldrich di sodium 99 %), lactic acid (Fluka solution) and butyric acid (Alfa Aesar solution 99 %) were used as standard compounds. Phosphoric acid (Sigma Aldrich 85 % solution), citric acid (Sigma Aldrich ≥99.5 %) and sulfuric acid (Sigma Aldrich 95 % solution) were used to check identification hypothesis.

2.3.2. BL sample preparation

Samples of each liquor were diluted (1:1000) in ultra-pure water prior to analysis by ion chromatography connected with tandem mass spectrometry (IC-MS/MS). They were analyzed without any pH adjustment.

2.3.3. Equipment

The IC-MS/MS instrument involved was ICS-5000 + Ion Chromatography System combined with TSQ Fortis Triple Quadrupole Mass Spectrometer (Thermo Scientific), employing Chromeleon 7.2.10 as monitoring software. The IC system was equipped with an AS-AP autosampler, a Dual Pump analytical gradient system, a suppressor ADRS 600 (2 mm, Thermo Scientific), which neutralizes the ions in the eluent, and a conductivity detector (P/N 061830). The conductivity detector

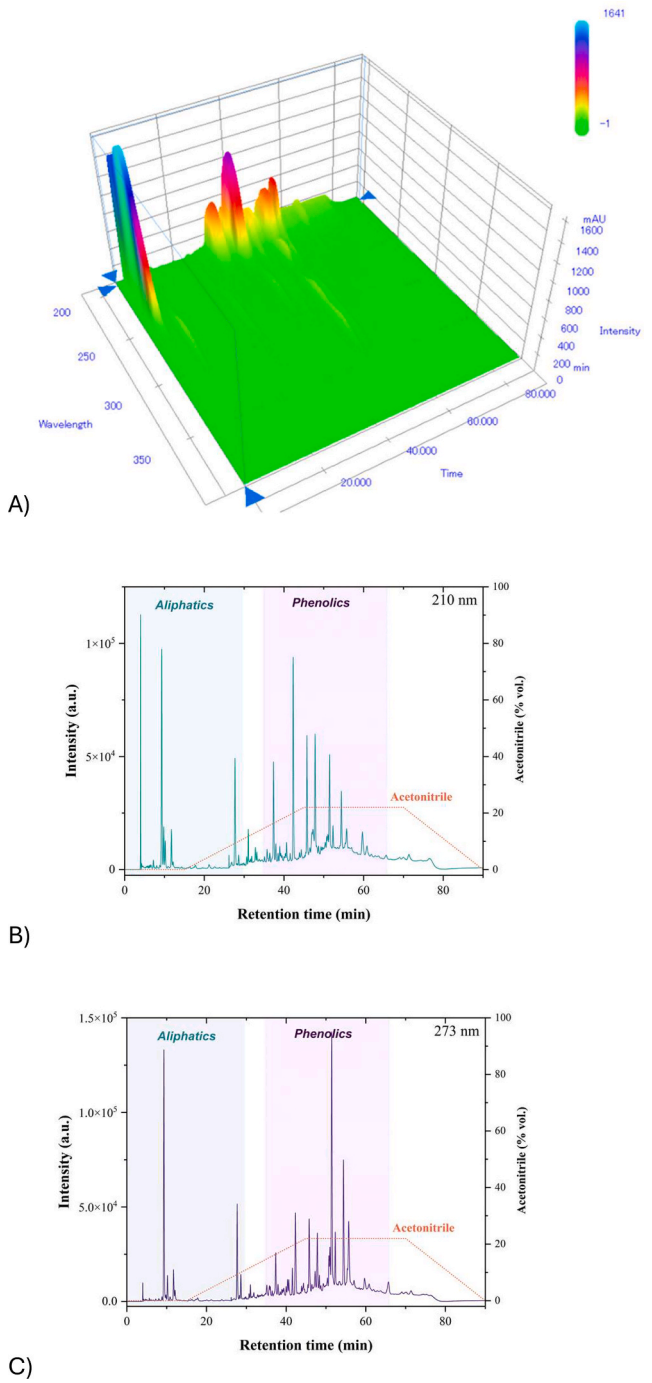

Fig. 3. Chromatograms of BL-1 analysis: full range of UV wavelength (A); 210 nm (B); 273 nm (C).

was used to check that the suppressor is operating normally. The equipment is depicted on Fig. S1 in ESI (adapted from [31]).

2.3.4. Ion chromatography separation – Operating conditions

The IC separation was carried out employing an anion-exchange column IonPac AS11-HC-4 μm (2 × 250 mm, Thermo Scientific) preceded by a guard column IonPac AG11-HC-4 μm (2 × 50 mm, Thermo Scientific), operating at 30 °C. An injection volume of 20 μL was used. The system was working with an aqueous mobile phase at a flow rate of 0.35 mL/min composed of potassium hydroxide (KOH), produced by an eluent generator. Before analysis, the system was equilibrated with a solution at 1 mM KOH for 10 min, thereafter a mobile phase composition gradient was applied from 1 mM of KOH to 100 mM of KOH (details of the gradient in Table S6 in ESI).

Suppression was performed by an electrochemical anion exchange suppressor at 96 mA using an external regeneration with water at 0.5 mL/min.

2.3.5. Conductivity and mass spectrometry detectors – Operating conditions

For the conductivity detection, the temperature was set at 30 °C. The MS electrospray source parameters such as spray tension, gases flow rate and source temperature as well as tube lens voltage and collision energy were optimized by direct infusion and analysis of standard solutions in MS. The standard solutions employed for the HPLC calibration (see Table S4 in ESI) were infused in the MS detector through a syringe pump. A tee adapter allowed to connect the syringe pump (50 μL/min), the ion chromatograph (0.30 mL/min) and the MS detector, as shown on Fig. S1 in ESI. Then, the parameter value allowing the highest signal intensity was selected with the help of Chromeleon software. To realize simultaneous measurements, a compromise was taken for the source parameters between all the optimized values of the different compounds; however, the tube lens voltage and the collision energy were still compound dependent.

The heated electrospray ionization source was used in negative mode with the following optimized parameters: negative ion spray voltage 3.0 kV, sheath gas 54 Arb, auxiliary gas 7 Arb, sweep gas 2 Arb, ion transfer tube temperature 275 °C and vaporizer temperature 375 °C. Compound-specific parameters (*i.e.* tube lens, transitions and collision energies) are given in Table S7 in ESI. Acquisitions were performed in full scan and in selected reaction monitoring modes (SRM).

For molecules able to fragment themselves, only the two most intensive transitions from precursor ion (deprotonated molecule) to product ion were kept for the SRM method (see Table S7 in ESI for details). One is used for quantification transition to calculate the peak area and the other one as confirmation transition for identification. For compounds that do not fragment themselves such as propionic acid, only the precursor-to-precursor transition was considered.

2.3.6. Determination of aliphatic and phenolic compounds in liquors

For the identification, the retention time, transitions and ratio were considered. Each multi-sample run was preceded by a blank run (water injection) and included the analysis of a standard mix. A comparison between the results of samples, blank and standard mixes was established. Quantification transition and confirmation transition, for molecules able to fragment themselves, allowed to confirm the compound identification. In order to calculate a peak area, only the quantification transition was used.

2.3.7. Method validation and quantification for ten carboxylic acids

Ten carboxylic acids, identified in BL-1, were chosen to carry out a quantification study: formic, acetic, glycolic, oxalic, propionic, lactic, butyric, succinic, malic, maleic acids. In order to estimate the matrix influence on quantification, BL-1 was diluted by a factor 1000 with ultra-pure water and spiked at 10 ppm with standard mix. Recovery rates were calculated with the following equation:

$$Recovery\ rate(\%) = \frac{peak\ area\ of\ sample\ spiked\ at\ 10\ ppm - peak\ area\ of\ sample}{peak\ area\ of\ standard\ at\ 10\ ppm} \times 100$$

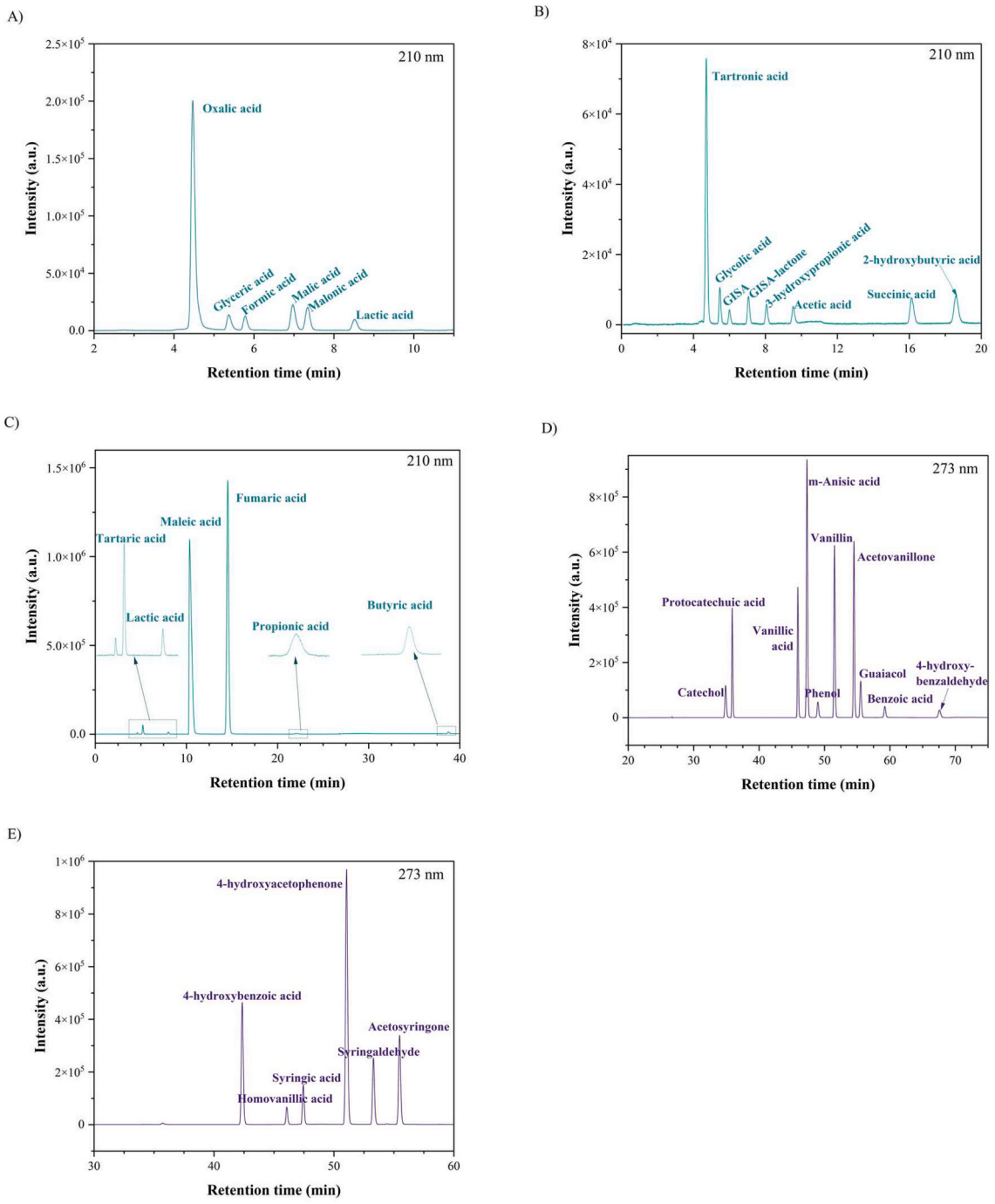

Fig. 4. Examples of chromatograms of standard solutions. Carboxylic acids standard (A/B/C) and aromatics standard solutions (D/E).

Generally, recovery rates too far from 100 % highlight an overestimation or underestimation of compound concentration in the sample. For recovery rates between 80 and 120 %, the quantification is considered satisfying. The recovery rates of the selected 10 carboxylic acids are illustrated on Fig. 2. All the acids had recovery rates between 80 % and 120 %. Therefore, for all acids, matrix effects could be considered as negligible. This could be explained by the important dilution of samples before analysis.

The limit of quantification (LOQ) was estimated with signal/noise ratio of 10. Linear calibration curves, from LOQ to 100 ppm, were obtained with correlation coefficient above 0.99. The repeatability was established by analyzing the BL-1 sample in triplicates. For butyric and maleic acids, the variation coefficients were estimated at 17 and 18 % respectively, which is consistent with the fact that they are present in lower concentration than other acids. Indeed, for the other acids, the variation coefficients were below 6 %, showing that this analytical method is accurate. Standard deviations were calculated, and uncertainties were estimated as two times the standard deviation, to get into the 95 % confidence interval.

2.3.8. Identification of unknown compounds

MS acquisition was performed in full scan mode in negative polarity, from *m/z* 30 to 500. Some of the identification hypotheses were validated using standard mix at 100 ppm and at 10 ppm. BL-1 sample was diluted by 1000 with ultra-pure water and spiked at 10 ppm of standard (15 µL of 100 ppm standard solution and 135 µL of the diluted sample).

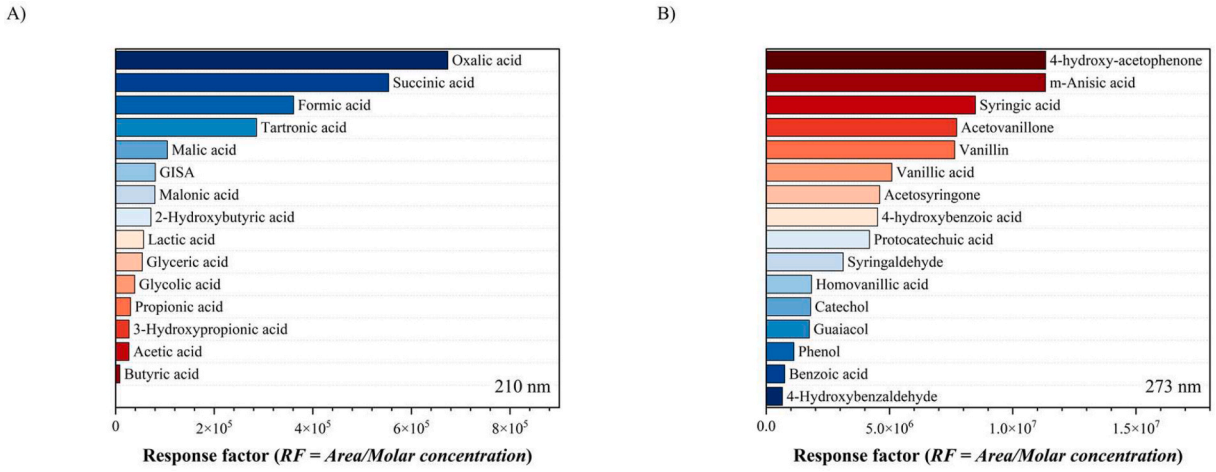

Fig. 5. Response factors for standard aliphatic compounds at 210 nm (A) and for phenolic compounds at 273 nm (B).

3. Results and discussion

The black liquors studied in this work were produced in an industrial Kraft pulp mill using pine wood (softwood) as feedstock. The liquors have different pH values ranging between 4 and 13 pH units; this is linked to the delignification treatments applied to BL-2 and BL-3, as previously mentioned (*cf*. Fig. 1). Crude black liquor BL-1 contains relatively high concentrations of lignin, followed by BL-2, and finally BL-3, where no lignin was detected. The liquors also have different contents of dry matter from 24 to 30 wt%. This should be considered when comparing the concentration of compounds, aliphatic and aromatic, between the different samples, since a higher concentration of solids generally translates into a higher concentration of compounds in solution. Additionally, all liquors also contain large amounts of inorganic elements such as Na and S, which comes from Kraft cooking ingredients. Their properties are summarized in Table S1 in ESI.

3.1. HPLC analysis of liquors

3.1.1. Typical chromatograms of black liquor

Typical chromatograms of the HPLC analysis of BL-1, diluted ten times, are shown on Fig. 3. On the 3D data, it is observed that intense signals are present at low wavelength (190–220 nm), that could correspond to carboxyl functions, whereas at high wavelength (270–280 nm) these signals could correspond to aromatic functions. Chromatograms extracted at 210 nm and 273 nm are shown on Fig. 3-B and -C with the variation of the ACN percentage over the analysis time. Two zones (colored sections) were determined, corresponding to polar compounds (<30 min, intense peaks at 210 nm) and apolar compounds (>40 min, intense peaks at 273 nm). It is also observed that after 60 min, no significant peaks are detected. For BL-1 sample, 21 peaks were detected between 0 and 25 min at 210 nm (aliphatic compounds) and 58 peaks were detected between 25 and 70 min at 273 nm (phenolic compounds).

3.1.2. Preparation of standard solutions and calibration of HPLC

Five stock solutions of commercial compounds were prepared by dissolving or diluting commercial compounds in buffered water (solutions S1, S2, S3) or in 20 % ACN/80 % buffered water (solutions S4, S5). The stock solutions were composed of compounds with different retention times to avoid co-elution. For each series, four standard solutions with four levels of concentrations from 0.5 to 10 mM were obtained by preparing two stock solutions and diluting each stock solution by a factor 5. Table ESI-S3 summarizes the composition of the 5 standard solutions employed for the HPLC calibration.

The standard solutions were analyzed by HPLC in triplicates, examples of chromatograms are shown on Fig. 4. The integration of peaks was performed at 210 nm for most aliphatic compounds except for fumaric and maleic acids which were integrated at 230 nm to limit the drift of baseline. For aromatic compounds, integration was performed at 210, 230, 254 or 273 nm. The choice of wavelength for integration was made as to have the maximum intensity value without saturating the UV detector and avoiding the risk of co-elution (*i.e.* two compounds with similar retention times were integrated at different wavelengths).

Calibration curves were built as linear regression of peaks areas as a function of their respective concentrations at a given wavelength. The response factor, standard deviation, R^2, LOD and LOQ of each compound is given in Table ESI-S3.

Response factors estimated for the aliphatic molecules are lower compared to the phenolic ones (Fig. 5). For aliphatic compounds, response factors vary with the number of carboxylic functions, the presence of hydroxyl groups, and the size of carbon chain, being the short chain, di-acids, the molecules with the higher response factors. Response factors of phenolic compounds vary with molecular structure: conjugated systems have higher response factors, *e.g.* vanillic acid has a response factor twice as high as homovanillic acid. Moreover, conjugation with carbonyl leads to higher response factors than conjugation with carboxylic acid, and with the presence of methoxy groups, although no clear trend could be established.

3.1.3. HPLC analysis of liquors – Determination of aliphatic and phenolic compounds

Fig. 6 presents examples of chromatograms obtained from the analysis of three black liquors. Variations in peaks numbers and height are visible from one liquor to another, indicating some changes in the monomer composition. Particularly, peaks are much more intense in the polar region for BL-2 and BL-3 whereas in the apolar region, peaks are more intense for BL-1. These observations are in agreement with the nature and treatment of liquors. Crude black liquor (BL-1) as it was not treated is richer in phenolic compounds compared to BL-2 and BL-3 that were partially delignified, and therefore both contain less phenolics and accordingly more aliphatic molecules.

The compounds identification was based on retention time and similarity of the UV spectra. The results of compounds identification and quantification are summarized on Table S3 in ESI. The main aliphatic and phenolic compounds identified and quantified by HPLC are presented on Fig. 7.

The most concentrated compounds are small aliphatic acids such as formic acid, acetic acid glycolic acid, lactic acid, together with *gluco*-isosaccharinic acid (Fig. 7-A). The identified aliphatic compounds as well as the concentration values correspond to the ones already described in literature [2]. The concentrations obtained for each acid are similar to those listed in literature for softwood-type BLs obtained by

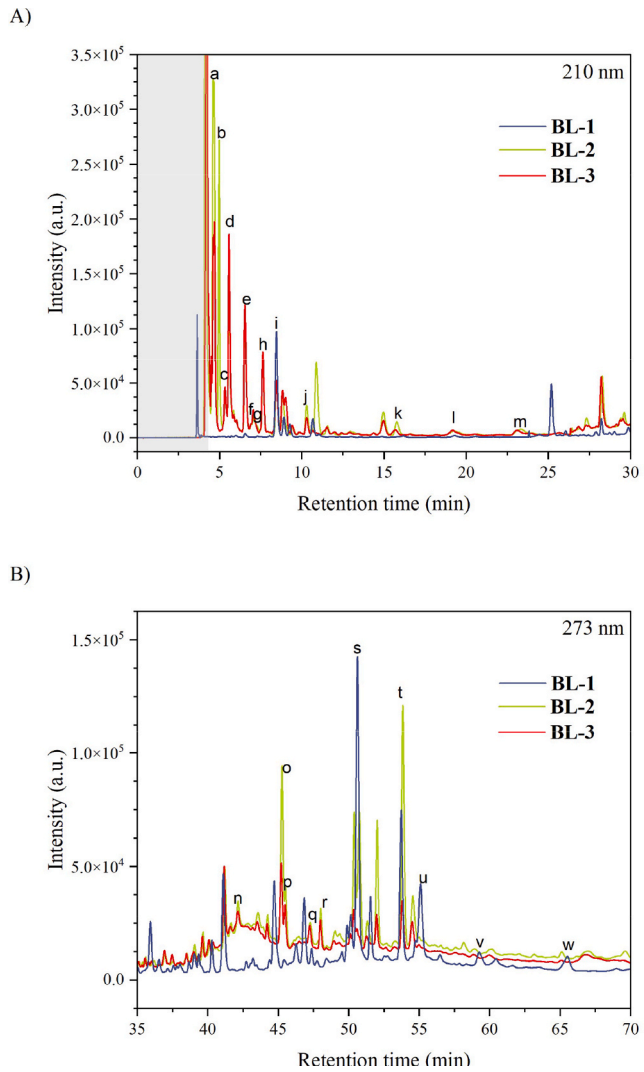

Fig. 6. Chromatograms of HPLC analysis of liquors at 210 nm (A) and 273 nm (B). *a: tartronic acid; b: tartaric acid; c: glycolic acid; d: formic acid; e: GISA; f: malic acid; g: malonic acid; h: lactic acid; i: acetic acid; maleic acid; k: succinic acid; l: 2-hydroxybutyric acid; m: propionic acid; n: 4-hydroxybenzoic acid; o: vanillic acid; p: homovanillic acid; q: anisic acid; r: phenol; s: vanillin; t: acetovanillone; u: guaiacol; v: benzoic acid; w: 4-hydroxybenzoic acid. Some identified peaks were not labelled because their retention time is between 30 and 35* min*, or because they overlap with other peaks.*

Kraft treatment. The range of reference values are: formic acid (5–7 g. L^{-1}), acetic acid (4–5 g L^{-1}), Glycolic (1–3 g L^{-1}), lactic acid (3–4 g L^{-1}) and *gluco*-isosaccharinic acids (GISA) (7–11 g L^{-1}). In general, the trends of the acid group type as well as among the acids themselves are in line with what could be expected. Formic and lactic acids are the major compounds in the volatile and hydroxy-acid groups, respectively. It is important to note that the concentration depends strongly on the percentage of dry matter in the solution, *i.e.* the higher total concentration of acids in BL-2 and BL-3 is linked with a higher dry matter content. However, the HPLC analysis was not conclusive for the identification and quantification of other reported compounds, such as oxalic acid (too low retention time) or propionic acid (too low response factor).

Phenolic compounds were also detected, in very low concentrations (Fig. 7-B). The main phenolic compounds are the ones associated with softwood lignin, with structures derived from guaiacyl moieties: vanillin, vanillic acid, homovanillic acid, acetovanillone. The total content of phenolic compounds is higher in BL-1 Crude. The absence of phenolics in BL-2 and BL-3 is explained by the delignification treatments applied to these liquors. Phenolic monomers were possibly extracted through adsorption on solid precipitated lignin.

In the polar zone, for BL-1, 21 peaks were detected at 210 nm and among them 10 were identified with a good similarity of UV spectra, *i.e.* 11 peaks correspond to compounds that could not be certainly identified. For BL-2, 28 peaks were detected, among them 11 compounds were identified certainly. For BL-3, 27 peaks were detected at 210 nm, among them 8 were certainly identified. The area ratio of peaks close to dead time (before 5 min), that could not be attributed certainly because of low retention, is 69 % for BL-1, the raw black liquor, and 63 % for both delignified liquors. The area ratio of identified peaks in all peaks after 5 min is 85 % for BL-1, 67 % for BL-2 and 75 % for BL-3.

In the apolar zone, for BL-1 58 peaks were detected at 273 nm, among them 9 could be certainly identified, *i.e.* 49 peaks correspond to compounds that could not be accurately identified. For BL-2, 37 peaks were detected at 273 nm, among them 8 peaks were identified with a good similarity of UV spectra. Finally, for BL-3 30 peaks were detected and from which 10 were certainly identified. The sum of areas of identified compounds represents 61 % of total integrated areas for BL-1, 36 % for BL-2 and 53 % for BL-3.

Finally, HPLC analysis was effective for the identification and quantification of the main aliphatic and phenolic compounds in the investigated black liquors. However, the complexity of chromatograms makes the identification of some compounds uncertain. Moreover, an important number of peaks corresponding to unknown compounds were also detected without successful identification and quantification. The HPLC method is also limited for very polar compounds which are poorly retained on the column.

In the studied liquors, the main identified acids were simple carboxylic acids (formic, acetic acids), hydroxy-acids (glycolic, lactic acids), di-acids (succinic acid) and isosaccharinic acids. All these molecules are formed by peeling reactions degrading sugars coming from hemicelluloses [2]. The main identified phenolic compounds were derived from guaiacyl moieties present in softwood lignins: guaiacol, vanillic and homovanillic acids, acetovanillone, vanillin. These compounds were already identified in BL by other techniques [6].

3.2. IC-MS/MS analysis

3.2.1. Determination of aliphatic and phenolic compounds in liquors

The identification of compounds in liquor samples was confirmed by comparison with analysis of aqueous solution of commercial compounds. The compounds identification was validated ("Id." In Table 1) if the following conditions are fulfilled: the retention time is within the tolerance range (± 0.2 min), a SRM transition is observed, the peak area for this transition is at least three times higher than the blank signal, the ratio (quantification peak area divided by confirmation peak area) is similar to the ratio obtained for standard (± 25 %). The tolerance values range were chosen after numerous experiments in IC-MS/MS. The identification conclusions for each compound in black liquors (BL-1, BL-2 and BL-3), are summarized in Table 1. Some SRM chromatograms are shown on Fig. S2 in ESI.

It was observed that 17 compounds were present in all black liquors analyzed. More compounds were identified in BL-1 (24 compounds). For BL-1, no peaks corresponding to benzoic acid and m-anisic acid were observed, indicating the absence of these molecules in the sample. For adipic acid, in all samples the ratio (quantification peak area divided by confirmation peak area) calculated was considered as too far from the ratio found in standard injection. It could be possible that an interfering or unknown compound coelutes with the acid causing the ratio to shift. For some compounds such as phthalic acid and 4-hydroxybenzaldehyde, it was not possible to conclude on the identification as their peak area was 3 times lower than the blank peak area.

Most of the searched aliphatic acids were present in all three liquors and their identification was certain. Regarding phenolic compounds, they were present mainly in BL-1 and more difficulties were

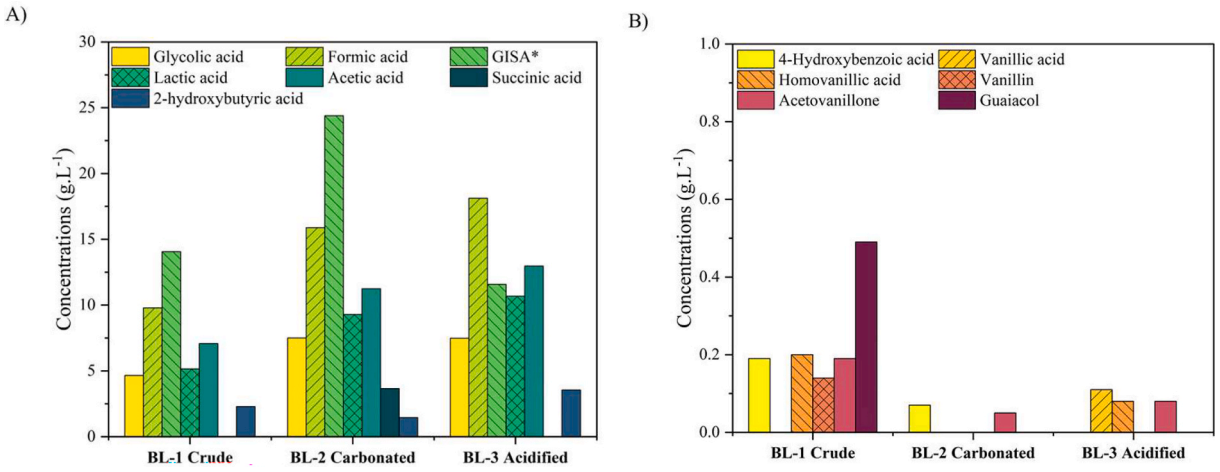

Fig. 7. Main aliphatic compounds (A) and phenolic compounds (B) identified and quantified in black liquors. Concentrations correspond to concentration values before dilution for HPLC analysis.

Table 1
IC-MS/MS identification of compounds in BL samples.

Sample/Compound	BL-1	BL-2	BL-3
Oxalic acid	Id.	Id.	Id.
Tartaric acid	Id.	Id.	Id.
Glyceric acid	Id.	Id.	Id.
Formic acid	Id.	Id.	Id.
Malic acid	Id.	Id.	Id.
Malonic acid	Low area	Id.	Low area
Lactic acid	Id.	Id.	Id.
Tartronic acid	Id.	Id.	Id.
Glycolic acid	Id.	Id.	Id.
GISA	Id.	Id.	Id.
3-hydroxypropionic acid	Low area	Low area	Id.
Acetic acid	Id.	Id.	Id.
Succinic acid	Id.	Id.	Id.
2-hydroxybutyric acid	Id.	Id.	Id.
Acrylic acid	Id.	No peak	Id.
Propionic acid	Id.	Id.	Id.
2-hydroxy-2-methylbutyric acid	Id.	Id.	Id.
Methylsuccinic acid	Id.	Id.	Id.
Adipic acid	No ratio	No ratio	No ratio
Butyric acid	Id.	Id.	Id.
Maleic acid	Id.	No ratio	No ratio
Fumaric acid	Id.	No peak	No peak
Phtalic acid	Low area	Low area	Low area
p-coumaric acid	Id.	No peak	No peak
Vanillic acid	Id.	Id.	Id.
m-anisic acid	No peak	No ratio	No peak
Vanillin	Id.	No ratio	No peak
Guaiacol	Id.	No ratio	No ratio
Benzoic acid	No peak	No ratio	Id.
4-hydroxybenzaldehyde	Low area	Low area	Low area
4-hydroxybenzoic acid	Id.	Low area	Id.

Id. = Compound was identified in the sample.
Low area = area inferior to 3 times the area of a blank run.
No ratio = the ratio between quantification and confirmation peaks does not correspond to the compound.

encountered in their identification (low area, ration different from standard).

3.2.2. Quantification of carboxylic acids

The quantification of ten carboxylic acids identified in BL-1 was carried out by IC-MS/MS. Calibration curves were built thanks to the analysis of standard samples. The parameters of calibration curves are summarized in Table 2, along with their respective uncertainties calculated with repeatability tests. Most of carboxylic acids had a LOQ of 0.05 ppm except for malic and butyric acids (<0.05 ppm), lactic and propionic acids (0.1 ppm) and acetic acid (0.5 ppm). The concentrations of acids in BL-1 sample varied from very low concentrations (malic, maleic, butyric, propionic, succinic acids) to concentrations superior to 5 g L^{-1} (formic, acetic, lactic acids). It should be noted that the IC-MS/MS allows to discriminate, identify and quantify acids with similar retention times.

Table 2
IC-MS/MS analysis – Concentration, limit of quantification, retention time and uncertainties for 10 carboxylic acids previously identified in BL-1.

	Retention time (min)	LOQ (mg/L)*	BL-1 concentration (g/L)**	Uncertainties (mg/L)
Maleic acid	22.7	0.05	<LOQ	–
Acetic acid	8.3	0.5	6.5	±0.2
Butyric acid	11.5	<0.05	0.005	±0.002
Formic acid	9.4	0.05	6.56	±0.07
Oxalic acid	24.1	0.05	1.3	±0.1
Glycolic acid	8.1	0.05	2.84	±0.06
Lactic acid	7.7	0.1	5.9	±0.5
Malic acid	21.3	<0.05	0.48	±0.02
Propionic acid	10.1	0.1	0.1	±0.01
Succinic acid	21.2	0.05	0.71	±0.02

* In diluted samples.
** In raw liquor (before dilution).

3.2.3. Identification of unknown compounds

To further investigate the liquors composition, a full scan acquisition was realized on BL-1, as described in the materials and methods section. The *m/z* obtained are summarized in Table ESI-S5 along with identification hypotheses found with NIST (National Institute of Standards and Technology) data base assuming that compounds were detected in their deprotonated form. For instance, 97 and 191 *m/z* values were detected in BL-1. According to NIST, sulfuric and phosphoric acids have a molecular weight of 98 g/mol and citric acid of 192 g/mol. These three hypotheses were directly checked by analyzing standards and spiked BL-1. Transitions were optimized with standards to use their fragmentation information in analytical methods. The analysis of the spiked sample aimed to confirm the retention time in the BL-1 sample as the matrix effect for these compounds were not previously investigated. The increase in peak intensity in the spiked sample confirms the identification, whereas the appearance of a second peak in the spiked sample cancels

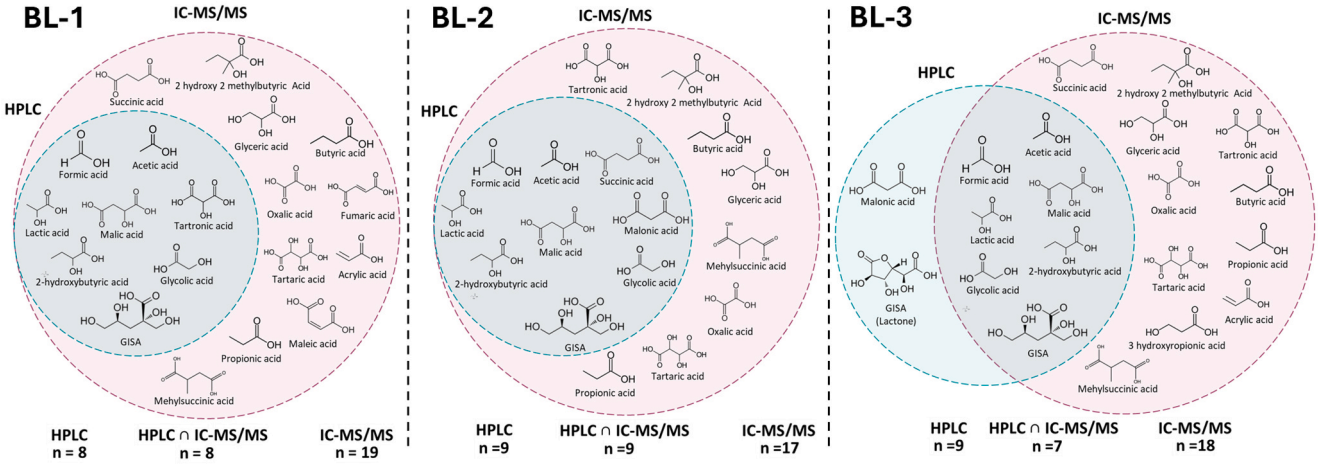

Fig. 8. Venn diagrams for the aliphatic acids present in each type of liquor. HPLC-DAD (light blue); IC-MS/MS (purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

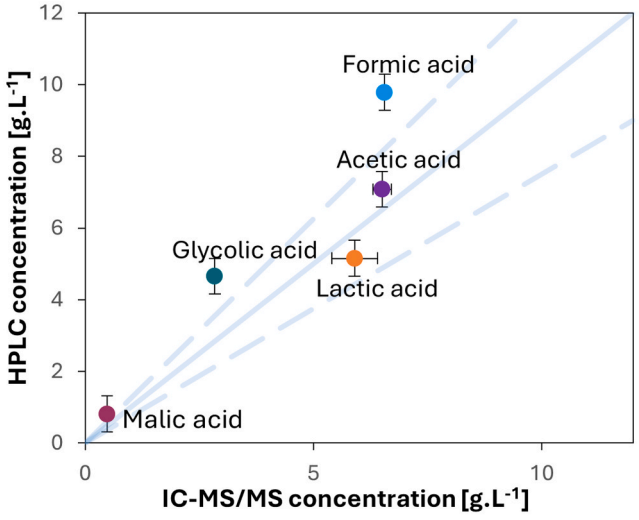

Fig. 9. Parity plot of concentration values between HPLC-DAD and IC-MS/MS methods.

the identification hypothesis. A correct identification was obtained for phosphoric, sulfuric and citric acids in the BL-1 sample. Even though the triple quadrupole allows using fragmentation information to confirm identification hypotheses, the *m/z* results were obtained in low resolution quality. To further investigate the presence of other molecules, a high-resolution mass detector such as quadrupole time-of-flight could be more suitable.

3.3. Comparison between HPLC-DAD and IC-MS/MS

3.3.1. Analysis of carboxylic acids

In general terms, the IC-MS/MS technique allowed to accurately identify a higher number of carboxylic acid compounds in each of the liquor samples compared to HPLC-DAD. Indeed, between 17 (BL-2) and 19 (BL-1) carboxylic acids can be identified by the first technique and between 8 (BL-1) and 9 (BL-2, BL-3) by HPLC analysis. Even though on average the same number of carboxylic acids were effectively identified with each chromatographic technique, their distribution varies according to the sample analyzed. For liquor BL-3, the techniques seem to be complementary, while for BL-1 and BL-2 the IC-MS/MS analysis seems to be sufficient to unveil most of the compounds present in the sample. By combining the two techniques, it was possible to accurately identify and confirm the presence of at least 19, 17 and 20 aliphatic molecules, respectively for BL-1, BL-2 and BL-3. Fig. 8 illustrates the complementarity of HPLC-DAD and IC-MS/MS techniques.

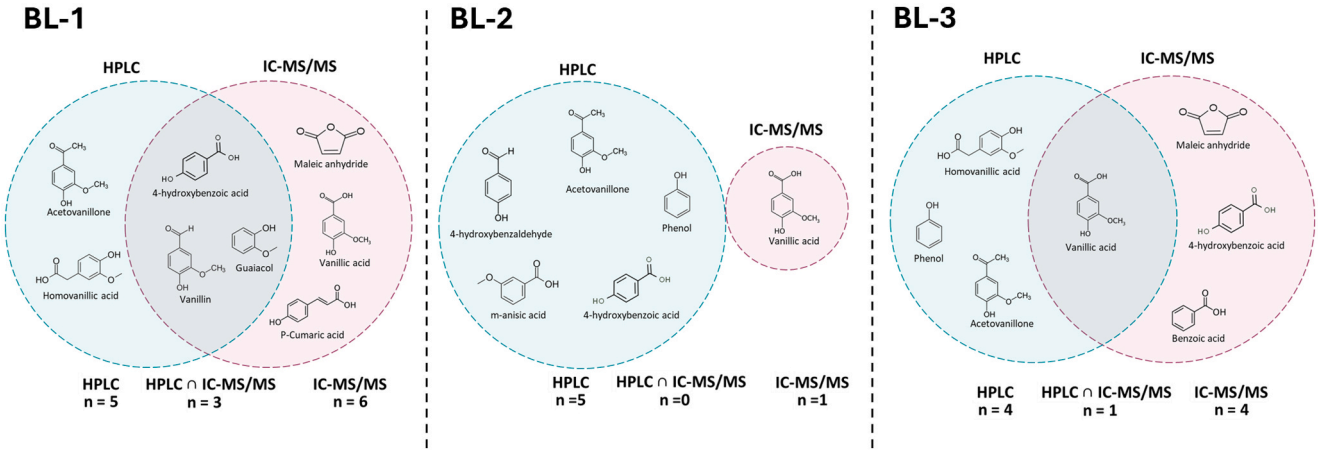

Fig. 10. Venn diagrams for the phenolic molecules present in each type of liquor. From left to right BL-1, BL-2 and BL-3. HPLC (light blue); IC-MS/MS (purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Among the 10 aliphatic acids accurately quantified by IC-MS/MS, only 5 were also found and quantified in the BL-1 by HPLC-DAD technique. Fig. 9 shows a parity plot showing the results of the quantification of the molecules by the two chromatographic techniques. The graph shows that the values of the quantification are consistent and are in the same order of magnitude for both techniques. Moreover, the values estimated for 3 of the 5 molecules studied are within the 20 % range (dashed lines). Still, there seems to be a trend to overestimate the concentration of glycolic and formic acid quantified by HPLC, or to underestimate these concentrations by IC-MS/MS. One should note that the pH of sample is different from one technique to another (acid pH for HPLC and alkaline pH for IC-MS/MS) and also that the sample preparation is different between techniques (dilution by ten, acidification and filtration of precipitate before HPLC, versus dilution by 1000 and filtration before IC-MS/MS). This could induce some bias in the analysis of some of the aliphatic acids.

3.3.2. *Analysis of phenolic compounds*

In contrast to the results for the carboxylic acids, where most compounds could be confirmed with IC-MS/MS, the phenolic compounds were rather equally or mostly confirmed by HPLC analysis. For liquors BL-1 and BL-3, it seems that the use of both methods is required for a good description of the media in terms of aromatic compounds, whereas for liquor BL-2 HPLC is the dominant technique for its correct analysis (Fig. 10). It seems that this difference between the two groups of molecules, carboxylic and phenolic acids, in terms of IC-MS/MS analysis is linked to the detection limits. Indeed, of the 31 compounds studied by this technique (Table 2), the acids have an occurrence of 4 incidents in which the area is less than 3 times the peak of the blank, versus 11 for the aromatic compounds (all liquors included). This limitation in quantification was also evidenced in the HPLC analyses, in which at least 5 compounds (gallic acid, protocatechuic acid, catechol, phthalic acid and p-coumaric acid) were qualitatively corroborated without ever being able to be effectively quantified (*i.e.* traces) in any of the liquors (see Table ESI-S4).

In general terms, it seems that the two techniques are rather complementary for the identification of both aliphatic and phenolic compounds in black liquor samples. HLPC-DAD allows the detection and quantification of semi-polar aliphatic and phenolic compounds but is limited for the analysis of very polar compounds whereas IC-MS/MS is efficient for the detection and analysis of polar compounds but not for phenolics with lower polarity. However, a systematic limitation was also identified in both techniques for the accurate quantification of phenolic compounds, most probably linked to the low concentration of these compounds in the medium. Nonetheless, regarding the quantification of the concentration of carboxylic acids in the liquors, it was observed that the two techniques present similar values, and these are within the normal ranges described in the literature for this type of Kraft liquor from Softwood.

4. Conclusions

Methods of two chromatographic techniques, HPLC-DAD and IC-MS/MS were successfully developed and employed for the accurate detection and quantification of various aliphatic and phenolic species in black liquor. The range of values found by HPLC-DAD seems to be in agreement with the literature and the treatments applied to each of the liquors. By HPLC analysis with DAD detection, 8 to 9 aliphatic molecules were identified resulting in concentrations of 47.12, 78.06 and 81.57 g L^{-1} for liquors 1, 2 and 3 respectively. The differences in concentration among the liquors could be explained a higher concentration in dry matter in 2 and 3 with respect to 1, between 11 and 20 % less dry matter. On the contrary, the concentration of phenolics is more important in BL-1 (1.38 g L^{-1}) than in BL-2 (0.23 g L^{-1}) and BL-3 (0.41 g L^{-1}) due to the fact that the two latter were delignified.

On the other hand, the IC-MS/MS method seems to be slightly more effective than HPLC in the identification of carboxylic acids, with an average of 18 molecules identified. However, it can be said that as far as phenolic molecules are concerned, the two methods are rather complementary. It was also observed that in general IC-MS/MS was somewhat more limited than HPLC due to quantification limits. This, as addressed above, can be leveraged by a high-resolution mass detector or by adapting the detection ranges. However, the latter alternative has the major challenge of establishing a wider range of concentration quantification without loss of resolution or causing detector saturation, while keeping a low uncertainty.

The analysis of complex mixtures coming from biomass and biomass waste, such as black liquors, requires the application of complementary chromatography techniques to detect and identify as much compounds as possible. In the future, the development of coupled detection (for example DAD and MS/MS) and mixed mode columns, would be necessary.

CRediT authorship contribution statement

Laura Reyes: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Caroline Bourgeois:** Writing – review & editing, Methodology, Investigation, Data curation. **Guillaume Gautier Renard:** Investigation. **Patrick Jame:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Xavier Saupin:** Writing – review & editing, Methodology, Investigation, Data curation. **Clémence Nikitine:** Supervision. **Léa Vilcocq:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Data curation, Conceptualization.

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.

Acknowledgments

The authors address their recognition to the Paper Technical Center (CTP), The FCBA technological institute and Smurfit Kappa members: Frédérique Bertaud, Sandra Tapin-Lingua and Jérôme Moindrot for black liquor supply and intellectual input. Ircatech platform is acknowledged for their help on ICP analysis. Kai Szeto (CP2M) is acknowledged for his help on CHNS analysis. Pierre Bolteau (CP2M) is acknowledged for his work on liquors characterization. Nicolas Duguet (ICBMS) and Valentin Fiancette (ICBMS) are acknowledged for the synthesis of gluco-isosaccharinic acid.

Laura Reyes thanks the Ecole Doctorale de Chimie de Lyon for her PhD grant. This work was also supported by the LABEX iMUST of the University of Lyon (ANR-10-LABX-0064), created within the « Plan France 2030 » set up by the French government and managed by the French National Research Agency (ANR).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2024.124442.

Data availability

The data supporting this article has been included as part of the ESI.

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