



A validated chiral LC-MS/MS method for enantioselective quantification of aromatic lactic acids in human faeces

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ABSTRACT

Bifidobacterium, a key genus of the infant gut microbiome, produces D- and L- enantiomers of aromatic lactic acids that may influence early-life immune development through stereochemistry-dependent biological activity. No validated analytical methods currently enable their accurate enantio-separation and quantification in human biological samples. We report the first validated, targeted, liquid chromatography-mass spectrometry method using a chiral column for the enantioselective separation and quantification of D- and L- forms of phenylactic acid (PLA), 4-hydroxyphenylactic acid (4OH-PLA), and indolelactic acid (ILA) in faecal samples. The method achieves baseline separation of all enantiomers within 10 min, with resolution values of 2.66 (PLA), 1.77 (4OH-PLA), and 2.42 (ILA). Solid-phase extraction reduces matrix effects (>80 %) and improves analyte recovery (>80 %). Limits of quantification range from 2.9 to 6.7 ng mL⁻¹, and calibration curves show excellent linearity (R² > 0.99). Inter- and intra-day precision expressed as %RSD are < 15 % for most analytes. The method was successfully applied to infant faecal samples, enabling sensitive and stereospecific quantification of aromatic lactic acids, thus establishing a foundation for exploring the biological relevance of these enantiomers in infant health.

1. Introduction

Metabolites produced by the gut microbiota are important mediators of host-microbial cross-talk [1,2]. Examples of such molecules include phenylactic acid (PLA), 4-hydroxyphenylactic (4OH-PLA) acid and indolelactic acid (ILA), which are produced from the catabolism of phenylalanine, tyrosine and tryptophan, respectively, by certain *Bifidobacterium* species in the human gut via an aromatic lactate dehydrogenase (ALDH) [3] as well as by other lactic acid bacteria in fermented foods [4]. These metabolites, also called aromatic lactic acids, have been increasingly recognized for their bioactive properties including antimicrobial and immune modulating effects [5,6]. Notably, they can influence human immune function and gut barrier integrity by activating the Hydroxycarboxylic acid Receptor 3 (HCA3) and the aryl hydrocarbon receptor (AhR) [4,7]. Measuring aromatic lactic acids in infant faecal samples provides a non-invasive means to assess *Bifidobacterium* metabolic activity within the gut lumen and its health effect during early life.

Recently, an in vitro study showed that *Bifidobacterium* species produce a mixture of D- and L- enantiomers of aromatic lactic acids, with the α-carbon in the aromatic lactic acids acting as a stereogenic centre. The study further demonstrated that D-enantiomers of aromatic lactic acids

possess a higher HCA3 activating potential than their L- counterparts [8]. This phenomenon, known as stereoselectivity, occurs when one enantiomer has a preferable three-dimensional spatial orientation for receptor site interaction, leading to greater ligand-receptor binding affinity. This may have consequential downstream effects influencing absorption, metabolism, and excretion [9].

Several analytical methods to quantify aromatic lactic acids in biological matrices have been previously published. These include chromatography-based separation methods such as Gas Chromatography - Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC) and Ultra-high-Pressure Liquid Chromatography tandem Mass Spectrometry (UPLC-MS/MS) [10–14], as well as electrokinetic separation methods including Capillary Electrophoresis [15]. However, these methods do not provide enantiomeric resolution and therefore cannot distinguish between the D- and L- forms of these analytes. Sakurai et al. [8] developed a HPLC-MS method to analyse the optical isomers of PLA, 4OH-PLA, and ILA in bacterial cell culture supernatants. However, this method was neither quantitative nor suitable for high-throughput analysis, as it required different chiral columns and mobile phases for each analyte.

Therefore, a single analytical method to precisely quantify aromatic

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lactic acid enantiomers in human biological matrices and investigate their metabolic fate is lacking.

The present study aimed to develop and validate a single, fully quantitative LC-MS/MS method for the direct enantiomeric separation, identification and quantification of PLA, 4OH-PLA, and ILA enantiomers in infant faeces. A cinchona alkaloid-based chiral stationary phase (CSP) was selected for its strong enantioselectivity toward acidic chiral compounds via a weak anion exchange mechanism. The optical identity of each analyte was confirmed using enantiomerically pure reference standards. Furthermore, a Solid Phase Extraction (SPE) sample preparation strategy using Oasis® Hydrophilic-Lipophilic Balance (HLB) cartridges was optimized to minimize matrix effects (ME) and maximize analyte recovery (RE) from faecal samples, ensuring high sensitivity and reproducible quantification. Finally, to demonstrate real-world applicability, the validated LC-MS/MS method was applied to nine infant faecal samples to quantify the D- and L- enantiomers of aromatic lactic in this complex matrix using a single analytical workflow. This work provides a sensitive, quantitative, and high-throughput analytical method that enables the scientific community to study relationships between aromatic lactic acids enantiomers, gut microbial species, and receptor-dependent immune-modulating effects.

2. Experimental section

2.1. Chemicals and materials

Optima LC-MS grade acetonitrile (ACN), methanol (MeOH), formic acid (FA), phosphoric acid (H_3PO_4), triethylamine (TEA), and ammonium hydroxide (NH_4OH) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). DL-PLA, DL-4OH-PLA, DL-ILA, and L-PLA were obtained from Merck (St. Louis, Missouri, USA). (R)-3-(4-Hydroxyphenyl)-2-hydroxypropionic acid (D-4OH-PLA) was purchased from Activate Scientific GmbH (Prien, Germany). (R)-2-Hydroxy-3-(1H-indol-3-yl) propanoic acid (D-ILA) was purchased from BLD pharm (Reinbek, Germany). Isotopically labelled standards, including DL-PLA d_3 , DL-4OH-PLA d_3 , and DL-ILA d_3 , were acquired from LGC Standards GmbH (Wesel, Germany). Solid-phase extraction cartridges, Oasis® HLB (60 mg, 30 μm), Oasis® MAX (30 mg, 30 μm), One mL and two mL LCMS Certified Clear Glass 12 $\times$ 32 mm Screw Neck vials were purchased from Waters Corporation (Milford, MA, USA). Milli-Q water (resistivity: 18.2 M Ω -cm, total organic carbon [TOC]: 2.0 ppb) was used for all aqueous preparations and sample dilutions and was obtained from a Milli-Q IQ 700 Water Purification System (Merck, Darmstadt, Germany).

2.2. Standards and solutions

Stock solutions (1 mg mL⁻¹) were prepared by dissolving 4 mg of each standard in 4 mL MeOH. Racemic standards (DL-PLA, DL-4OH-PLA, DL-ILA), and their isotopically labelled analogues (DL-PLA d_3 , DL-4OH-PLA d_3 , DL-ILA d_3) were used for method development and validation, while pure enantiomers (L-PLA, D-4OH-PLA, D-ILA) were used to confirm enantiomeric identity. For racemic standards, each enantiomer had an effective concentration of 0.5 mg mL⁻¹. Working solutions of DL standards and isotopically labelled Internal Standards (IS) mixes were prepared at 0.02 mg mL⁻¹.

2.3. Sample collection and preparation for analysis

2.3.1. Faecal sample collection and pretreatment

Faeces for SPE optimisation and Limit of Detection/Limit of Quantification (LOD/LOQ) calculations were donated by parents of a healthy, 1.5-month-old breastfed infant. Parents collected samples from diapers and stored them at -20 °C at home. Samples were then transported in a cold bag (4 °C) to the Department of Nutrition, Exercise and Sports, University of Copenhagen, and stored at -80 °C until preparation. Prior to analysis, samples were thawed at 4 °C. Approximately 100 mg faeces

were transferred to a 2 mL Eppendorf tube and mixed with 400 μL Milli-Q water (1:4 w/v dilution). The mixture was vortexed for 30 s, then centrifuged at 16,000 $\times$ g (RCF) for 5 min at 4 °C. The supernatant was transferred to a new tube and centrifuged twice more under the same conditions. Finally, 100 μL of the clarified supernatant was mixed with 100 μL Milli-Q water (1:1 v/v dilution), resulting in a final tenfold dilution relative to neat faeces. Samples were stored at -80 °C until analysis with SPE (see below).

For method application, nine faecal samples were obtained from three infants enrolled in the MOTILITY Mother-Child cohort [16]. The MOTILITY study has been approved by the Research Ethics Committee for the Capital Region of Denmark (H-21063016). All parents provided informed consent. As previously described [16], infant faecal samples were collected biweekly from 2 to 3 days after birth until 12 months of age at home, and immediately stored at -20 °C in the freezer of the participants. Samples were transported in a cooler bag with freezer packs to the study site. Faecal samples (100–500 mg) were diluted in Milli-Q water at a 1:1 ratio (w/v) and homogenized. The homogenates were centrifuged at 12,000 rpm for 5 min, and the resulting supernatants were transferred to cryotubes and stored at -80 °C. On the day of analysis, samples were thawed at 4 °C, a 50 μL aliquot was diluted with 200 μL of Milli-Q water and centrifuged at 16,000 $\times$ g and 4 °C for 5 min to yield a tenfold dilution before SPE (see below).

2.3.2. Optimized SPE-based sample preparation in faecal samples

Initially, a protein precipitation procedure was performed by mixing 50 μL of diluted faecal sample with 375 μL of MeOH. The mixture was incubated at -20 °C for 20 min to induce protein precipitation, followed by centrifugation at 16,000 $\times$ g and 4 °C for 10 min. The supernatant was transferred to a clean Eppendorf tube, evaporated to dryness under vacuum, and reconstituted in 200 μL of MeOH prior to analysis. To evaluate ME and RE, 10 μL of IS solution (0.02 mg mL⁻¹) was spiked before addition of MeOH (pre-spike) and after reconstitution (post-spike). Due to high ME the precipitation procedure was replaced by SPE.

For SPE, 50 μL of diluted sample was acidified with 300 μL of 4 % (v/v) aqueous H_3PO_4 and vortexed for 10 s. SPE was carried out using Oasis® HLB cartridges. The cartridges were conditioned with 2 mL of MeOH and equilibrated with 2 mL of Milli-Q water. The acidified sample was loaded under gentle vacuum. To optimize wash and elution conditions, two wash solvents (5 % MeOH in Milli-Q water and Milli-Q water only) and two elution solvents (100 % MeOH and 5 % NH_4OH in MeOH) were evaluated. For assessment of ME and RE, 10 μL of IS solution (0.02 mg mL⁻¹) was added either prior to extraction (pre-spike) or post-reconstitution (post-spike) as explained below.

The final optimized SPE protocol applied to the MOTILITY cohort was as follows: All sample handling steps were performed on ice unless otherwise stated. 50 μL of diluted faecal sample was spiked with 10 μL of IS mix (0.02 mg mL⁻¹), acidified with 300 μL of 4 % H_3PO_4 (v/v), and vortexed for 10 s. The sample was loaded onto conditioned and equilibrated HLB cartridges (2 mL MeOH, 2 mL Milli-Q water respectively). After loading, the cartridges were washed with 1 mL of Milli-Q water, and analytes were eluted with two sequential volumes of 750 μL of 5 % NH_4OH in MeOH. Elution was performed under gravity flow, with low vacuum (<5 in Hg) applied only after partial solvent drainage to ensure complete eluent recovery. The eluate was evaporated to dryness under vacuum centrifugation, reconstituted in 200 μL of MeOH, vortexed (10 s), and centrifuged at 16,000 $\times$ g and 4 °C for 5 min. The supernatant was transferred to an LC vial and stored at -20 °C until LC-MS analysis.

For metabolite quantification, an IS corrected external calibration curve was prepared by spiking MeOH with a constant amount of IS mix (1000 ng mL⁻¹) and authentic standards at 2.5, 5, 12.5, 25, 50, 125, 250, 375, 500, and 1000 ng mL⁻¹ per analyte enantiomer. The ratio of the authentic standard intensity to the IS intensity and known concentration was then used to perform linear regression using least squares fitting and the concentration calculated from the resulting line equation.

2.4. LC-MS/MS analysis of aromatic lactic acid enantiomers

2.4.1. Chromatographic method optimisation

Chromatographic separation of aromatic lactic acids was achieved using an Acquity H—Class Ultra Performance Liquid Chromatography (UPLC) (Waters Corporation), equipped with a CHIRALPAK QD-AX column (150 × 4.6 mm I.D., 5 µm particle size; Chiral Technologies Europe). The column temperature, flow rate and mobile phase composition were systematically optimised to achieve baseline separation, improved peak shape and sensitivity.

The chromatographic method was optimized using a One-Factor-at-a-Time (OFAT) approach with each condition tested in triplicate injections of standard solutions. Seven ACN:MeOH ratios (100:0, 90:10, 70:30, 50:50, 30:70, 10:90 and 0:100, v/v) were tested at a constant 0.5 % FA concentration to evaluate elution strength and analyte retention. FA was evaluated at 0.125, 0.25, 0.375, 0.5 and 0.625 % (v/v) under a fixed ACN:MeOH ratio of 70:30 (v/v). TEA was tested at 0.25 %, 0.5 %, 0.75 % and 1 % (v/v) under a constant mobile phase composition of ACN:MeOH (70:30, v/v) with 0.5 % FA. Flow rates of 0.6, 0.8, 1 and 1.1 mL min⁻¹ and column temperatures of 20 °C, 25 °C, 30 °C, 35 °C and 40 °C were also evaluated.

Chromatographic performance was evaluated using key parameters, including resolution (Rs), retention time (t_R), and Tailing Factor (TF). Rs was calculated using Eq. (1) where t_{R1} and t_{R2} are the t_Rs of the first and second eluting peaks, and FWHM 1 and FWHM 2 are their respective full widths at half maximum. TF was determined using Eq. (2) where a is the distance from the peak's leading edge to its midpoint, and b is the distance from the midpoint to the trailing edge, both measured at 5 % of the peak height. Signal response was also a critical consideration during optimization.

$$R_s = \frac{(t_{R2} - t_{R1})}{1.7 * 0.5 * (FWHM1 + FWHM2)} \quad (1)$$

$$TF = \frac{(a + b)}{2a} \quad (2)$$

The final optimised chromatographic conditions used to analyse all six analytes were selected based on the best balance of Rs, t_R, TF, and sensitivity. These included an isocratic mobile phase consisting of ACN:MeOH (70:30, v/v) with 0.375 % FA, a flow rate of 1 mL min⁻¹, a column temperature of 25 °C, no addition of TEA, and a column equilibration time of 30 min with the optimised mobile phase. Standard solutions and faecal extracts were analysed under these final conditions.

2.4.2. Mass spectrometric detection and MRM transition optimization

Mass spectrometric analysis was carried out using a VION Quadrupole Time-of-Flight Ion Mobility Spectrometry (QTOF-IMS) High Resolution Mass Spectrometer (HRMS) (Waters Corporation), interfaced with the UPLC system via a LockSpray™ Electro Spray Ionization (ESI) source (Waters Corporation). All analyses were conducted in negative mode and in triplicates. Various instrument parameters were evaluated using an OFAT approach. Desolvation temperature, cone gas flow, desolvation gas flow, capillary voltage, source temperature, and cone voltage were each varied across the following ranges: 310–600 °C, 50–200 L h⁻¹, 400–800 L h⁻¹, 1–5 kV, 90–150 °C, and 10–80 V, respectively. Final parameter selection used to analyse all six analytes was based on maximum signal response and reproducibility across multiple injections of standard solutions. The optimised tune parameters were as follows: capillary voltage 1.00 kV, cone voltage 40 V, source temperature 130 °C, desolvation temperature 600 °C, cone gas flow 50 L h⁻¹, and desolvation gas flow 1000 L h⁻¹.

Fragmentation patterns were acquired in Mass Spectrometry Elevated Energy (MS^E) mode in negative ESI, with low-energy (6.00 eV) and high-energy (20–30 eV) collision settings. Fragment ions were confirmed by comparison with high-resolution MS/MS spectra from the mzCloud spectral library (www.mzcloud.org) and the most abundant

fragment was selected for quantification. All fragments/transitions were extracted at 10 ppm mass accuracy. Fragments, collision energies and scan times for each analyte of interest are listed in Supporting Information (Table S1).

2.5. Method validation

2.5.1. Calculation of matrix effects (ME) and recovery (RE)

ME and RE were evaluated using post-spiked, pre-spiked, and neat blank samples. The isotopically labelled IS mix was added to achieve two concentration levels (250 and 2500 ng mL⁻¹) of the isotopically labelled analytes in all sample types. For the post-spiked samples, IS mix was spiked into the faecal extracts after drying and reconstitution. In the pre-spike samples, the IS mix was added to infant faecal extracts prior to SPE or MeOH protein precipitation as previously mentioned. The neat blank samples were prepared by adding the IS mix to pure solvent (MeOH) at concentrations of 250 and 2500 ng mL⁻¹ to serve as a reference free of matrix interferences. All samples were prepared and analysed in triplicate to ensure reproducibility. The ME and RE were calculated using the following equations:

$$ME = \frac{\text{Post spike}}{\text{Neat blank}} * 100 \quad (3)$$

$$RE = \frac{\text{Pre spike}}{\text{Post spike}} * 100 \quad (4)$$

2.5.2. Limit of linearity (LOL), limit of detection (LOD), and limit of quantification (LOQ)

Calibration curves were generated by spiking pooled faecal extracts with IS at final concentrations of 2.5, 5, 12.5, 25, 50, 125, 250, 375, 500, and 1000 ng mL⁻¹ per analyte enantiomer. All samples were prepared in triplicate. Instrument response (Y) was plotted against known concentration (X), and linear regression was performed.

LOD and LOQ values were calculated on the lowest five concentrations (2.5, 5, 12.5, 25 and 50 ng mL⁻¹) using Eqs. (5 and 6) respectively, where *b* is the slope of the calibration curve and *s* is the standard deviation of the intercept.

$$LoD = 3.3 \times \frac{s}{b} \quad (5)$$

$$LoQ = 10 \times \frac{s}{b} \quad (6)$$

The LoL was assessed by calculating relative residuals across all calibration points, including higher concentrations (125, 250, 375 and 1000 ng mL⁻¹). The LoL was defined as the highest concentration with a residual within ±15 %, following FDA and EMA bioanalytical validation guidelines [17,18].

2.5.3. Specificity and precision

Specificity was evaluated by analysing solvent (MeOH) blanks and analyte-free matrix samples to assess potential endogenous or exogenous interferences at the retention times and accurate masses of the target analytes.

The precision of the LC-MS/MS method was evaluated in a single laboratory using SPE extracted pooled faecal water samples spiked with deuterated analytes (IS) at 10 ng mL⁻¹, 125 ng mL⁻¹, and 1000 ng mL⁻¹ concentrations per analyte enantiomer. Intra-day precision (%RSD) was calculated from the average peak area of six consecutive injections of each sample on the same day, while Inter-day precision (%RSD) was assessed over three different days within a five-day period.

3. Results and discussion

We aimed to develop and validate a targeted LC-MS/MS method for the absolute quantification of aromatic lactic acid enantiomers in infant

faeces. A sample preparation method was optimized to minimise ME and maximise RE. Moreover, an OFAT approach was chosen to systematically optimise both chromatographic separation and mass spectrometric detection, with the goal of achieving optimal resolution, sensitivity, and reproducibility. Finally, the method was validated using established bioanalytical method validation criteria.

3.1. Sample handling and preparation optimisation

To minimize any potential alteration of the analyte D/L enantiomer ratios, samples were immediately frozen (-20°C), transported at 4°C , stored at -80°C , and extracted on ice. Although literature on the interconversion of aromatic lactic acid enantiomers is lacking, it is known that bacterial species possess amino acid racemases that are able to interconvert D- and L- enantiomers of precursor aromatic amino acids [19], this could potentially alter sample D/L ratio. Therefore, strict thermal control was employed to suppress bacterial activity. While we cannot fully exclude handling-induced interconversion, these precautions are expected to mitigate bacterial enzymatic activity.

An SPE based sample preparation method was developed for reducing ME and increasing sensitivity. As a matter of fact, a simple sample preparation method consisting of 1:4 (w/v) dilution of faecal samples in Milli-Q water followed by protein precipitation using $375\ \mu\text{L}$ of cold methanol (added to $50\ \mu\text{L}$ of diluted sample) indicated severe ionization suppression due to ME ($> 70\%$ suppression for all analytes), suggesting the need for a more thorough sample clean-up (Fig. 1a). Moreover, peaks corresponding to L-PLA d_3 exhibited significant asymmetry, precluding reliable peak integration. To optimize sample cleanup, two SPE cartridges (Oasis® HLB and MAX) were initially compared. The HLB cartridge demonstrated superior performance, achieving higher RE and greater reduction in ME, and was therefore selected for subsequent analyses (data not shown). Using a generic SPE HLB protocol involving conditioning with $2\ \text{mL}$ MeOH and $2\ \text{mL}$ Milli-Q water, sample loading, washing with $1\ \text{mL}$ of 5% MeOH in water, and elution with pure MeOH, ME was reduced to 80% for all analytes (Fig. 1c) but RE significantly decreased (Fig. 1d). This was due to analyte loss during the washing step since MeOH-containing washes were found to prematurely elute analytes as confirmed by LC-MS analysis of wash

fractions (Supporting Information Fig. S1). Optimizing the wash step by replacing 5% MeOH in water with water alone and eluting with 5% NH_4OH in MeOH instead of pure MeOH further enhanced RE to $>80\%$ for most analytes (Fig. 1e). The basic environment created by NH_4OH increases the polarity of the analytes, disrupting their interactions with the stationary phase of the HLB sorbent and facilitating their elution. The optimised wash and elute conditions resulted in ME% and RE% $>80\%$ for most analytes, consistent with accepted bioanalytical performance criteria [17,18].

3.2. Chromatographic method optimization

3.2.1. Chiral column and mobile phase

The enantioseparation of the aromatic lactic acids was achieved using a cinchona alkaloid-based CSP, CHIRALPAK QD-AX, which has previously demonstrated excellent performance in the separation of weak acids via weak anion-exchange mechanisms [20,21]. The main chiral selector of the CSP is the quinuclidine moiety, which contains a tertiary amino group that becomes protonated under acidic conditions. The resulting positively charged nitrogen facilitates long-range electrostatic interactions with the negatively charged functional groups of the acidic analytes. Additionally, hydrogen bonding may occur between the analyte's carbonyl group and the CSP's amide functionality, while π - π stacking interactions can take place between the aromatic moieties of both the analyte and the CSP. (Supporting information Fig. S2). This potential multi-interaction mechanism justified the choice of this column for enantioseparation of aromatic lactic acids rather than the widely used polysaccharide-based CSPs, which rely mainly on hydrogen bonding [22,23].

Non-aqueous polar organic mobile phases, typically composed of ACN and MeOH, are preferred when working with this class of CSPs [20, 21,24] since they provide appropriate solvation properties while maintaining the protonation state of the selector. We selected isocratic flow based on manufacturer recommendations and previous reports on similar CSPs [20,21]. Maintaining a constant mobile phase composition preserves ionic interactions between the CSP's protonated quinuclidine moiety and the analyte's carboxyl group, which are essential for chiral recognition.

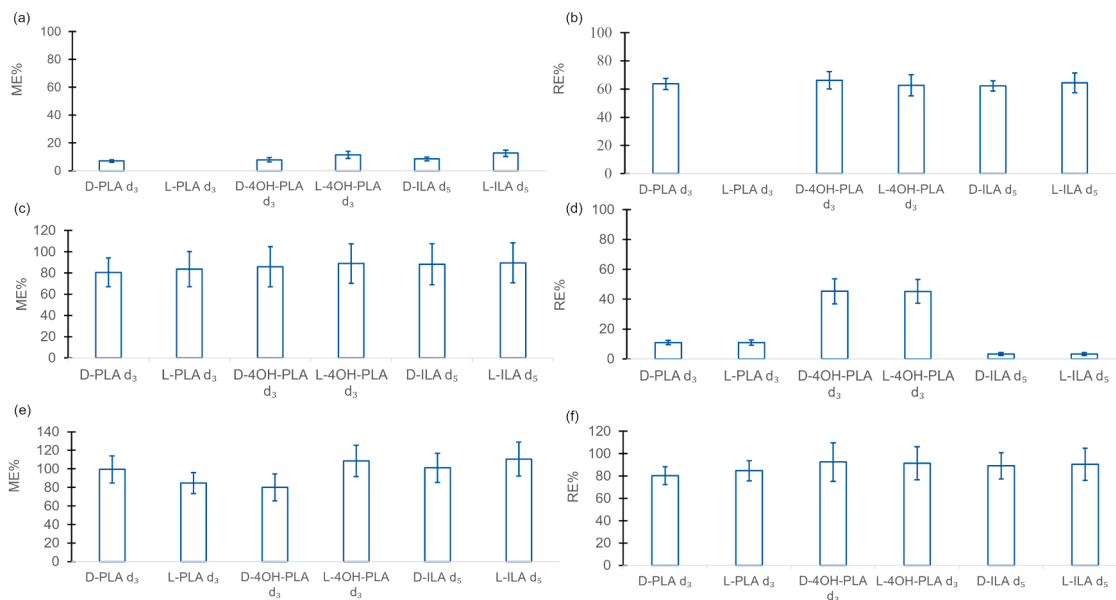


Fig. 1. (a) ME% and (b) %RE% of deuterated aromatic lactic acid enantiomers obtained using protein precipitation with MeOH. (c) ME% and (d) RE% achieved with SPE using 5% MeOH in water wash and eluting with MeOH only. (e) ME% and (f) %RE achieved with SPE using Milli-Q water only wash and eluting with 5% NH_4OH in MeOH. Error bars represent propagated standard deviation based on triplicate measurements ($n = 3$). Full numerical data in Supporting Information (Table S2).

3.2.2. ACN:MeOH ratio optimization

To optimize chromatographic performance, seven mobile phase mixtures with varying ACN:MeOH ratios (100:0 to 0:100) (v/v) were evaluated to ensure sufficient elution strength and maintain the protonated form of the CSP. A constant FA concentration of 0.5 % was kept since, in the absence of FA, none of the target analytes were eluted within 15 min.

The highest Rs between peaks corresponding to D and L enantiomers was observed for 4OH-PLA and ILA using 70:30 ACN:MeOH. For PLA the highest Rs was achieved with 10:90 ACN:MeOH mobile phase, although 70:30 still showed a good Rs. (Fig. 2a).

Peak tailing factors (TF) were generally far from the ideal value of 1, indicating suboptimal peak symmetry for all analytes. TF values varied depending on the ACN:MeOH ratio, with no single condition optimising all compounds simultaneously. Notably, 0:100 ACN:MeOH resulted in significant tailing for L-PLA and L-ILA, suggesting this condition should be avoided. Overall, a balanced composition of ACN and MeOH improved chromatographic performance compared to pure solvents, but differences remained between analytes (Fig. 2b).

The t_R of the analytes decreased with increasing MeOH content, reaching their lowest values at a 30:70 ACN:MeOH ratio, except for D-ILA and L-ILA where further increasing the MeOH content beyond 70 % resulted in a slight increase in t_R (Fig. 2c). Nonetheless a 70:30 ACN:MeOH ratio allowed to obtain $t_R < 10$ min for all analytes.

Sensitivity generally decreased as the ACN:MeOH ratio approached 50:50 (Fig. 2d). Although ACN:MeOH (70:30, v/v) yielded the second-lowest intensity for most metabolites, it was selected as the optimal mobile phase because it provided the maximum Rs for 4OH-PLA and ILA while maintaining acceptable Rs for PLA, and allowed to elute all the analytes within 10 min.

Mobile phases consisting entirely of ACN resulted in prolonged t_R , as previously reported by Tanacs et al. [24]. Additionally, these conditions led to low Rs for all analytes, and a low sensitivity for D- and L-PLA.

The balance between ACN and MeOH was critical for optimal separation of aromatic lactic acid enantiomers, as each solvent interacts differently with both the analytes and the CSP. A ratio of 70:30 (ACN:MeOH) provided the best compromise, possibly where ACN enhanced ionic interactions and disrupted π - π stacking, while MeOH suppressed hydrogen bonding and nonspecific hydrophobic effects [25]. The

increase in retention factors with higher ACN content likely results from reduced solvation of ionizable basic nitrogen groups on the CSP. As a polar aprotic solvent, ACN forms thinner solvation shells around charged sites compared to protic solvents like MeOH. This brings analyte ions closer to the stationary phase, enhancing electrostatic interactions which get stronger as the distance between charges decreases. In contrast, MeOH stabilizes ionic groups more effectively, increasing the effective distance and reducing interaction strength, as previously published [24].

3.2.3. Effect of acidic additive

The effect of an acidic additive on chromatographic performance and sensitivity was investigated to optimize the mobile phase composition (Fig. 3). FA was evaluated at various concentrations at a constant ACN:MeOH ratio (70:30, v/v).

The Rs of PLA and ILA did not remarkably change with varying FA%, whereas 4OH-PLA Rs decreased progressively with increasing FA content (Fig. 3a).

The effect of FA on peak tailing strongly varied among the analytes and thus did not suggest a specific optimal FA%.

As previously demonstrated for an anion-exchange mechanism, t_R decreased with increasing FA concentration (Fig. 3c), due to enhanced elution strength [20,26].

The addition of FA to the mobile phase significantly influenced signal intensity. Signal intensity initially increased with FA concentration, reaching a maximum at 0.375 % (Fig. 3d). However, beyond 0.375 % FA, a decline in signal intensity was observed. This effect might be due to a combination of factors such as improved ionization efficiency, through favourable changes in droplet formation, surface tension, or charge distribution during ESI in contrast to ion suppression caused by excessive FA and reduced deprotonation efficiency of the analyte. Based on these observations, a mobile phase containing 0.375 % FA in ACN:MeOH (70:30, v/v) was deemed optimal for this method because it facilitated the elution of all analytes of interest within 10 min, yielding high sensitivity and allowing acceptable chromatographic performance.

3.2.4. Effect of basic additive

Previous work has reported the benefits of using basic amines as a mobile phase co-modifier [27]. Amines added to an acidic mobile phase

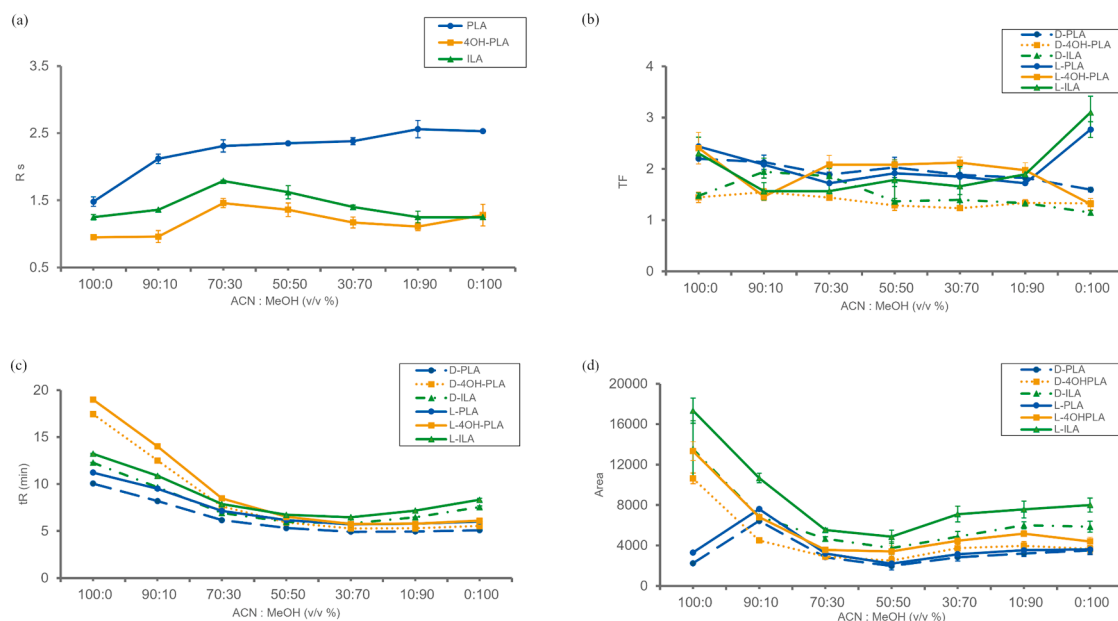


Fig. 2. Effect of varying ACN:MeOH ratio (100:0 to 0:100, v/v) on (a) chromatographic resolution (R_s), (b) Tailing Factor (TF), (c) retention time (t_R), and (d) peak area. Resolution was calculated between D- and L-enantiomers of PLA, 4OH-PLA, and ILA. Values represent the mean of three repeated measurements ($n = 3$), with error bars indicating standard deviation. Full numerical data in Supporting Information (Tables S3–S6).

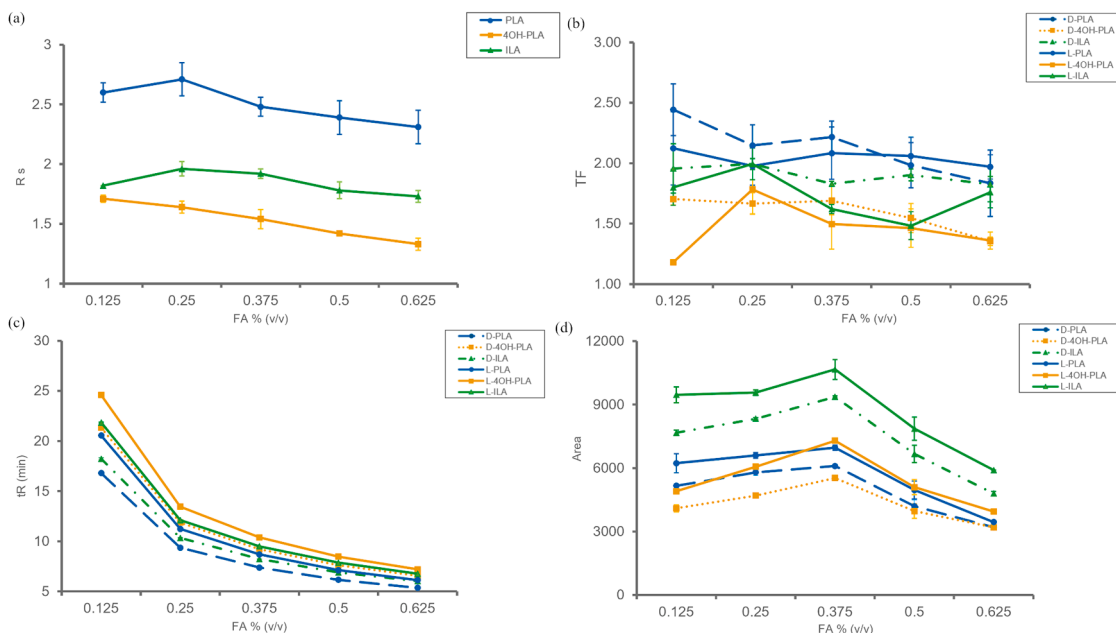


Fig. 3. Effect of varying FA concentration (0.125 %–0.625 %) on (a) chromatographic resolution (R_s), (b) Tailing Factor (TF), (c) retention time (t_R), and (d) peak area. Resolution was calculated between D- and L-enantiomers of PLA, 4OH-PLA, and ILA. Values represent the mean of three repeated measurements ($n = 3$), with error bars indicating standard deviation. Full numerical data in Supporting Information (Tables S7–S10).

become protonated and compete with the CSP for ionic interaction with the deprotonated acidic analytes, thus enhancing the elution strength of the mobile phase. In the study from Xiong et al. [27] using a similar CSP, TEA demonstrated the highest elution strength compared to other amines, probably due to its structural resemblance to the CSP moiety. TEA is also recommended by the column manufacturer guidelines and prior studies using similar CSPs [20,24]. Therefore, various TEA concentrations were tested under a fixed mobile phase composition of ACN: MeOH (70:30, v/v) with 0.5 % FA. The R_s of PLA and 4OH-PLA showed a slight improvement with higher TEA concentrations. In contrast, the R_s of ILA was variable at different TEA concentrations (Fig. 4a). The effect

of TEA on peak tailing strongly varied among the analytes, thus not suggesting a specific optimal TEA% (Fig. 4b). As expected, t_R decreased with increasing amounts of TEA (Fig. 4c). Signal sensitivity, however, was significantly reduced with the addition of TEA, reaching maximum suppression at a 0.5:0.5 FA: TEA (v/v) (Fig. 4d).

The severe negative impact on sensitivity, combined with the minimal effect of TEA on chromatographic performance, led us to exclude the use of a basic additive in the mobile phase.

3.2.5. Effect of flow rate and column temperature

Flow rates between 0.6 and 1.1 mL min^{−1} were evaluated, exhibiting

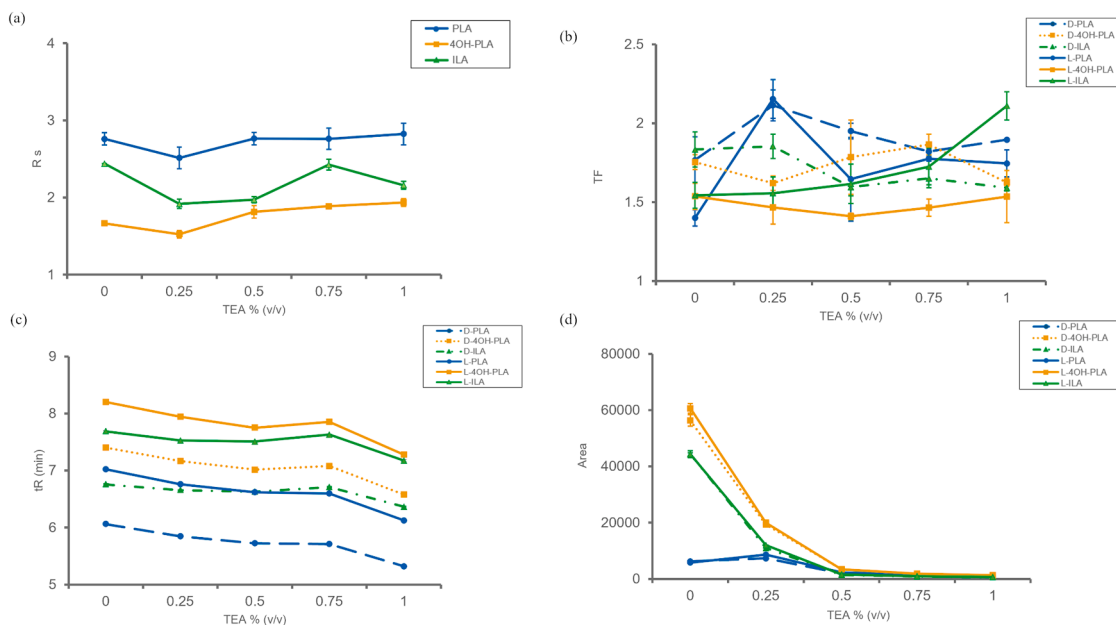


Fig. 4. Effect of varying TEA concentration (0 %–1 %) on (a) chromatographic resolution (R_s), (b) Tailing Factor (TF), (c) retention time (t_R), and (d) peak area. Resolution was calculated between D- and L-enantiomers of PLA, 4OH-PLA, and ILA. Values represent the mean of three repeated measurements ($n = 3$), with error bars indicating standard deviation. Full numerical data in Supporting Information (Tables S11–S14).

minimal impact on R_s (Supplementary Information Table S15 and Fig. S3a). TF was observed to decrease with increasing flow rate, with the most notable impact seen in D-PLA (Supplementary Information Table S16 and Fig. S3b). As expected, higher flow rates decreased t_{RS} (Supplementary Information Table S17 and Fig. S3c). Sensitivity initially increased for all analytes when the flow rate was raised from 0.6 to 0.8 mL min⁻¹. However, further increases in flow rate led to a decline in signal response (Supplementary Information Table S18 and Fig. S3d). This decrease in sensitivity is likely due to reduced ionization efficiency, a well-documented effect at higher flow rates in ESI-based LC–MS systems [28]. The chosen flow rate was 1 mL min⁻¹ since it showed a good compromise between chromatographic parameters and sensitivity, and it is the recommended flow rate by the column manufacturer.

Several column temperatures were tested showing a slight decrease in PLA and ILA R_s with increasing the temperature (Supplementary Information Table S19 and Fig. S4a). In addition, TFs were lowest at lower column temperatures and increased with rising temperature. (Supplementary Information Table S20 and Fig. S4b). t_{RS} decreased only when the temperature was raised from 20 °C to 25 °C, remaining constant with further temperature increases except for L-ILA (Supplementary Information Table S21 and Fig. S4c). Additionally, column temperature had a minor but consistent negative effect on signal intensity, leading to a slight decrease as the temperature increased (Supplementary Information Table S22 and Fig. S4d). Therefore, the manufacturer recommended column temperature of 25 °C was selected for the final optimized chromatographic method, as it provided best R_s , TF, t_{RS} and sensitivity. With a flow rate of 1 mL min⁻¹ at 25 °C, all analytes were eluted within 10 min, achieving favourable resolution, peak shape, and sensitivity.

The final chromatographic conditions included an isocratic elution at a flow rate 1 mL min⁻¹, at 25 °C and using ACN:MeOH, 70:30 v/v, 0.375 % FA as mobile phase. The extracted ion chromatograms (XICs) of the 6 analytes in the final condition are shown in Fig. 5. This is the first method to achieve baseline separation of all aromatic lactic acid enantiomers in under 10 min using a single chromatographic column, representing a > 80 % reduction in run time compared to the only other previously reported method (≥60 min, using three different columns) [8], thereby enabling high-throughput quantification in a complex biological matrix.

3.3. Mass spectrometric parameters optimisation

Mass spectrometric parameters were optimized using an OFAT approach after chromatographic conditions had been finalized to achieve optimal sensitivity. Increasing the desolvation temperature improved sensitivity for all analytes up to 450 °C, likely due to enhanced solvent evaporation, with no significant further gains at higher temperatures (Supporting Information Table S23 and Fig. S5a). Raising the source temperature produced only a slight increase in sensitivity for L-4OH-PLA and ILA enantiomers (Supporting Information Table S24 and Fig. S5b). The desolvation gas flow rate resulted in the highest sensitivity at 600 L hr⁻¹ for all analytes. Higher flows slightly reduced sensitivity, possibly due to spray destabilization (Supporting Information Table S25 Fig. S5c). Conversely, increasing cone gas flow consistently decreased sensitivity (Supporting Information Table S26 and Fig. S5d), likely by dispersing ions before efficient transfer into the MS. Optimal capillary voltages differed among analytes: 1 kV maximized sensitivity for PLA enantiomers, while 3 kV was optimal for 4OH-PLA and ILA enantiomers. Higher voltages reduced sensitivity (Supporting Information Table S27 and Fig. S5e), possibly due to increased in-source fragmentation or discharge effects. Finally, cone voltages between 25 and 40 V provided the highest sensitivity for all enantiomers, with higher voltages leading to signal suppression (Supporting Information Table S28 and Fig. S5f), likely from excessive in-source fragmentation.

For both the analytes of interest and their corresponding isotopically labelled IS, a single fragment ion corresponding to the deprotonated molecular ion $[M-H]^-$ was selected for detection and quantification, allowing all compounds to be analysed in a single negative ion mode, whereas the only previously reported method [8] required acquisition of ILA in positive mode. Moreover, racemic standards (DL-PLA, DL-4OH-PLA, DL-ILA), and pure single enantiomers (L-PLA, D-4OH-PLA, D-ILA) were used for the unequivocal identification of the D and L forms of each analyte (Supporting information Figs. S6–S8). This contrasts with the above mentioned method- [8], which identified the optical identity only for PLA due to the limited commercial availability of single-enantiomer standards for 4OH-PLA and ILA at the time of that study.

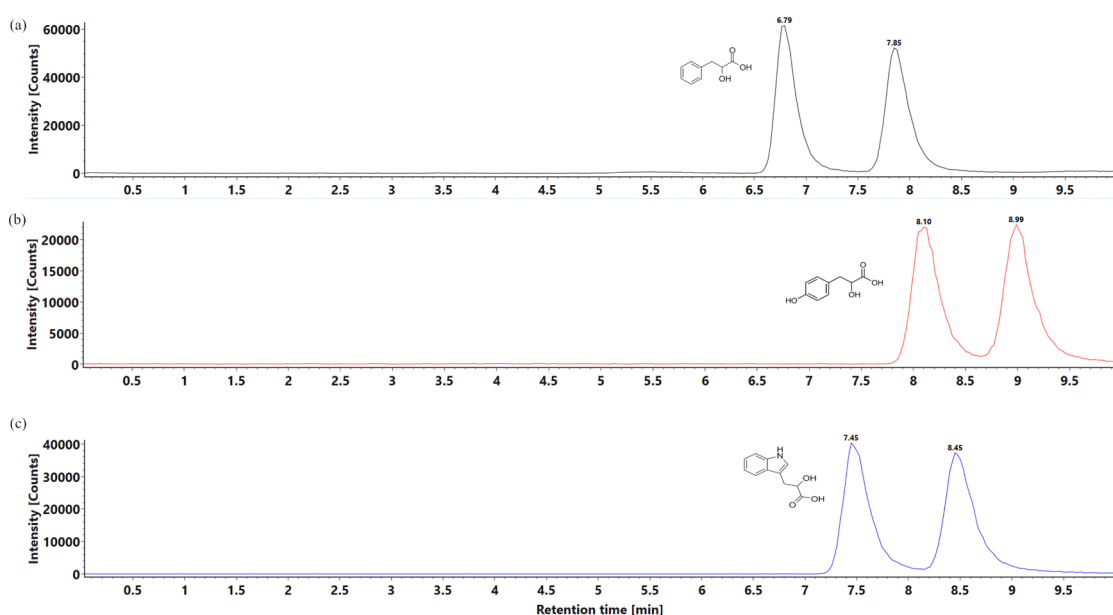


Fig. 5. Extracted ion chromatograms (XIC)s showing baseline separation of D (first peak) and L (second peak) enantiomers of (a) PLA, (b) 4OH-PLA, and (c) ILA and their respective chemical structure. Separation was achieved on a CHIRALPAK® QD-AX column (150 × 4.6 mm, 5 μm) under isocratic conditions (ACN:MeOH, 70:30 v/v, 0.375 % FA) at 1 mL min⁻¹ and 25 °C.

3.4. Method validation

3.4.1. Matrix effects and recovery

ME and RE were evaluated at two analyte concentrations (250 ng mL⁻¹ and 2500 ng mL⁻¹) with the final optimised sample preparation, chromatographic method and mass spectrometric parameters (Fig. 6). RE% and ME% were $\geq 80\%$ for all analytes at both concentrations. Notably, positive ME% was observed for L-4OH-PLA d₃ and both enantiomers of ILA d₅ across both concentrations, possibly due to co-elution of ionization enhancing matrix components under the optimised SPE conditions. The minimised MEs enable reliable quantification using IS-corrected external calibration in solvent, avoiding more laborious approaches such as standard addition.

3.4.2. Determination of LoL, LOD and LOQ

The LoL, LOD and LOQ were determined by using calibration curves based on known concentrations of the isotopically labelled IS in a pooled sample matrix versus instrument response (Supporting Information Figs. S9–S11). The resulting regression analysis revealed excellent linearity, with correlation coefficients (R^2) exceeding 0.99 for all analytes. Relative residuals were randomly distributed around zero and generally below 20 % (Supplementary Information Figs. S9–S11). Limits of linearity remained sufficiently broad for all the analytes except D-4OH-PLA d₃ (3.3–375 ng mL⁻¹) and D-ILA d₅ (5.8–125 ng mL⁻¹), suggesting potential limitations in its reliable quantification across a broad dynamic range.

LOD and LOQ were calculated from the calibration curves and using Eqs. (Eqs. 5 and 6), respectively, as recommended by previous study reports to give conservative estimates [29]. The method exhibited high sensitivity, with LOD values ranging from 1 to 2.2 ng mL⁻¹ and LOQ values from 2.9 to 6.7 ng mL⁻¹, enabling reliable quantification at low concentrations. Full information can be found in Supporting Information Table S30. The LOQ values are well above the previously reported physiological ranges for DL-PLA, DL-4OH-PLA, and DL-ILA in human infant faeces [3], highlighting the method's high sensitivity. This is particularly notable as no validated method currently exists for the enantioselective quantification of these metabolites in faecal matrices.

3.4.3. Specificity and precision

Specificity was evaluated by analysing solvent blanks and analyte-free matrix samples. No co-eluting or interfering peaks were observed in any of the blanks or analyte-free matrix samples at the specific retention times with accurate masses (± 10 ppm) of the target analytes or IS (data not shown). HRMS combined with chiral chromatography and optimized SPE, effectively minimized isobaric and matrix interferences, confirming the method's specificity for simultaneous quantification of all six analytes in infant faeces.

Intra and inter-day precision was evaluated in pooled infant faecal samples spiked with deuterated IS to achieve concentrations of 10 ng mL⁻¹, 125 ng mL⁻¹ and 1000 ng mL⁻¹ per analyte enantiomer, covering the calibration range from near the LOQ to the upper limit of linearity. Due to the limited linear range D-4OH-PLA d₃ (3.3–375 ng mL⁻¹) and D-ILA d₅ (5.8–125 ng mL⁻¹), precision for these analytes was assessed only at 10 and 125 ng mL⁻¹. Full information can be found in Supporting Information Table S31. Intra-day RSD was $\leq 13.5\%$ across all analytes and Inter-day RSD $\leq 17.68\%$, both below the values recommended by FDA bioanalytical method validation guidelines (intra- and inter-day precision $\leq 15\%$ RSD and $\leq 20\%$ RSD, respectively, near the LOQ [17]).

3.5. Metabolite quantification

To demonstrate the applicability of the developed LC-MS/MS method in a real-world setting, the method was applied to nine faecal water samples from infants aged 1–10 months to quantify aromatic lactic acid enantiomers. External calibration in MeOH was possible due to minimal ME and maximised RE after SPE optimization. Calibration methods that account for ME and RE (e.g. Standard addition) were excluded due to being time consuming, laborious, and necessitating a relatively large sample volume, making it unsuitable for high throughput analysis. The IS-corrected external calibration curve used for metabolite quantification demonstrated excellent linearity for all analytes ($R^2 > 0.99$) and relative residuals $\leq 20\%$ for all calibrants (Supporting Information, Figs. S12–S14), meeting the acceptance criteria outlined in current bioanalytical method validation guidelines [17]. Fig. 7 presents the concentrations of all six enantiomers in each sample. Most reported concentrations were above the LOD and LOQ, except for D-ILA in samples 2–01 (below LOD) and 3–10 (below LOQ), demonstrating the method's ability to analyse a complex biological matrix with satisfactory sensitivity. In two samples, L-4OH-PLA and D-ILA exceeded the upper limit of linearity. To ensure accurate quantification in such high-concentration cases, samples surpassing the linear dynamic range would be diluted and re-analysed to bring analyte levels within the validated linear range. Significant variability was observed across the analysed samples with metabolite concentrations ranging from 1.43 ng mL⁻¹ to 1279.22 ng mL⁻¹. Previous work has shown that faecal concentrations of aromatic lactic acids is highly variable and depend on breastfeeding and abundance of *Bifidobacterium* among the infants [3]. Notably, we observed that L-enantiomers were more abundant than their D- counterparts in most samples. However, D-PLA exceeded L-PLA in several samples, and D-4OH-PLA was higher than its L- form in one sample. This is the first time that the relative concentration of D- and L- aromatic lactic acids in infant faeces has ever been reported in the literature, showing the importance of the method for application in future human studies. While the limited sample size (n = 9) prioritised

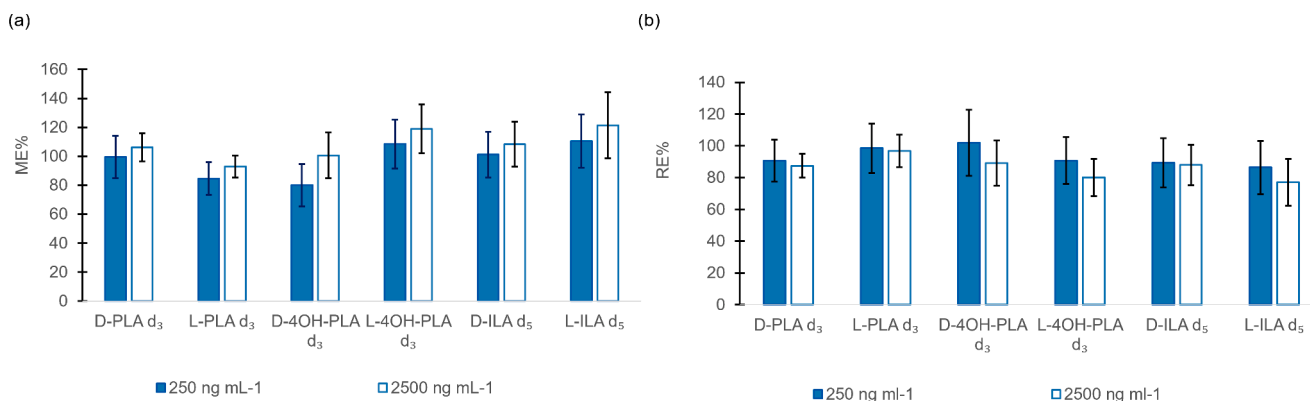


Fig. 6. (a) ME% and (b) RE% at 250 ng mL⁻¹ and 2500 ng mL⁻¹ of deuterated aromatic lactic acid enantiomers using *An SPE* protocol with a water-only wash and elution performed with 5 % NH₄OH in MeOH. Error bars represent propagated standard deviation based on triplicate measurements (n = 3). Full numerical data in Supporting Information (Table S29).

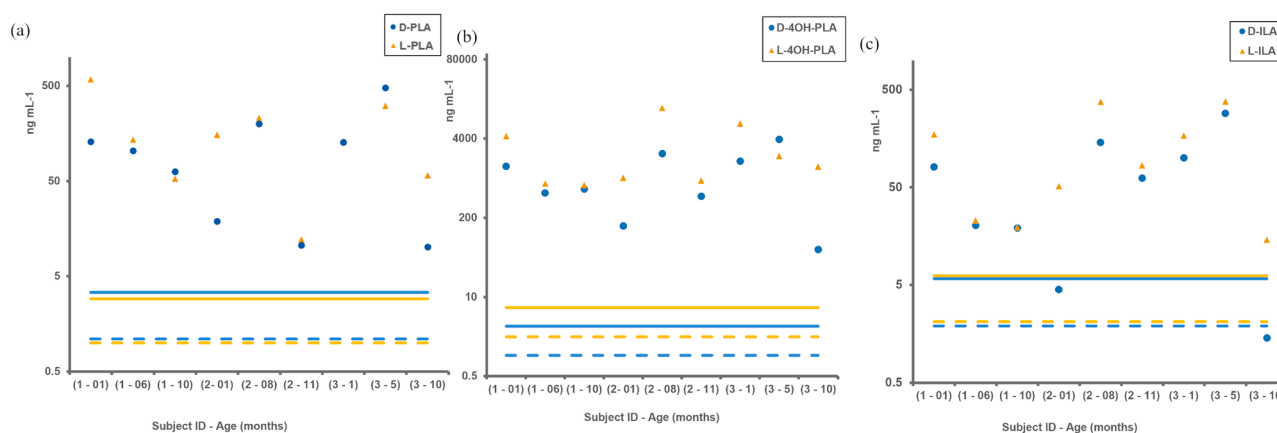


Fig. 7. Measured concentrations (ng mL⁻¹) of (a) D-PLA and L-PLA, (b) D-4OH-PLA and L-4OH-PLA, and (c) D-ILA and L-ILA across nine infant faecal samples. Horizontal lines indicate method validation thresholds: solid line = LOQ; dashed line = LOD. Data points represent individual measurements from separate subjects. Reported concentrations have not been corrected for the applied dilution factor. Full numerical data in Supporting Information (Table S32).

method validation; expanded cohorts are warranted to enable statistically powered biological investigations.

4. Conclusion

In the present study, we developed and validated a targeted chiral LC-MS/MS method for absolute quantification of aromatic lactic acid enantiomers in infant faeces, achieving high precision, low detection limits and excellent ME and RE. By enabling precise quantification of individual enantiomers in faeces, this method facilitates deeper exploration of potential *Bifidobacterium* dependent metabolic activity and its physiological consequences. Application to infant faeces revealed variability in enantiomer concentrations across different samples, reflecting the dynamic nature of early-life gut microbiota and suggesting that enantioselective profiling may provide important information for deducing aromatic lactic acid enantiomer-specific effects on immune development during infancy. This work contributes to the growing effort to bridge analytical innovation with microbiome research, offering a validated platform for future investigations into the emerging roles of aromatic lactic acids enantiomers in early life immune development.

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Notes

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.

CRediT authorship contribution statement

Ahmed M. Gaafar: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Gladys Stolberg-Mathieu:** Writing – review & editing, Resources. **Henrik M. Roager:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Giorgia La Barbera:** Writing – review & editing, Validation, Supervision, Methodology, 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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2026.466997](https://doi.org/10.1016/j.chroma.2026.466997).

Data availability

Data will be made available on request.

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