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Bio-Based Solvents Extraction and Enzyme-assisted Recovery of Furan Fatty Acid From Ammonia-Stabilized *Hevea* Latex

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Keywords: bio-based solvent | enzymes | furan fatty acid | *Hevea brasiliensis* | latex | lipid

ABSTRACT

The latex of *Hevea brasiliensis* is rich in furan fatty acid (FuFA) esterified in both neutral and polar lipids. This study evaluates bio-based solvents for selective extraction of lipids and FuFA from low-ammonia (LA) and high-ammonia (HA) stabilized *H. brasiliensis* latex. Extraction was performed at room temperature using a latex-to-solvent ratio of 1:5 (v/v). For LA-stabilized latex, ethyl acetate was the most efficient solvent for trifuranoylglycerol (TG-FuFA) extraction, outperforming traditional chloroform–methanol mixture (2:1, v/v), with or without NaCl addition, achieving a TG-FuFA yield of 0.28% (w/w latex). Isopropanol (iPrOH) was also effective, presenting a fair alternative. For HA stabilized latex, ethyl acetate maintained its efficacy, achieving 0.24% (w/w latex), similar to chloroform–methanol mixture (2:1, v/v) with NaCl solution washing (0.25% w/w latex). iPrOH matched this TG-FuFA yield but was less effective at excluding polar lipids. For total-FuFA extraction, iPrOH outperformed chloroform–methanol mixture, reaching 0.21% (w/w latex). Several lipases were tested for their aptitude to hydrolyzing FuFA-containing lipids. Guinea pig pancreatic lipase-related protein 2 and *Fusarium solani* cutinase proved to be effective enzymes for releasing free FuFA from triacylglycerols, galactolipids, and phospholipids in latex, indicating their potential for greener FuFA recovery. Further research is required to optimize enzyme-assisted FuFA extraction from stabilized latex.

Promoting eco-friendly product of bioactive molecules—from sourcing to processing—is essential for sustainable resource management and for reducing reliance on fossil fuels. Fresh latex from *Hevea brasiliensis* offers significant bioactive potential,

Abbreviations: ASE, accelerated solvent extraction; C/M mixture, chloroform/methanol mixture; CRRC, Chachoengsao Rubber Research Center; DES, deep eutectic solvents; DG, diacylglycerol; DGDG, digalactosyl diacylglycerol; ESG, esterified steryl glucoside; FFA, free fatty acids; FuFA, furan fatty acid; GC-FID, gas chromatography with flame-ionization detection; GL, glycolipid; GPLRP2, guinea pig pancreatic lipase-related protein 2; HA, high-ammonia stabilized latex; LA, low-ammonia stabilized latex; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; MAG, monoacylglycerol; MGDG, monogalactosyl diacylglycerol; NADES, natural deep eutectic solvents; NL, neutral lipid; NMR, nuclear magnetic resonance; NR, natural rubber; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; RC, regenerated cellulose; Rdgl, recombinant dog gastric lipase; SG, steryl glycoside; TG, triacylglycerol; TLC, thin-layer chromatography; YLLIP2, *Yarrowia lipolytica* LIP2 lipase.

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containing up to 0.4% (w/w) of furan fatty acid (10, 13-epoxy-11-methyl-octadecan-10,12-dienoic, named FuFA-F2). FuFA-F2, part of the FuFA lipid family found in fruits, vegetables, and oils, is of growing interest for its health benefits, such as cardioprotection and obesity prevention. Natural rubber production (14.5 million tons in 2023) mainly originates from Southeast Asia (72%) and small-scale farmers who depend on rubber for livelihood. Thus, the rising demand for environmentally friendly rubber-derived products, combined with the untapped therapeutic potential of *Hevea* latex FuFA-F2, offers significant socio-economic opportunities in both Asia and Europe, underscoring the importance of exploring efficient extraction strategies, especially from stabilized latex.

1 | Introduction

Lipids are versatile organic compounds found in living organisms (plants, animals, fungi, algae, etc.) characterized by their solubility properties and categorized into two main groups on the basis of the polarity [1]—(i) reserve lipids (neutral and nonpolar), which lack charged groups, include triacylglycerols (TG), diacylglycerol (DG), carotenoids, sterols, and certain high molecular weight hydrocarbons, and (ii) membrane lipids (polar or complex) further classified into phospholipids (PL) and glycolipids (GL). Total lipids also include terpenes, waxes, and other ether-soluble compounds. Lipid extraction involves the recovery of these molecules using solvents, either with or without additional energy (heat, ultrasound, microwave, etc.), or under supercritical or subcritical conditions, as well as through enzymatic or mechanical pressing techniques, depending on the material. Solvent extraction is the most widely applied method, wherein lipids are transferred to a solvent in contact with the matrix (e.g., agricultural biomass) without involving chemical reactions. Traditionally, organic solvents of fossil origin are used in laboratories to extract dehydrated biomass [2]. The Bligh and Dyer [3] and Folch [4] methods are the most used methods to simultaneously extract neutral and polar lipids, both relying on a mixture of chloroform and methanol [5]. To minimize the negative impact of traditional fossil-derived solvents and to promote harmless and more eco-efficient processing, greener alternatives solvents were introduced in the early 2000s [6]. This category includes bio-based organic solvents, supercritical CO₂ fluids, ionic liquids [2], deep eutectic solvents (DES) [7], natural DES (NADES) [8], or renewable feedstock-based solvents. These latter, derived from biomass like cellulose and starch, are often alcohols or ethers. Various solvents, including ethanol (EtOH), ethyl acetate, isopropanol (iPrOH), *n*-propanol, and others, have been used to extract lipids from sources like spent coffee grounds, yeast, egg-based materials, animal tissues, and microalgae, achieving results comparable to traditional methods [5, 9–11]. Terpenes like D-limonene, α -pinene, and *p*-cymene, derived from citrus peels or tree leaves, have recently been utilized in oil extraction but are currently too expensive [2]. Other green lipid extraction methods, such as ionic liquids (for algae) and supercritical CO₂ (for argan seed, grape seed, soybean seed, microalgae, and oats), have demonstrated a key advantage extraction, whereas consideration needed on high instrumentation cost and high energy requirement [2]. NADES and DES represent the latest class of green solvents under exploration. Although they share some similarities with ionic liquids, they possess distinct intrinsic properties that set them apart [7, 8, 12–14]. However, industrial application has been challenging due to high viscosity, solute

separation issues, and the need for toxicity and biodegradability assessments [15–18].

Lipids are fundamental components in the complex network of biochemical processes underlying human physiology. Among these, furan fatty acids (FuFA), which are oxylipins biosynthesized through uncommon biosynthetic pathways, which include sequential oxidation, cyclization, and methylation reactions, have recently garnered scientific interest [4]. FuFAs represent a distinctive group of fatty acids featuring a furan ring, substituted at the α' -position with an alkyl chain and at the α -position with a straight chain containing a carboxylic group. The β -position of the furan ring is consistently occupied by a methyl group, whereas the β' -position is variably substituted by either a methyl group or hydrogen ion (Figure 1A). FuFAs were first identified in northern pike (*Esox lucius*) by Glass et al. [19, 20], who established the initial FuFAs nomenclature on the basis of GC retention time (Figure 1B). Later, Vetter et al. [21] introduced an alternative naming system on the basis of carbon number and methyl group position (Figure 1C). FuFAs are found in various fish tissues or blood and are mostly concentrated in the liver [19, 20, 22, 23]. They have also been detected in several plants, including rosemary, spinach, flat-leaf parsley, curly-leaf parsley, and dill, among others [24]. The richest known source of FuFAs is cod fillet, containing 175 mg of total FuFA/100 g lipid, with 117 mg of 11D5 (FuFA-F6) per 100 g of lipid. The second ranking is fish oil at 108 mg of total-FuFA/100 g lipid, and the most detected FuFA in fish oil is 9M5 (F2) at 70 mg/100 g lipid.

Although they are minor lipids (<25 mg/100 g lipid), 9M5 (F2) and 11D5 (F6) are the most abundant FuFAs identified in marine organisms [25]. The first non-edible FuFA source was found in *Hevea* latex by Hasma and Subramaniam (RRIM501 genotype, 2500 μ g/g latex) [26–28]. Although many *Hevea* genotypes exhibit lower FuFA content [29], the PB235 genotype contains 9M5 (F2) [30], shown in Figure 1D, and ranks among the highest FuFA-producing genotypes, with a concentration of 6000 ± 1000 μ g/g of latex and a yield 2.4 g/tree/tapping. This makes PB235 the most concentrated natural source of FuFA available [29]. FuFAs demonstrate significant bioactivity as radical scavengers, anti-inflammatory agents, and potential modulators of cardiometabolic health, with evidence suggesting stronger anti-inflammatory effects than eicosapentaenoic acid (EPA) and promising cardiovascular benefits [26, 31, 32]. Recent studies show that the FuFA extracted from *Hevea* latex can promote muscle anabolism and enhance insulin sensitivity in murine models, supporting their potential as biomarkers for

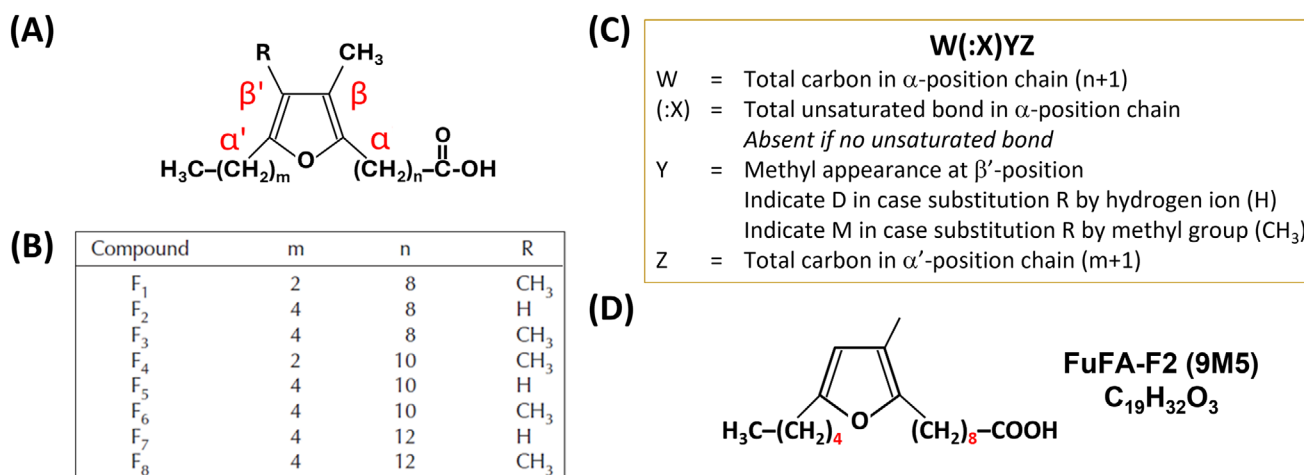


FIGURE 1 | (A) FuFA structure, (B) FuFA nomenclature by Glass et al. [19], (C) FuFA nomenclature from Vetter et al. [21], and (D) FuFA-F2 (9M5) structure. FuFA, furan fatty acid.

metabolic syndrome risk and highlighting the need for further investigation in human clinical trials [33, 34].

Mixture of chloroform/methanol (C/M mixture, 2:1, v/v) has been widely used for lipid extraction from *Hevea* latex, with or without aqueous sodium chloride (NaCl (aq.), 0.9% w/v) solution washing [35]. One of the main challenges in lipid extraction from *Hevea* latex is the presence and potential co-extraction of the predominant poly-*cis*-1,4-isoprene component [36–38]. For instance, coagulation that occurs during extraction with C/M mixture may trap some NL in the rubber coagulum [35]. To address this, Liengprayoon et al. [35] developed a method where *Hevea* latex is continuously diluted dropwise onto the solvent and washed with 0.9% NaCl (aq.). Currently, no comparative research has explored FuFA-containing lipid extraction from latex using bio-based solvents. Moreover, the impact of ammonia-stabilized latex—commonly used to extend the storage life of fresh latex—on FuFA recovery has never been investigated.

In this study, we aim to evaluate different approaches for lipid extraction from stabilized latex collected from a PB235 *Hevea* genotype. To achieve this, bio-based solvent extractions were conducted on PB235 stabilized latex (0.3% or 0.6% ammonia, w/w) at a 1:5 sample-to-solvent ratio (v/v), exploring alternatives to the traditional C/M mixture. The FuFA content in each lipid extract as well as the trifuranoylglycerol (TG-FuFA) content were evaluated. An alternative approach involves investigating enzyme-catalyzed hydrolysis of FuFA-containing molecules. This way could eliminate the need for solvents and expand the perspective for isolating FuFA as free fatty acid (FFA) in higher amount from stabilized latex, as it may recover FuFA grafted to all lipid classes.

2 | Materials and Methods

2.1 | Chemicals and Enzymes

Blue molybdenum reagent (1.3% [w/v] molybdenum oxide in 4.2 M sulfuric acid), copper(II) acetate monohydrate

(100%), formic acid ($\geq 98\%$, ACS), methanol ($\geq 99.8\%$, ACS), methyl red ($\leq 100\%$), orcinol monohydrate ($\geq 99\%$), deuterated chloroform (99.8 atom % D, CP), sodium methoxide (25% [w/v] in methanol), sodium taurodeoxycholate hydrate ($>97\%$), sulfuric acid ($\geq 95\%$), Trizma hydrochloride buffer solution (pH 7.2 $\pm$ 0.1), methyl *cis*-9-octadecenoate (C18:1ME, 99%), methyl heptadecanoate (C17:0ME, $\geq 99\%$), methyl icosanoate (C20:0ME, $\approx 99\%$), methyl octadecenoate (C18:0ME, 99%), ammonia (28%–30% [w/v] in water), diethyl ether ($\geq 99.7\%$, ACS), heptane ($\geq 99\%$), hydrochloric acid (37% [w/w] in water), sodium chloride ($>99.99\%$), dimethyl carbonate (DiMeCO₃) ($\geq 95\%$), 2-methylbutyl acetate (MeAc) (natural, $\geq 95\%$), ethyl acetate (natural, $\geq 99\%$), EtOH ($\geq 99.8\%$), 2-methyl tetrahydrofuran (MeTHF) ($\geq 99\%$), 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane (eucalyptol [Euc], natural, $\geq 99\%$), ethyl lactate (EtLac) (natural, $\geq 98\%$), and iPrOH ($\geq 99\%$, ACS) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Methyl *cis,cis*-9,12-octadecadienoate (C18:2ME, $>99\%$), methyl *cis*-9-hexadecenoate (C16:1ME, $>99\%$), methyl dodecanoate (C12:0ME, $>99\%$), methyl *cis,cis,cis*-9,12,15-octadecatrienoate (C18:3ME, $>99\%$), and thin layer chromatography (TLC) Mix 34 ($>99\%$) were obtained from Larodan (Solna, Sweden). *n*-Butanol (BuOH) ($\geq 99.5\%$, AR) was from Carlo Erba (Val de Reuil, France). Acetic acid ($\geq 99.8\%$, ACS), chloroform ($\geq 99.8\%$, ACS), hexane ($\geq 95\%$, LR), methyl hexadecanoate (C16:0ME, $\geq 95\%$), methyl tetradecanoate (C14:0ME, $>99.5\%$), and diethyl ether ($\geq 99.5\%$, AR) were purchased from Honeywell (Charlotte, USA). Guinea pig pancreatic lipase related protein 2 (GPLRP2) and *Fusarium solani* cutinase were produced as recombinant enzymes in the yeast *Pichia pastoris* as previously described [39]. The LIP2 lipase from *Yarrowia lipolytica* (YLLIP2) was produced and purified according to Aloulou et al. [40]. Recombinant dog gastric lipase (rDGL) was produced in transgenic maize [41].

2.2 | Source of Natural Rubber (NR) Latex, Collection, and Stabilization

The NR latex samples were collected at Chachoengsao Rubber Research Center (CRRCC) in Chachoengsao province (Thailand).

The latex sample was collected from *Hevea* genotype PB235 in October 2023 and sampled at least 30 min after tapping in the early morning (around 6 am) by filtering latex through sieve filter onto plastic bottle. The total collection volume was more than 1 liter. The latex bottle was immediately stored in an ice bucket and transferred to the laboratory. The field latex was preserved with ammonia either 0.3% w/w (low ammonia stabilized latex—LA) or 0.6% w/w (high ammonia stabilized latex—HA), which are typically used in industrial latex stabilization processes after centrifugation.

2.3 | Selection of Bio-Based Solvents

The selected natural solvents were ethyl acetate (EtOAc), EtOH (EtOH), MeTHF, iPrOH, DiMeCO₃, BuOH, MeAc, EtLac, and Euc. In addition, Folch's extraction method (C/M mixture, 2:1, v/v) with or without salting purification with NaCl (aq., 0.9% w/v) was also tested for comparison efficiencies. The extraction by C/M mixture (2:1, v/v) follows the method described by Liengprayoon et al. [35] and was used to extract lipids in Sections 2.7, 2.8, and 2.12.

2.4 | Lipid Extraction From Stabilized Latex

The study design is depicted schematically in Figure 2. A two-step extraction method was implemented to evaluate the extraction efficiency of FuFA-containing lipids from stabilized latex. The first extraction step (yielding the “first extract”) was performed using a bio-based extraction process. In the second extraction step (yielding the “second extract”), the dried residue from the first step was dissolved in chloroform, and the soluble part was dried again.

The first-step extraction was performed from stabilized latex (4 mL) drop-wised onto the selected solvents (20 mL) with three repetitions. Sample dropping rate was controlled at 1.05 ± 0.05 mL/min. Stirring rate varied depending on bio-based solvent viscosity and latex homogeneity, but the stirring must form a small vortex with latex dropping on the side of vortex. The rest of the latex was purged by 2 mL of the solvent. The magnetic bar was washed by 1 mL of the solvent. The coagulum part was squeezed and extracted by 5 mL with the same solvent. Then, the coagulum was withdrawn and dried ($100^\circ\text{C} \pm 5^\circ\text{C}$) to get steady dry coagulum weight. The liquid part was filtered (regenerated cellulose [RC], $0.45 \mu\text{m}$). After filtering, the solvent was removed by rotary evaporator ($40^\circ\text{C} \pm 5^\circ\text{C}$, 100 ± 20 rpm, BUCHI—R300). The evaporation was completed by flowing nitrogen gas for 5 min/sample.

2.4.1 | First-Step Extraction Yield With Bio-Based Solvent

The first step extraction content was calculated on fresh latex weight in stabilized latex (W_0 , g), dry weight of initial extract (W_1 , g), and NH₃ concentration (C_w , g/L) as follows:

$$W_0 = 4 - (\% \text{NH}_3 \text{ in latex} \times 4) / C_w$$

$$1^{\text{st}} \text{step extract (\%w/w of latex)} = W_1 \times 100 / W_0 \quad (1)$$

2.4.2 | Second-Step Extraction in Chloroform

The first step extract was dissolved by chloroform (4 mL), and the soluble part was transferred to amber vial. After transferring, chloroform was dried until steady weight (W_2 , g). This second step extract was expressed against initial latex weight, as follows:

$$2^{\text{nd}} \text{step extract (\%w/w of latex)} = W_2 \times 100 / W_0 \quad (2)$$

2.5 | Lipid Profile Analysis of Lipid Extract by TLC

The second step extracts in chloroform (adjusted to 1 mg/mL), TLC Mix34, GL standard, PL standard, and standard TG-FuFA (5 concentrations, see Section 2.7) were deposited on TLC plate ($20 \times 10 \text{ cm}^2$, silica gel 60G HPTLC plate) $10 \mu\text{L}$ as a 5 mm wide band at 10 mm apart from the bottom edge of the plate using an ATS4 sample applicator (CAMAG) under a flow of nitrogen gas. The plates were developed in an ADC2 automatic developing chamber (CAMAG) previously saturated with mobile phase at room temperature. The first elution was done with a polar mobile phase ($\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$, 85:20:25, v/v/v) on the plate height 0–40 mm then, after drying, a second elution with hexane/diethyl ether/acetic acid (80:20:1, v/v/v) was performed on the plate height 0–80 mm. After drying, the plate was immersed in 40% orthophosphoric acid and aqueous solution saturated with copper acetate (1:1, v/v) using a CAMAG chromatogram immersion device III (CAMAG—22.6606). The plate was then put in the oven (Mettmert—U10) at 180°C for 10 min. The lipid spots appeared brownish on a white background.

2.6 | Determination of TG-FuFA Content by TLC-Densitometry

To get the TG-FuFA content, the RF of TG-FuFA based on TG-FuFA standard (see Section 2.7) was identified, and the spot intensity was scanned by TLC scanner 3 (CAMAG—22.985) at 500 nm. The result was analyzed by visionCAT software (version 3.0). The calibration curve for TG-FuFA was created by plotting the integrated area at 500 nm on the y-axis against five different concentrations of the standard TG-FuFA on the x-axis. The data were then fitted with a linear function to allow for the prediction of unknown concentrations.

2.7 | Purification of TG-FuFA From Lipid Extract

TG-FuFA was purified from PB235 lipid extract by liquid chromatography (BUCHI, Reveleris X2). The lipid (400 mg) was deposited on silica60A column (RediSep Rf, packing size 4 g, silica particle size 40–63 μm). The first mobile phase was *n*-hexane that was eluted for 30 min at a flow rate of 1 mL/min. Then, the second mobile phase made of *n*-hexane/diethyl ether mixture (90:10, v/v) was eluted for 1 h at a flow rate of 5 mL/min. The lipid-containing fractions detected through a

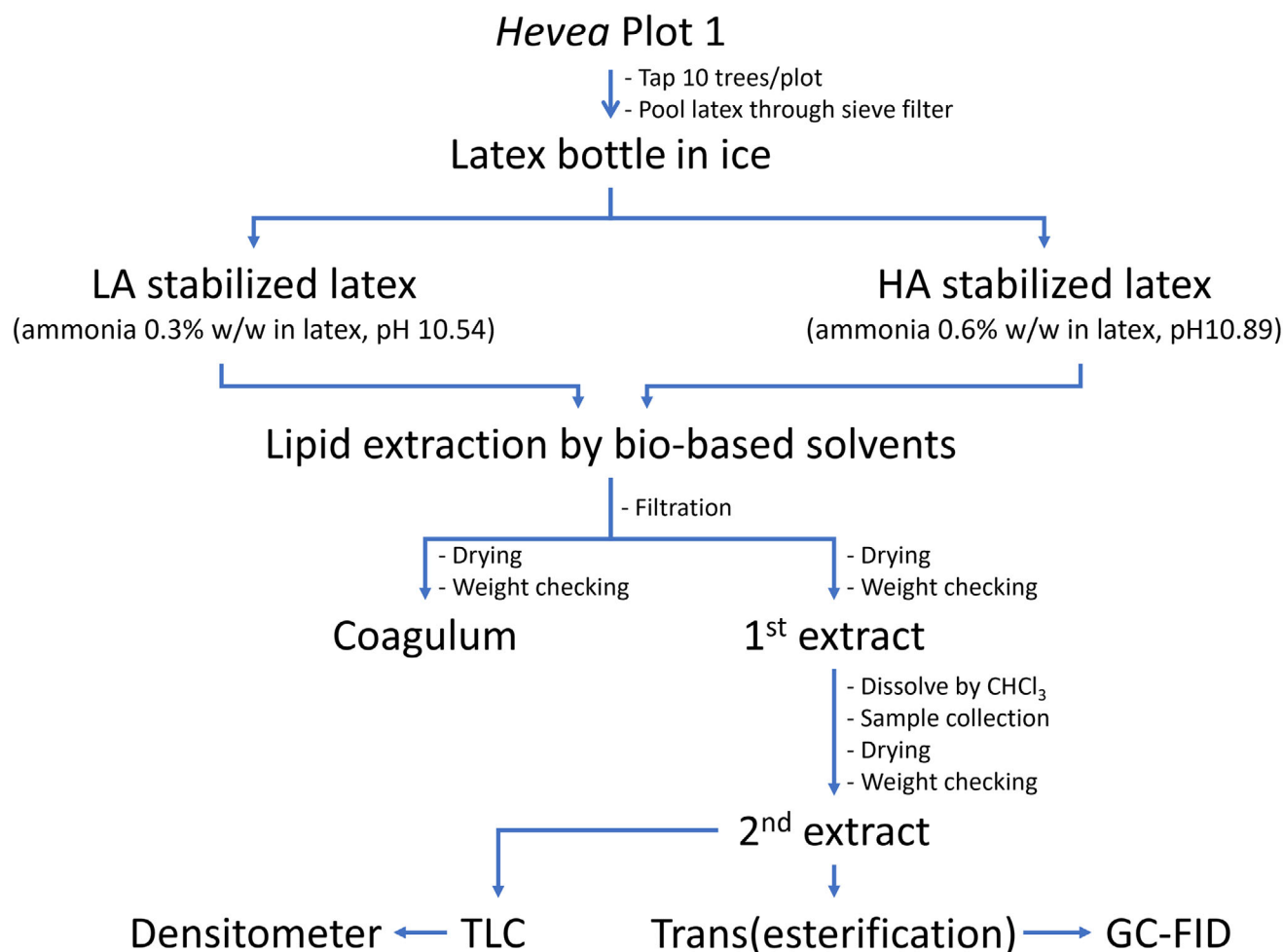


FIGURE 2 | Diagrammatic illustration of the study design. GC-FID, gas chromatography with flame-ionization detection; HA, high-ammonia; LA, low-ammonia; TLC, thin-layer chromatography.

high signal on evaporative light scattering detector (ELSD) and UV1 detector (230 nm) were characterized by thin layer chromatography proposed by Liengprayoon et al. [30] with PB235 lipid extract as a control deposit. The fraction with the highest relative intensity TG-FuFA spot was selected and further analyzed to assess the purity (see Sections 2.8, 2.9, 2.10, and 2.11).

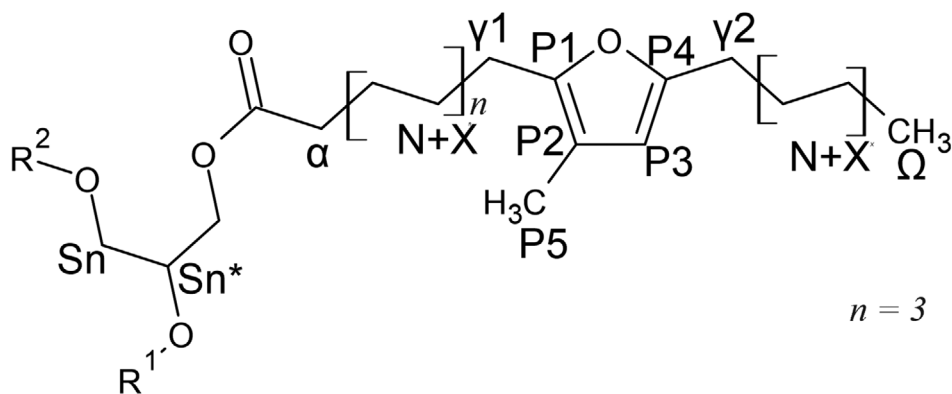
2.8 | Nuclear Magnetic Resonance (NMR) (1D and 2D experiments) of TG-FuFA

NMR spectra were recorded on a 600 MHz Bruker Avance II spectrometer using a cryoprobe at 298 K. All spectra were analyzed and processed in topspin. The TG-FuFA fraction was diluted with 10% CDCl₃ (deuterated chloroform) for lock. Shimming and calibration were done following standard protocols. The 1D experimental data was acquired using a watergate solvent-suppression for the chloroform. The acquisition time was 1.36 s, collecting 32 K points, for which 128 scans were acquired with 3 s in between scans. For 2D experiment, HSQC acquisition time was 0.42 s, collecting 8 K points (P2) for 456 (P1) increments of 24 scans with 1.2 s delay in-between scans. HMBC acquisition

time was 0.34 s, collecting 8 K points for 456 increments of 24 scans with 1.2 s delay in-between scans. TOCSY acquisition time was 0.08 s, collecting 2 K points (P2) for 456 (P1) increments of 24 scans with 1.2 s delay in-between scans. Single FuFA-F2-containing TG schematic structure with carbon position labels, and NMR identification (from ¹H, ¹³C, HMBC, and HSQC) was represented below (Figure 3 and Figures S1 and S2). N and X are -(CH₂)- chain on the ester-side and alkyl-side, on both sides of the furan ring.

2.9 | Preparation of Fatty Acid Methyl Ester (FAME)

The dry lipid extract (15 ± 5 mg) and internal standard (1 mg of methyl heptadecanoate—C17:0 FAME) were transesterified in screw-cap test tube with 1 mL of 0.5 M sodium methoxide/methanol (10:90, v/v; sodium methoxide [25% w/v in methanol]) at 65°C ± 5°C for 15 min. Test tube was placed on ice for 10 min, and the sample was then esterified by adding 1 mL of 7.4% (v/v) of acetyl chloride in methanol and heating at 65°C ± 5°C for 15 min. The test tube was placed on ice for 10 min; then, the reaction mixture containing FAME



Name	¹ H NMR ppm (multiple) (integral)	¹³ C NMR (ppm)	Expected proton
N+X	1.2 – 1.6 (O*) (54.9)	15 – 35	54
P1	No	114	
P2	No	149	
P3	5.76 (s) (3.3)	153	3
P4	No	107	
P5	1.88 (s) (9.1)	9	9
γ1	2.48 (O) (10.5 /2)	25	6
γ2	2.50 (O) (10.5 /2)	27	6
Sn	4.28 – 4.13 (dd dd) (1.7+1.7)	61.8	4
Sn*	5.28 (m) (1 cal)	68.6	1
Ω	0.88 (t) (10.1)	13.8	9
α	2.30 (t*) (5.3)	33.7	6
RCOOR	No	173.0	

s : singlet ; d : doublet ; m : multiplet ; O overlap; * weird shape

FIGURE 3 | Single FuFA-F2-containing TG schematic structure with carbon position labels and NMR identification (from ¹H, ¹³C, HMBC, and HSQC). NMR, nuclear magnetic resonance.

was added with 1 mL of water and extracted with 1 mL of *n*-hexane. The supernatant phase was collected for further analysis.

2.10 | Analysis of FAME by Gas Chromatography With Flame-Ionization Detection (GC-FID)

FAME were analyzed by GC-FID (Thermo Scientific FOCUS GC) from Italy. The GC-column phase was CP-Sil 88 for FAME (Agilent Technologies) with highly substituted and stabilized cyanopropyl stationary phase. Column length was 50 m with 0.25 mm of inner diameter, 0.25 μm of film thickness. Sample injection volume was 1 μL with split ratio of 15. Flow rate was adjusted to provide a constant pressure of 241 kPa. The oven temperature started from at 160°C for 0.5 min, then it was increased by 10°C/min rate for 6 min and held at 220°C for 5 min. Injector and detector temperatures were set to 250°C and 270°C, respectively. Total elution time was 11.5 min.

2.11 | Calculation of Fatty Acids Content, Including FuFA, in Lipid Extracts

The concentration of detected fatty acids in lipid extracts was determined using 8-level calibration curves obtained by GC-FID for each of the following standard FAMES: C12:0ME, C14:0ME, C16:0ME, C16:1ME, C17:0ME, C18:0ME, C18:1ME, C18:2ME, C18:3ME, C20:0ME, and FuFAME (in-house). FuFAME was obtained from transesterification (cf. 2.9) of the purified TG-FuFA (cf. 2.7). Total fatty acid content in lipid (TFA/lipid) was estimated by the difference between the initial lipid weight before (*trans*)esterification step and the total weight of detected fatty acids by GC-FID (the TFA weight was calculated on the basis of the measured weight of FAME). It is summarized in the following equation:

$$\text{TFA/lipid (\%TFA w/w lipid)} = \frac{(\text{Total fatty acid weight (mg)} \times 100) / (\text{2}^{\text{nd}} \text{ step extract weight for (trans) esterification (mg)})}{1}$$

TFA content in latex (TFA/latex) was calculated by multiplying TFA in lipid from Equation (3) by lipid extract content in latex from Equation (1), as in the following equation.

$$\begin{aligned} \text{TFA/latex (\%w/w latex)} \\ = (\text{TFA/lipid}) \times (2^{\text{nd}} \text{step extract}(\% \text{latex})) / 100 \end{aligned}$$

2.12 | Enzymatic Hydrolysis of Lipid Extract and TG-FuFA

FuFA-containing lipid was extracted from PB235 latex by using a C/M mixture following the method given by Liengprayoon et al. [35]. Purified TG-FuFA (98% FuFA in TFA) was separated by following Section 2.7. Lipid fraction or purified TG-FuFA were incubated with various lipases with distinct substrate specificities, GPLRP2, *F. solani* cutinase, YLLIP2, and rDGL. All enzymes were used in the presence of bile salts (4 mM sodium taurodeoxycholate, NaTDC) as emulsifiers for dispersing latex lipids and purified TG-FuFA in 7 mM Tris-HCl buffer at pH 7.0. The incubations were performed at 37°C, under stirring at 300 rpm for 3 h. The reaction mixture was extracted by C/M mixture (2:1, v/v) with a sample/solvent ratio of 1.5:3, v/v. Then, HCl (1 N, 100 µL) was added for fatty acid protonation and further extraction of lipids for TLC analysis. The experiments were performed in triplicates for each enzyme, and a blank without enzyme was also incubated under the same conditions.

2.13 | Qualitative NL, GL, and PL Analysis by TLC

Qualitative analysis of residual substrate and lipolysis products was performed by TLC. Lipid extracts (5–10 µL) and lipid standards were spotted on TLC silica plates. The mobile phases depended on lipid class. For NL analysis, the mobile phase was heptane/diethyl ether/formic acid (55:45:1, v/v/v), followed by developing and drying (cf. 2.5). For GL analysis, the mobile phase was chloroform/methanol/water mixture (95:20:2.5, v/v/v). After drying with hot air, the TLC plate was dipped into orcinol sulfuric reagent. The orcinol sulfuric reagent was prepared by dissolving orcinol monohydrate (2 g) in 70% (v/v) sulfuric acid (1 L). The plate was then put in the oven (Binder—FP115) at 100°C ± 5°C for 12.5 ± 2.5 min. The GL profile appeared as orange and purple spots on white background. For PL analysis, mobile phase was chloroform/methanol/30% NH₃ mixture (65:25:5; v/v/v). After drying with hot air, the TLC plate was dipped into blue molybdenum reagent. The PL profile appeared as blue spots on a white background. The blue color gradually disappeared within 25 ± 5 min.

2.14 | Statistical Analysis

The data were analyzed using All pairs—Tukey—Kramer HSD (honestly significant difference) test, $p < 0.05$, and Pearson correlation coefficient (PCC) analysis on JMP Pro program (SAS Institute, Caru, NC, USA, version 14).

3 | Results and Discussion

3.1 | Exploring Bio-Based Solvents Method for Extracting FuFA From Stabilized Latex

3.1.1 | Lipid Extraction

A preliminary lipid extraction from the LA sample (0.3% w/w) showed that MeTHF, MeAc, EtLac, and Euc were not suitable solvents. Indeed, when mixed with the stabilized latex, a glue-like homogeneous mixture forms, making it impossible to separate the lipid-containing solvent even after centrifugation (Figure S3). These results highlight the challenge of selectively extracting FuFA-containing lipids from *Hevea* latex while avoiding contamination from polyisoprene. Indeed, the microstructure and composition of the latex make extracting FuFA-containing lipids particularly difficult, as (i) these lipids are primarily located in the rubber particle centrifugation fraction (both small and large particles with density lower than water) [42], and (ii) the presence of polyisoprene, which constitutes about 35% (w/v) of latex, is highly susceptible to coagulation, which significantly complicates the FuFA extraction process. Here, it is likely that these solvents have high affinity and solubility for polyisoprene, leading to significant swelling of the polymer chains—similar to how rubber adhesive is produced by immersing dry rubber in organic solvents such as toluene, hexane, or heptane. In these cases, alternative methods to separate the lipid-containing solvent from the polyisoprene matrix would be required. Therefore, MeTHF, MeAc, EtLac, and Euc were excluded for the further experiments.

The first extract content in latex was obtained after drying the initial extract from each bio-based solvent; it is represented by the dark blue bar (LA) and dark green bar (HA) in Figure 4. This dried extract was then re-dissolved in chloroform. The chloroform was selected as the solvent for the second extraction step to enhance the detection of lipids, thereby facilitating a more accurate qualitative and quantitative comparison of lipid yields across the bio-based solvents. This choice was also made to ensure consistency in lipid solubility and extraction efficiency, for which other bio-based solvents, particularly alcohol-based options, were unable to achieve reliably (data not shown). After drying the soluble part, the lipid content in chloroform (second extract) was calculated, and the data are presented by the light blue bar (LA) and light green bar (HA) in Figure 4. The solvent extraction using a C/M mixture (2:1, v/v) revealed significant differences when NaCl (aq., 0.9% w/v) was introduced during the first extraction step. The addition of NaCl (aq.) created a biphasic system with a salting-out effect, which aided in the separation of partially hydrophobic components such as proteins, carbohydrates, polyphenols [43–45] from the organic phase to the aqueous phase [3, 42]. This process resulted in a thin layer formation between the two phases, which was discarded when the lipid-enriched chloroform phase was collected. As a result, this likely also led to the exclusion of molecules such as proteins, carbohydrates, or complex and co-aggregated lipids (e.g. lipoproteins), as evidenced by the decreased content for C/M mixture after the second step of extraction in the chloroform (Figure 4). A similar large difference between the first and second extract content was observed from iPrOH, EtOH, and BuOH

Extract content (%w/w latex)

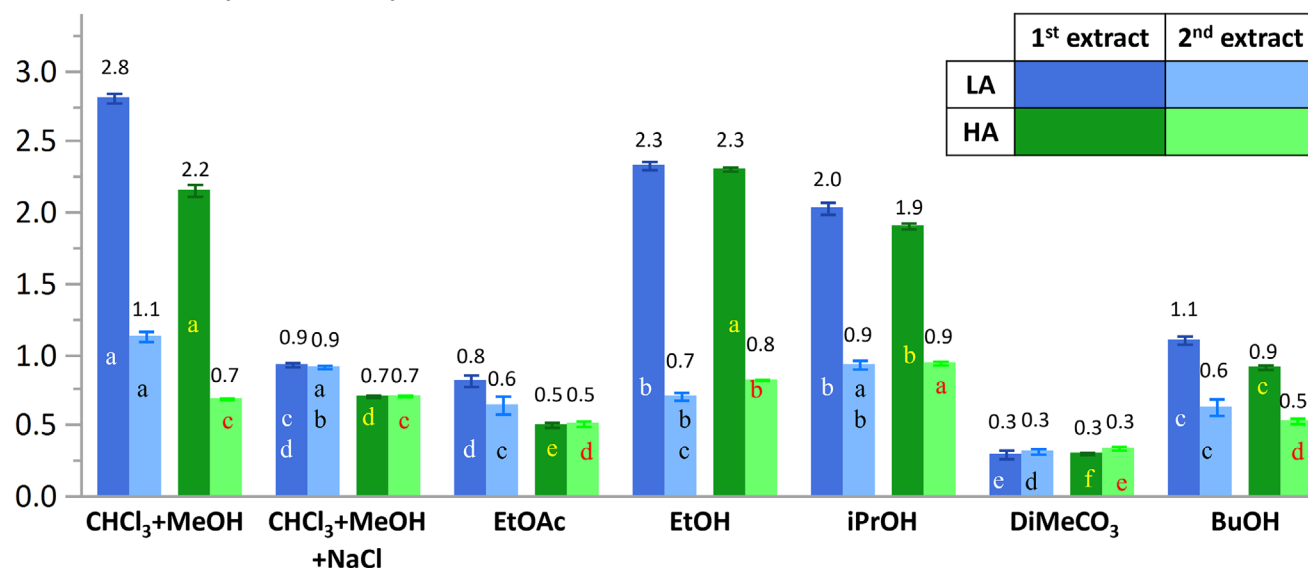


FIGURE 4 | Initial extract content in bio-based solvent (first extract [% w/w latex]) and extract content in chloroform (second extract [% w/w latex]) in LA sample (0.3% w/w NH₃ in fresh latex) and HA sample (0.6% w/w NH₃ in fresh latex)—by using C/M mixture (2:1, v/v)—with/without washing, ethyl acetate, ethanol, isopropanol, dimethyl carbonate, and *n*-butanol. Significant difference of average within each NH₃ concentration in latex sample and extraction by Tukey–Kramer test, $p < 0.05$, are indicated by letters. HA, high-ammonia; LA, low-ammonia.

extractions. However, DiMeCO₃ extractions did not provide significant differences between the first and second extraction steps.

This pattern was also observed in the HA sample (0.6% w/w NH₃ in latex), as shown in Figure 4, with an exception for EtOAc extractions that did not provide significant differences between the first and second extraction steps. Thus, it is important to note that the polarity of alcohol-derived solvents, particularly due to the presence of hydroxyl groups, likely played a role in unwanted extractions, as compared to ester solvents like EtOAc or carbonate ester solvents like DiMeCO₃. Comparing Figure 4, blue bar and green bar, we can note that the NH₃ concentration did not significantly affect the extraction rate for alcohol-derived solvents (difference <15%). However, an increase in NH₃ concentration resulted in decreased extraction rate for C/M extract without or with NaCl addition (39% and 23% differences, respectively) and EtOAc extract (20% difference).

3.1.2 | Lipid Profile

The composition of lipid extracts obtained from the second extract was investigated to evaluate the selectivity of bio-based solvents for lipid extraction, with the goal of assessing TG-FuFA content purity in comparison to other lipids. The lipid profile for all bio-based solvents extraction is illustrated in the TLC plates presented in Figure S4. C/M mixture, EtOH, iPrOH, and BuOH demonstrated a higher capacity for extracting polar lipids compared to EtOAc and DiMeCO₃. A high-intensity FFA spot, with the same R_f as the oleic acid (FA-C18:1) standard, was detected. Moreover, no spots corresponding to typical triglycerides, as referenced by the triolein (TG-C18:1) standard, were observed. The model proposed by Kumarn et al. [46] for NR particles in

ammonia-stabilized latex indicates a higher sensitivity of polar lipids to hydrolysis, resulting in the formation of negatively charged fatty acids that stabilize the latex by promoting uniform dispersion. Thus, one may suppose that fatty acids are primarily non-FuFA molecules, which were potentially hydrolyzed from polar lipids that are low in FuFA content [30, 47]. This assumption is supported by findings from Hasma [48] and Bottier et al. [49], who reported the hydrolysis of fatty acid-containing lipids, in addition to oxidation of α - and γ -tocotrienols in HA-stabilized latex across various genotypes. Hasma observed complete lipid hydrolysis within a month, whereas Bottier et al. noted that hydrolysis began after 4 days of incubation at 35°C in RRIM600 HA-stabilized latex. Interestingly, Hasma identified the presence of FuFA-F2-containing lipids from the RRIM701 genotype, where TG-FuFA hydrolysis started only after 9 days of storage. The delayed hydrolysis was attributed to the TG-FuFA being located away from the aqueous interface, rendering it less accessible to hydrolyzing agents, for instance, buried into lipid-like droplets or present inside rubber particles [42, 48].

3.1.3 | Evaluation of TG-FuFA in the Total Lipids From the Second Extract and Estimation of Its Concentration in Latex

The concentrations of TG-FuFA in lipid fraction extracts were evaluated (Figure 5A). The higher retention of TG-FuFA (lower R_f value) compared to other TG-bound fatty acids on the silica TLC plate ensures precise integration of the TG-FuFA spot during densitometer analysis. For LA samples, the C/M mixture, whether with or without NaCl washing, did not show a significant difference in TG-FuFA content. The lipid extract from LA samples showed that EtOAc yielded the highest TG-FuFA content in the second extract (43% w/w extract), followed by DiMeCO₃ (34%

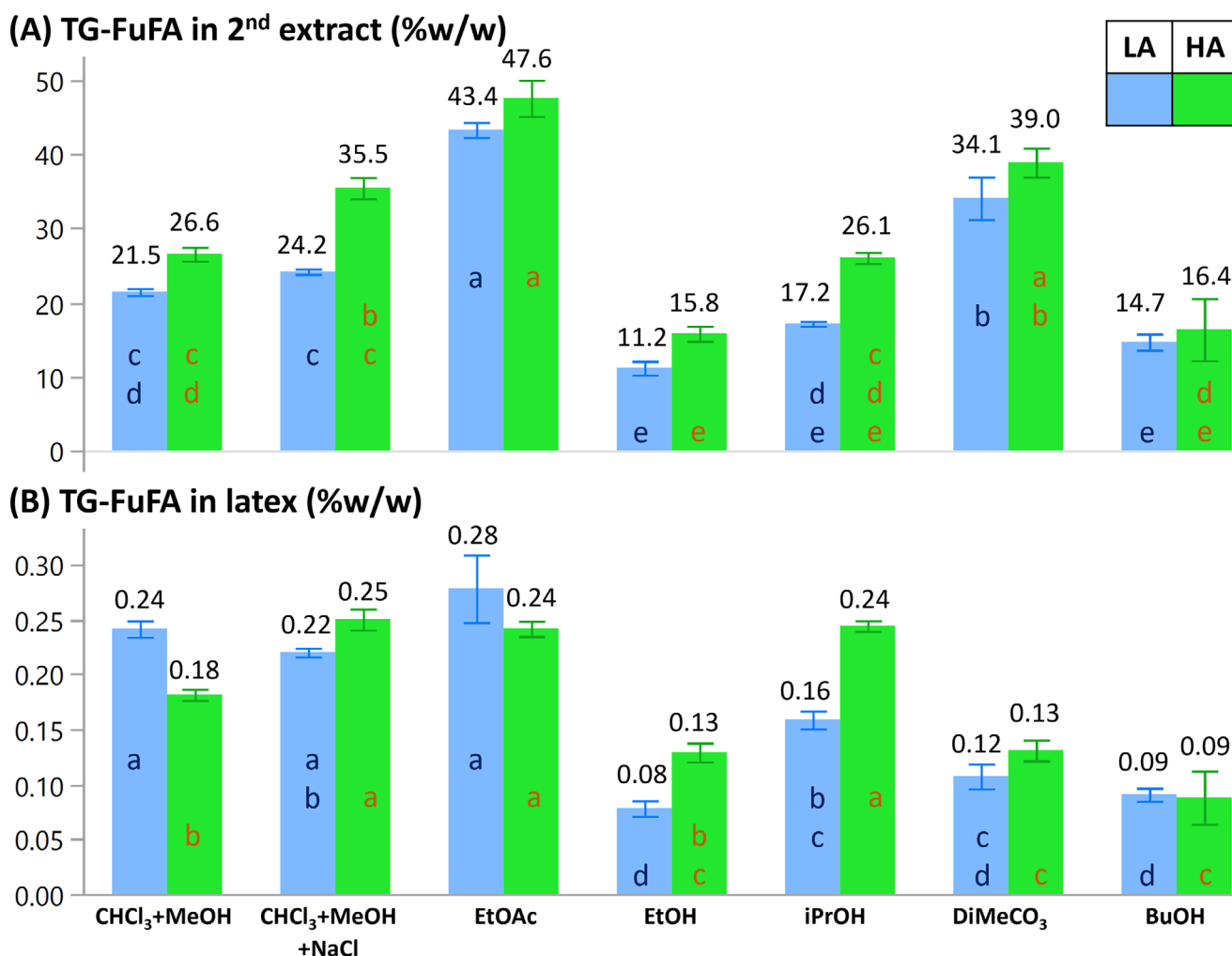


FIGURE 5 | Concentration of TG-FuFA in second extract (% w/w) (A), and in latex (% w/w) calculated by taking into consideration the extract content (% w/w latex) obtained in Figure 4 (B). Blue chart presents the result from LA sample (0.3% w/w NH₃ in latex) and green bar presents the result from HA sample (0.6% w/w NH₃ in latex). Solvents are C/M mixture with and without washing, ethyl acetate, ethanol, isopropanol, dimethyl carbonate, and *n*-butanol. Average comparison on different ammonia concentration in latex sample by Tukey–Kramer test, $p < 0.05$, are indicated by letters. HA, high-ammonia; LA, low-ammonia; TG-FuFA, trifuranoylglycerol.

w/w extract). iPrOH, EtOH, and BuOH resulted in the lowest TG-FuFA levels in the extract. For the HA samples, a similar trend was observed, with slightly higher TG-FuFA content.

Thus, the highest TG-FuFA levels in lipid fraction were statistically found in HA samples using EtOAc (47% w/w extract) and DiMeCO₃ (39% w/w extract) as bio-based solvents.

The concentration of TG-FuFA in LA and HA lattices extracted with different bio-based solvents were calculated and represented in Figure 5B. The maximum TG-FuFA content, approximately 0.22%–0.28% (w/w latex), was observed in the LA sample using C/M mixtures (both with and without washing) and EtOAc. For the HA sample, the highest TG-FuFA content was found in C/M with washing, as well as in EtOAc and iPrOH. EtOAc appeared to be the most effective bio-based solvent for extracting TG-FuFA from both HA and LA samples. Moreover, it was observed that alcohol-based solvents, especially EtOH and iPrOH, extracted more TG-FuFA from HA sample than from LA sample. HA sample exhibits lower viscosity than LA

sample due to its higher NH₃ content, which may also delay coagulation in alcohol and DiMeCO₃ solvents. This reduced coagulation rate prolongs latex-solvent contact time, thereby extending the extraction period. However, on the basis of TLC plates presented in Figure S4, these solvents demonstrated the ability to extract both TG-FuFA and FFA, along with polar lipids. Moreover, the higher viscosity of LA-stabilized latex may impede the extraction of FuFA-containing lipids; therefore, dilution could be an interesting option to further enhance the efficiency of extraction, as described by Liengprayoon et al. [35]. In conclusion, EtOAc yielded the highest concentration of TG-FuFA in lipid extracts, with minimal influence from ammonia concentration.

3.1.4 | Total-FuFA Content in Extracts and Comparison to Other Fatty Acids

The TFA content was quantified to evaluate the part of FuFA moieties present in the extracts. Figure 6 shows the TFA content

Content in latex (%w/w latex)

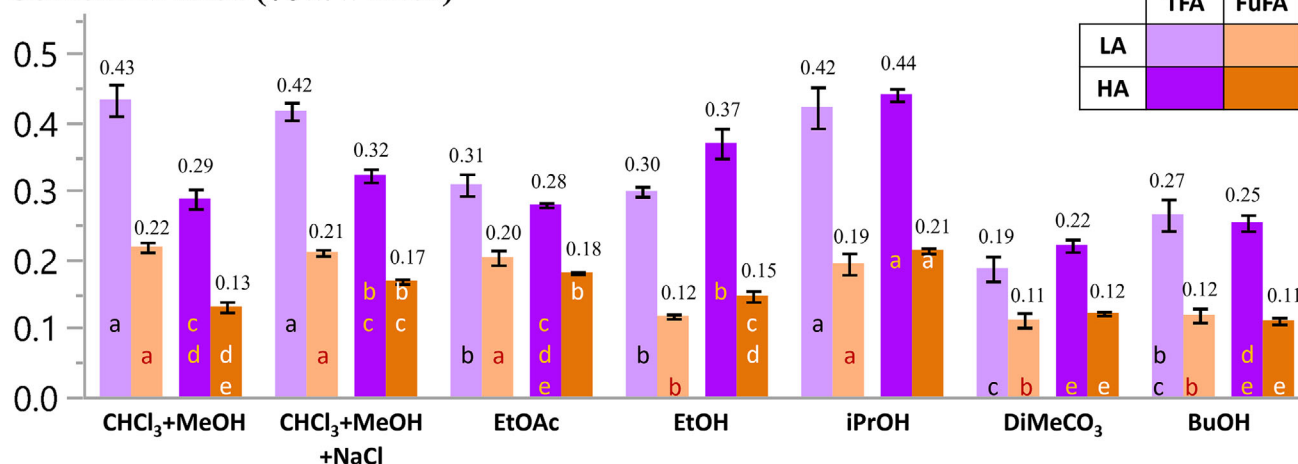


FIGURE 6 | Total fatty acid content (purple bar) and total-FuFA content (orange bar) in latex (% w/w) from LA sample (0.3% NH₃ in latex) and HA sample (0.6% NH₃ in latex). The solvents are C/M mixture—with/without washing, ethyl acetate, ethanol, isopropanol, dimethyl carbonate, and *n*-butanol. Statistical differences of average within each ammonia concentration in latex sample by Tukey–Kramer test, $p < 0.05$, are indicated by letters. FuFA, furan fatty acid; HA, high-ammonia; LA, low-ammonia; TFA, total fatty acid.

(% w/w latex) and total-FuFA content (% w/w latex) in both LA and HA sample. For LA sample, solvents that extracted the highest TFA content (0.42%–0.44% w/w latex) were iPrOH and C/M mixtures (both with and without aqueous salt washing). Medium TFA extraction yield (0.27%–0.31% w/w latex) was achieved with EtOAc, EtOH, and BuOH solvents. The lowest TFA extraction yield (0.19% w/w latex) was obtained with DiMeCO₃ (purple bar in Figure 6). Solvents with the highest TFA extraction yield also exhibited elevated total-FuFA content, ranging from 0.19% to 0.22% (w/w latex). In contrast, solvents with medium and low TFA extraction yield demonstrated statistically similar total-FuFA levels, approximately 0.12% (w/w latex) (orange bar in Figure 6).

In HA sample, the maximum TFA extraction was achieved with iPrOH (0.44% w/w latex), slightly higher than its value with LA sample (light purple chart in Figure 6, and in Figure S5). Yet, the TFA extraction increased with NH₃ stabilizer concentration for extracts from EtOH and DiMeCO₃. Conversely, TFA content from C/M mixtures (with or without washing) and EtOAc appeared to decrease with higher NH₃ stabilizer concentration. Comparing total-FuFA content between LA sample (Figure 6—light orange) and HA sample (Figure 6—dark orange), it was observed that increasing NH₃ stabilizer concentration reduced FuFA content in extracts from C/M mixtures and EtOAc. However, no negative effect on total-FuFA content was found with increasing NH₃ stabilizer concentration for EtOH, iPrOH, DiMeCO₃, and BuOH extracts. Moreover, EtOAc and DiMeCO₃ showed lower levels of other fatty acids (Figure S6). This finding supports the lipid profile interpretation (Section 3.1.2) and the fact that EtOAc yields the higher selectivity for extracting neutral FuFA-containing molecules (Figure S4).

3.1.5 | Evaluation of Lipid Extraction, Including FuFA, in Relation to Ammonia Concentration

In comparison to already published data [29], where the total-FuFA content extracted from the fresh PB235 latex sample was

0.67% (w/w latex), the FuFA content from stabilized latex was found at a lower content of around 0.22% (w/w latex, maximum reached in Figure 6A), representing a 67% reduction.

The observed difference could be attributed to the pH and surface charge effects from the NH₃ content. The lipid extract content from LA and HA sample is lower than that of fresh latex (see Table 1), aligning with the findings of Bottier et al. [49] from the RRIM600 genotype. The reduced lipid extract content correlates with a lower TFA content, with fresh PB235 latex containing 1.10% TFA/latex (w/w), whereas the LA sample has 0.42% and the HA sample is even lower at 0.32%. The FuFA content (in Figure 6) was comparable to, and often not statistically different from, the TG-FuFA amount (Figure 5B), indicating that (i) most of the recovered FuFA were grafted to TG and (ii) the majority of TG-FuFA were preserved from hydrolytic degradation.

In addition, if we gather and analyze all the results to highlight the effect of ammonia concentration, the data presented in Table 2 suggest the following findings:

- For the C/M mixture systems, increasing NH₃ concentration negatively impacts lipid extraction yields, affecting both polar and neutral lipids. However, there is a slight increase in the TG-FuFA/lipids ratio, which does not significantly reduce the overall TG-FuFA and FuFA contents ultimately recovered.
- For alcohol-based solvents, increasing NH₃ concentrations typically have little impact on the total lipid extraction yields, extracting both polar and NL. Nonetheless, there is a trend indicating an increase in the TG-FuFA and the total-FuFA ratios relative to other lipids or FFA, leading to a higher final content of TG-FuFA extracted from latex.
- For EtOAc and DiMeCO₃, NH₃ concentration has minimal impact. In addition, these solvents demonstrate the highest selectivity for NL, including TG-FuFA, resulting in an increased proportion of FuFA compared to other FFA. In

TABLE 1 | Comparison of lipid content, total fatty acid content, and total-furan fatty acid (FuFA) content in latex (% w/w) from chloroform/methanol mixture (C/M mixture) with salt washing extract of PB235 latex sample (fresh, low-ammonia [LA], and high-ammonia [HA]).

Sample type	Lipid/latex (% w/w)		TFA/latex (% w/w)	Total-FuFA/latex (% w/w)
	PB235	RRIM600	PB235	PB235
Fresh latex	1.74 ^a	0.90 ^a 1.10 ^b	1.10 ^a	0.66 ^a
LA stabilized latex	0.91	—	0.42	0.21
HA stabilized latex	0.71	0.73 ^b	0.32	0.17

Abbreviation: TFA, total fatty acid.

^aFrom Kitpaosong et al. [29].^bFrom Bottier et al. [49].**TABLE 2** | Comparison of bio-based solvent extraction efficiencies with respect to lipid yield, % ratio of trifuranoylglycerol (TG-FuFA)/lipids, % ratio of total-FuFA/ΣFFA, as well as TG-FuFA or total-FuFA contents in both low-ammonia [LA] and high-ammonia [HA] sample (% w/w).

Solvents	Latex	Lipid yield (%wt./latex)	% Ratio TG-FuFA/lipids	% Ratio total-FuFA/ΣFFA	TG-FuFA extracted (%wt./latex)	Total-FuFA extracted (%wt./latex)
CHCl₃/MeOH	LA	1.13 ± 0.04	21.5 ± 0.5	50.6 ± 1.1	0.242 ± 0.007	0.219 ± 0.007
	HA	0.68 ± 0.01	26.6 ± 0.9	45.4 ± 0.4	0.182 ± 0.005	0.131 ± 0.008
CHCl₃/MeOH + washing NaCl	LA	0.91 ± 0.01	24.2 ± 0.4	50.6 ± 0.5	0.220 ± 0.004	0.211 ± 0.005
	HA	0.70 ± 0.01	35.5 ± 1.4	52.3 ± 1.1	0.250 ± 0.01	0.169 ± 0.004
EtOAc	LA	0.64 ± 0.06	43.4 ± 1.0	65.7 ± 0.1	0.278 ± 0.031	0.203 ± 0.011
	HA	0.51 ± 0.02	47.6 ± 2.5	64.7 ± 0.5	0.242 ± 0.007	0.181 ± 0.002
EtOH	LA	0.70 ± 0.03	11.2 ± 0.9	39.2 ± 0.3	0.078 ± 0.007	0.118 ± 0.003
	HA	0.82 ± 0.00	15.8 ± 1.0	39.8 ± 0.4	0.129 ± 0.009	0.147 ± 0.008
iPrOH	LA	0.93 ± 0.03	17.2 ± 0.3	45.9 ± 0.4	0.159 ± 0.008	0.194 ± 0.016
	HA	0.94 ± 0.01	26.1 ± 0.8	48.5 ± 0.3	0.244 ± 0.005	0.214 ± 0.004
DiMeCO₃	LA	0.31 ± 0.02	34.1 ± 2.9	59.9 ± 1.3	0.107 ± 0.011	0.112 ± 0.011
	HA	0.34 ± 0.01	39.0 ± 2.0	55.3 ± 1.5	0.131 ± 0.009	0.122 ± 0.003
BuOH	LA	0.63 ± 0.06	14.7 ± 1.1	44.8 ± 0.0	0.091 ± 0.006	0.119 ± 0.010
	HA	0.53 ± 0.02	16.4 ± 4.2	43.8 ± 0.3	0.088 ± 0.024	0.111 ± 0.005

Abbreviations: BuOH, *n*-butanol; DiMeCO₃, dimethyl carbonate; EtOAc, ethyl acetate; EtOH, ethanol; iPrOH, isopropanol.

this context, a high lipid extraction yield is associated with a higher concentration of less-contaminated TG-FuFA, as seen with EtOAc. Conversely, low lipid extraction efficiency results in a lower yield of TG-FuFA, albeit with reduced contamination.

Currently, the price of fresh latex, on the basis of dry rubber content, is estimated at approximately \$1/L [50]. Given the growing recognition of FuFA's health benefits [26, 31–34], the potential added value to Hevea latex could significantly surpass its current market price. However, the economic feasibility remains uncertain, as it depends on the latex production volume of the PB235 genotype (or other high FuFA-producing genotypes) and their capacity for FuFA production. Solvent extraction offers

a scalable approach from laboratory to industrial production, with bio-based solvents such as EtOAc and iPrOH providing additional benefits due to their relatively low toxicity [2]. EtOAc and iPrOH offer a safer alternative to hazardous, fossil-based solvents like chloroform and, to a lesser extent, methanol, with reduced health risks. Additionally, they offer the potential for recyclability through evaporation and reuse. Further research is essential to determine how many reuse cycles are possible before their TG-FuFA extraction efficiency declines. Enhancing this scalability could significantly improve their economic feasibility. However, long-term viability will ultimately depend on production costs, highlighting—alongside FuFA application sectors—the importance of agronomic and economic studies to assess farming practices and production constraints.

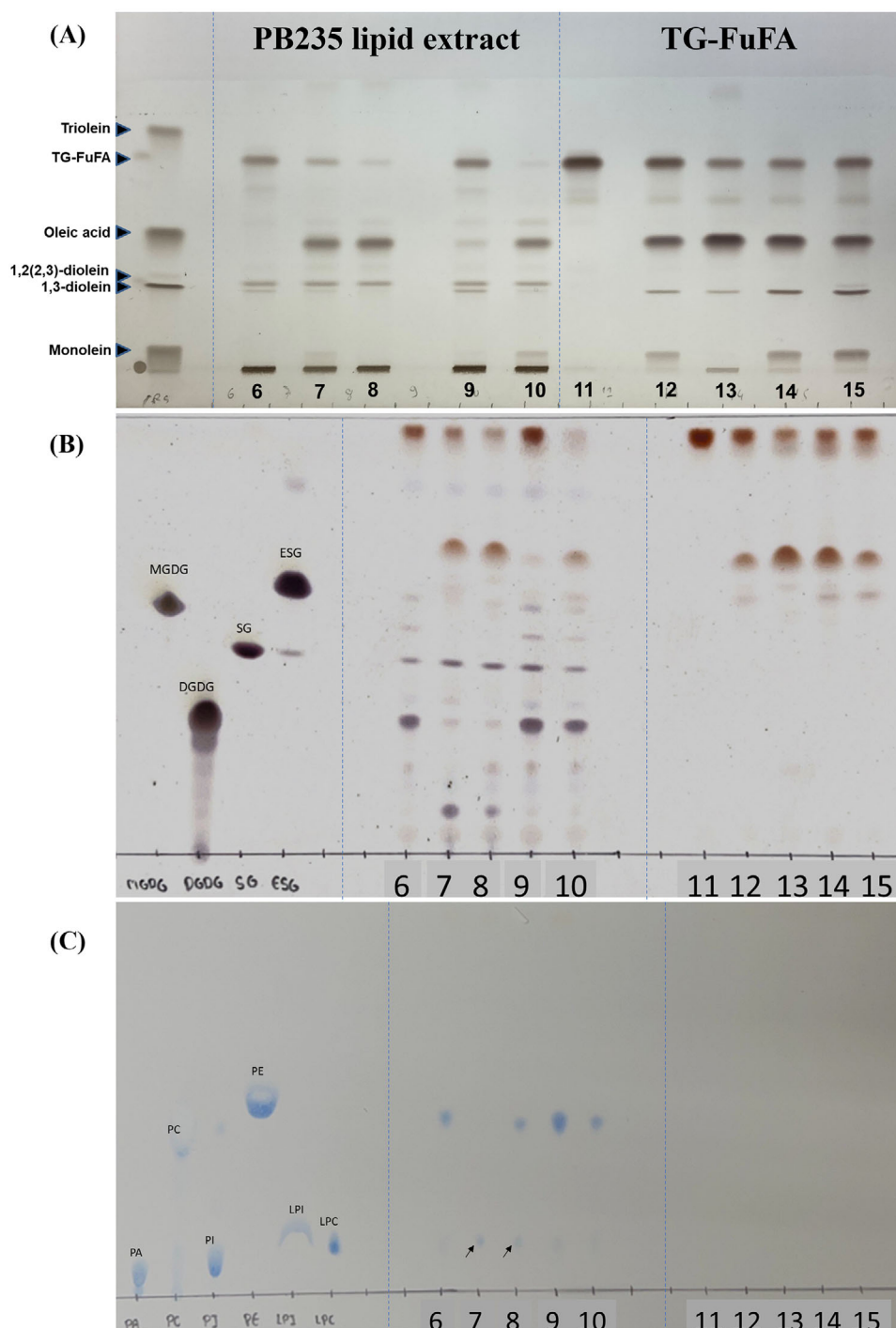


FIGURE 7 | TLC analysis of the enzymatic hydrolysis of FuFA-containing NL (A), GL (B), and PL (C) from PB235 fresh latex (Lane 6) and TG-FuFA (Lane 11), incubated with GPLRP2 (Lanes 7 and 12), Cutinase (Lanes 8 and 13), YLLIP2 (Lanes 9 and 14), and rDGL (Lanes 10 and 15). NB: Panels B and C, from purified TG-FuFA, confirm absence of polar lipids. Lipid references are DGDG, digalactosyl diacylglycerol; ESG, esterified steryl glucoside; FuFA, furan fatty acid; GL, glycolipid; GPLRP2, guinea pig pancreatic lipase-related protein 2; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; MGDG, monogalactosyl diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; rDGL, recombinant dog gastric lipase; SG, steryl glycoside; TG-FuFA, trifuranoylglycerol; TLC, thin-layer chromatography; YLLIP2, *Yarrowia lipolytica* LIP2 lipase.

3.2 | Exploring Enzymatic Approaches to Recover Higher Amount of FuFA as FFA

The feasibility of using enzymatic hydrolysis to break down all FuFA-containing molecules was explored to further isolate FuFA

from latex as FFA. As discussed in Section 3.1, the majority of the FuFA recovered from bio-based extraction solvents was grafted to TG, with EtOAc extraction yielding only up to 0.27% (w/w) of TG-FuFA from LA sample—about half of the total available FuFA [29].

FuFA may be isolated in its TG form, the main lipid structure found in *Hevea* latex, or by isolating it as a free form after preliminary hydrolysis to release FuFA from all the lipid classes. For instance, FuFA was isolated from a commercially stabilized and centrifuged latex in its free form after chemical hydrolysis of the lipid extract, utilizing pH-zone-refining countercurrent chromatography, followed by further purification with conventional countercurrent chromatography to achieve 99% purity [51]. On the basis of the findings of Liengprayoon et al. [30, 47] and with the knowledge of the structure of the main lipidic molecules present in lipid extract and their FA composition, we have estimated the FuFA content across different lipid classes in PB235 fresh latex: FuFA appears to be distributed approximately at 83% in NL, 10% in GL, and 7% in PL. Thus, even though its structure differs (FA instead of TG), enzyme-assisted extraction could be a promising alternative approach to enhance the yield of FuFA content recovery. Moreover, these results will provide insights into the stability of FuFA under enzymatic conditions and the effectiveness of different enzymes in hydrolyzing these lipid molecules. The enzymatic hydrolysis was studied on both a lipid extract from PB235 fresh latex (using the method by Liengprayoon et al. [35]) and purified TG-FuFA from the same lipid extract as described in Section 2.7.

3.2.1 | Qualitative Evaluating Hydrolysis Efficiency of Lipid Extract and Purified TG-FuFA From PB235 Latex Using Various Lipolytic Enzymes

The enzymatic hydrolysis of FuFA-containing lipid from the lipid extract of PB235 fresh latex and from purified TG-FuFA was studied using several lipases with different specificities. GPLRP2 and *F. solani* cutinase were used as enzymes broadly acting on TG, GL, and PL [39], whereas YLLIP2 and rDGL were used as TG lipases with 1,3-regioselectivity [40] and sn-3 stereoselectivity, respectively [41]. GPLRP2, cutinase, and rDGL were found to be highly active on TG-FuFA from the PB235 fresh latex lipid extract, as indicated by the formation of free FuFA (Figure 7A). *F. solani* cutinase can hydrolyze not only cutin and a wide range of esters but also exhibits lipase activity on emulsified TG, comparable to that of pancreatic enzymes [52, 53]. In contrast, YLLIP2 appeared inactive under these conditions. However, when using purified TG-FuFA as substrate, all enzymes, including YLLIP2, were active and released free FuFA. This suggests that some components, present in the lipid extract, may have inhibited YLLIP2 activity [51, 54]. It is worth noting that all enzymes were used under the same conditions for an initial and fast screening of lipases potentially active on these substrates. All appeared to be active at pH 7, although it is known that both rDGL and YLLIP2 show a higher lipase activity at lower acidic pH [40, 41].

The TLC analysis of the galactolipid components in PB235 revealed the presence of digalactosyl diacylglycerol (DGDG), steryl glycoside (SG), monogalactosyl diacylglycerol (MGDG), and esterified steryl glucoside (ESG) (Figure 7B). GPLRP2 (lane 7) and cutinase (lane 8) were effective in hydrolyzing DGDG, MGDG, and ESG, as shown by the disappearance of their respective bands and the appearance of a strong band of FFA. The hydrolysis activity on galactolipids is consistent with the result of Amara et al. [52] who reported the high rate of galactolipase

activity of cutinase and GPLRP2 on MGDG and DGDG. In contrast, YLLIP2 and rDGL have no activity on galactolipids. Regarding purified TG-FuFA substrate, the analysis confirmed its purity, with no presence of GL.

The TLC analysis of the phospholipid components in PB235 identified the presence of phosphatidylcholine (PC; Figure 7C). GPLRP2 effectively hydrolyzed PC to lysophosphatidylcholine (LPC; lane 7), whereas cutinase showed a lower activity (lane 8), indicated by black arrow. YLLIP2 and rDGL seemed to have no activity. These findings are consistent with the known substrate specificities of these lipases. GPLRP2 exhibits phospholipase A1 activity [55] with the release of the fatty acid at the sn-1 position of the glycerol backbone of PL. The phospholipase A1 activity of cutinase is, however, controversial. In a previous study, we were not able to see any activity on egg PC used as substrate [52]. Both YLLIP2 and rDGL are known to be inactive on polar lipids like PL and GL.

4 | Conclusions

In conclusion, the selected bio-based solvents demonstrated various selectivities for extracting total lipids and FuFA from LA and HA stabilized latex. For LA sample, ethyl acetate proved to be the most effective solvent for extracting TG-FuFA, outperforming the C/M mixture (2:1, v/v) with or without aqueous salt solution washing, achieving a TG-FuFA content of 0.28% (w/w latex). In addition, iPrOH emerged as an interesting alternative. For HA sample, EtOAc (0.24% w/w latex) remained the best solvent for TG-FuFA extraction, statistically comparable to C/M with aqueous salt washing (0.25% w/w latex). iPrOH also achieved a similar TG-FuFA content (0.24% w/w latex) but did not effectively exclude polar lipids from the extract. Regarding total-FuFA content, iPrOH was the best solvent at 0.21% (w/w latex), whereas the C/M mixture showed reduced efficacy. Regarding the enzymatic approach, the potential of GPLRP2 and cutinase for hydrolyzing all FuFA-containing lipids in latex has been demonstrated. However, further research is crucial to fully investigate the application of these lipolytic enzymes in a green extraction process, which could improve the recovery of FuFA as FFAs from stabilized latex. In this context, we are currently investigating the feasibility of performing this process directly on stabilized latex following dilution and pH adjustment to enable enzymatic activity process that may be challenged by potential polyisoprene coagulation, which can occur due to reduced surface charge and electrostatic repulsion. Regarding economic feasibility and production scalability, it is worth noting that viability will largely depend on product cost. However, considering the recent discoveries of FuFA's health benefits, the added value to *Hevea* latex could be substantial, far exceeding the current cost of latex. Further agronomic and economic study to know the farming and production limitations are interesting.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: 1ejlt70053-sup-0001-figureS1.png **Supporting**

File: 2ejlt70053-sup-0002-figureS2.png **Supporting**

File: 3ejlt70053-sup-0003-figureS3.png **Supporting**

File: 4ejlt70053-sup-0004-figureS4.png **Supporting**

File: 5ejlt70053-sup-0005-figureS5.png **Supporting File:**

6ejlt70053-sup-0006-figureS6.png