Supplementary Information

Standardization of Extraction Process Parameters and Solvent Reuse for Sustainable Extraction of Omega-3-Rich Flaxseed Oil

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1. Statistical Analysis (ANOVA):

Detailed ANOVA results for oil yield (%), solvent recovery (%), extraction efficiency, acid value (AV), and free fatty acid value (FFA) are included below.

1.1 Effect of solvent type, extraction time, and solid-liquid ratio on flax oil yield

Table S1. One-way ANOVA and Tukey's HSD test showing the effect of extraction time on oil yield for different solvents. Means values sharing the same lowercase letter are not significantly different ($p > 0.05$).

Solvent	ANOVA (F-value)	p-value	Time (h)	Mean oil yield (%)	Tukey group letter
Hexane	6.16	0.03511	16	43.44 ± 1.85	a
			12	40.25 ± 1.48	ab
			8	39.31 ± 1.11	b
Ethanol	774.39	< 0.001	16	38.22 ± 0.02	a
			12	34.36 ± 0.62	b
			8	26.14 ± 0.24	c
Ethyl acetate	23.17	0.0015	16	40.25 ± 0.43	a
			12	39.85 ± 0.02	a
			8	37.07 ± 0.99	b

Table S2: Two-way ANOVA for the effect of solvent type and extraction time on oil yield

Source	df	F-value	p-value
Extraction time	2	101.87	< 0.001
Solvent type	2	171.05	< 0.001
Interaction (time × solvent)	4	22.13	< 0.001

Table S3. One-way ANOVA and Tukey's HSD test showing the effect of solid-liquid ratio on oil yield for different solvents. Means values sharing the same lowercase letter are not significantly different ($p > 0.05$).

Solvent	ANOVA (F-value)	p-value	S-L Ratio	Mean oil yield (%)	Tukey group letter
Hexane	81.93	< 0.001	1:2.5	21.79 ± 0.53	c
			1:5	36.19 ± 2.76	b
			1:10	40.48 ± 1.3	ab
			1:15	45.56 ± 2.39	a
Ethanol	222.83	< 0.001	1:2.5	19.12 ± 1.73	d
			1:5	33.97 ± 0.18	c
			1:10	37.06 ± 0.35	b
			1:15	41.36 ± 1.37	a
Ethyl acetate	187.51	< 0.001	1:2.5	20.72 ± 1.1	d
			1:5	34.65 ± 0.26	c
			1:10	38.29 ±1.65	b
			1:15	43.7 ± 1.47	a

Table S4: Two-way ANOVA for the effect of solvent type and solid-liquid ratio on oil yield

Source	df	F-value	p-value
S-L ratio	3	398.99	< 0.001
Solvent type	2	13.31	< 0.001
Interaction (S-L ratio × solvent)	6	0.3329	0.92

Table S5. One-way ANOVA and Tukey's HSD test showing the effect of solid-liquid ratio on the extraction efficiency (%) for different solvents. Means values sharing the same lowercase letter are not significantly different ($p > 0.05$).

Solvent	ANOVA (F-value)	p-value	S-L Ratio	Mean oil yield (%)	Tukey group letter
Hexane	84.93	< 0.001	1:2.5	75.42 ± 1.27	d
			1:5	81.64 ± 2.56	c
			1:10	89.44 ± 2.55	b
			1:15	99.47 ± 0.74	a
Ethanol	36.70	< 0.001	1:2.5	60.96 ± 0.74	c
			1:5	74.94 ± 2.2	b
			1:10	81.84 ± 3.86	b
			1:15	91.95 ± 5.92	a
Ethyl acetate	88.77	< 0.001	1:2.5	66.87 ± 1.46	d
			1:5	76.2 ± 0.51	c
			1:10	86.8 ± 3.84	b
			1:15	96.93 ± 2.4	a

Table S6: Two-way ANOVA for the effect of solvent type and solid-liquid ratio on extraction efficiency (%)

Source	df	F-value	p-value
S-L ratio	3	168.68	< 0.001
Solvent type	2	31.72	< 0.001
Interaction (S-L ratio × solvent)	6	1.63	0.182

1.2 Solvent recycling study

Table S7: Solvent recovery and oil yield across ten Soxhlet extraction cycles. Values are expressed as mean (n = 3). Means sharing the same letter within each column are not significantly different ($p > 0.05$), as determined by one-way ANOVA followed by Tukey's HSD test.

Extraction Cycle	Solvent recovery (%)	Tukey group letter	Oil yield (%)	Tukey group letter
1	68 ± 1.5	c	42.5 ± 0.35	a
2	76 ± 1	ab	40.5 ±0.35	b
3	71 ± 2	c	42.5 ± 0.35	a
4	75 ± 1	b	40.5 ± 0.7	b
5	79 ± 1	a	42.5 ± 0.35	a
6	71 ± 2	c	40.5 ± 0.7	b
7	71 ± 1	c	42.5 ± 1.06	a
8	71 ± 1	c	42.5 ± 0.35	a
9	71 ± 1	c	42.5 ± 0.35	a
10	71 ± 1	c	42.5 ± 0.35	a

During the solvent recycling (hexane) study, the oil cake mass was recorded for each extraction cycle. For every 10 g of flaxseed processed, approximately 5–6 g of residual oil cake was obtained. Detailed oil cake data across extraction cycles are presented in **Figure S1**.

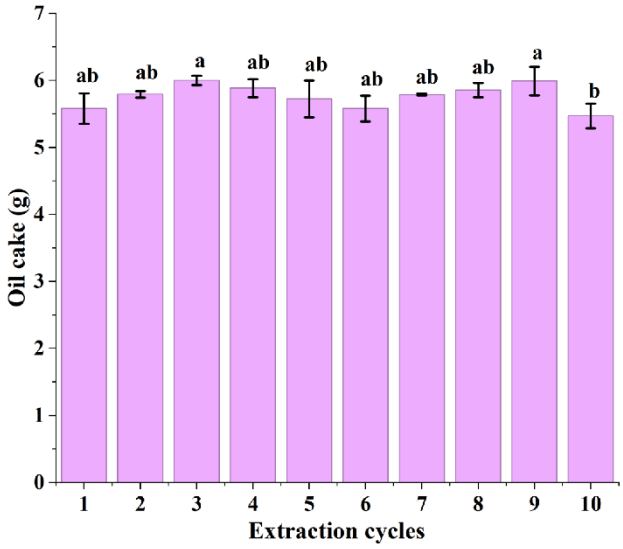

Figure S1. Amount of oil cake obtained over ten consecutive extraction cycles. Values are expressed as mean ± standard deviation (n = 3). Different lowercase letters indicate statistically significant differences ($p < 0.05$) among extraction cycles, as determined by one-way ANOVA followed by Tukey's post hoc test.

2. Gas Chromatography (GC) analysis:

The flax oil extracted and the hexane recovered at the end of each Soxhlet extraction cycle were analysed by GC-FID to assess the fatty acid composition and the presence of impurities in the recovered solvent. The protocol described in the GC-FID analysis in **Section 2.4** was used to analyse both oil and FAME standards. The retention time and corresponding area (%) of fatty acids present in the FAME standard are presented in **Table S8.**

The chromatograms of the oil obtained showed the presence of fatty acids such as palmitic acid, palmitoleic acid, cis-heptadecanoic acid, stearic acid, oleic acid, trans-9-elaidic acid, linoleic acid, linolenic acid, and gamma-linolenic acid. The fatty acids were identified by comparing the area percentages and retention times of fatty acids in the oil chromatogram (**Table S9**) with those in the FAME standard (**Table S8).**

Table S8: GC–FID analysis of fatty acid methyl ester (FAME) standards showing retention time and area percentage. Values are expressed as mean ± standard deviation (n = 3).

S. No	Fatty acid	Short name	Area (mean ± SD)	Area (%)	Retention time (min)
1	Butyric acid	C4:0	1492265.7 ± 2.14	3.22 ± 0.00	4.08 ± 0.00
2	Caproic acid	C6:0	1680481.6 ± 0.53	3.63 ± 0.00	8.10 ± 0.00
3	Capyrlic acid	C8:0	1800165.0 ± 2.05	3.89 ± 0.00	12.38 ± 0.00
4	Capric acid	C10:0	921792.12 ± 0.90	1.99 ± 0.00	14.60 ± 0.00
5	Undecanoic acid	C11:0	1888421.9 ± 0.84	4.08 ± 0.00	16.80± 0.00
6	Lauric acid	C12:0	960990.2 ± 0.56	2.07 ± 0.00	18.46± 0.00
7	Triundecanoic acid	C13:0	1982009.0 ± 1.14	4.28 ± 0.00	21.01± 0.57
8	Myristic acid	C14:0	955859.45 ± 0.60	2.06±0.00	21.81 ± 0.00
9	Myristoleic acid	C14:1	994407.38 ± 0.98	2.15±0.00	22.99± 0.01
10	Pentadecanoic acid	C15:0	969763.34 ± 3.79	2.09± 00	23.78 ± 0.00
11	*cis*-10-Pentadecenoic acid	C15:1	2957297.5 ± 1.50	6.39±0.00	24..91± 0.00
12	Palmitic acid	C16:0	991314.42 ± 0.56	2.14± 0.00	25.45± 0.00
13	Palmitoleic acid	C16:1	100854.18 ± 0.62	0.21±0.00	26.74 ± 0.00
14	Heptadecanoic acid	C17:0	998343.5 ± 1.17	2.15±0.00	27.27± 0.00
15	*cis*-10-Heptadecanoic acid	C17:1	2027160.4 ± 2.24	4.38±0.00	28.51± 0.01
16	Stearic acid	C18:0	3064543.3 ± 1.60	6.62±0.00	28.93± 0.00
17	Elaidic acid	C18:1 trans (n9)	2056423.7 ± 1.24	4.44±0.00	29.67± 0.00
18	Oleic acid	C18:1 cis (n9)			

19	Linolelaidic acid	C18:2 trans (n6)	1048160.2 ± 1.10	2.26±0.00	30.32 ± 0.02
20	Linoleic acid	C18:2 cis (n6)			
21	Gamma-Linolenic acid	C 18:3n6	1007678.4 ± 2.21	2.17 ± 0.00	30.93 ± 0.00
22	Alpha-linolenic acid	C18:3n3	2063120.5 ± 1.01	4.46 ± 0.00	32.08 ± 0.00
23	Arachidic acid	C20:0	1033274.4 ± 0.82	2.23 ± 0.00	32.57 ± 0.00
24	*cis*-11-Eicosenoic acid	C20:1(n9)	1003200.6 ± 0.54	2.17 ± 0.00	33.72 ± 0.01
25	*cis*-11,14-Eicosadienoic acid	C20:2	10271434.9±0.93	2.22±0.00	34.46 ± 0.00
26	*cis*-8,11,14-Eicosatrienoic acid	C20:3n6	1003002.5±0.50	2.16±0.00	34.46 ± 0.00
27	Heneicosanoic acid	C21:0			
28	*cis*-11,14,17-Eicosatrienoic acid	C20:3n3	992280.51 ± 0.61	2.14 ± 0.00	35.13 ± 0.01
29	Arachidonic acid	C20:4n6	995168.33 ± 0.58	2.15 ± 0.00	35.46 ± 0.01
30	*cis*-5,8,11,14,17-Eicosapentaenoic acid	C20:5n3	30753864.0 ±2.12	6.65 ± 0.00	37.1 ± 0.00
31	Behenic acid	C22:0	1009643 8 ± 0.84	2.18 ± 0.00	37.89 ± 0.01
32	Erucic acid	C22:1n9	1033815.0 ± 1.10	2.23 ± 0.00	39.66 ± 0.01
33	*cis*-13,16-Docosadienoic acid	C22:2	1065267.7 ± 0.65	2.30 ± 0.00	40.53± 0.00
34	Tricosanoic acid	C23.0	2105577.2 ± 0.40	4.55 ± 0.00	44.87 ± 0.01
35	Lignoceric acid	C24:0	1053386.8 ± 0.81	2.27 ± 0.00	46.19 ± 0.00
36	Nervonic acid	C24:1	868947.26 ± 0.67	1.87 ± 0.00	46.64 ± 0.02
37	*cis*-4,7,10,13,16,19-Docosahexaenoic acid	C22:6(n3) (DHA)			

Table S9: Retention time (RT) and area percentage of major fatty acids in flax oil across Soxhlet extraction cycles. Values are presented as mean ± standard deviation (n = 3).

Cycle	Parameter	PA	PAM	SA	CHA	OA	LA	LAA	GLA	TEA
1	**RT (min)**	25.64 ± 0.01	–	29.02 ± 0.01	28.95 ± 0.56	29.18 ± 0.58	29.88 ± 0.00	31.12 ± 0.00	–	–
	Area (%)	6.20 ± 0.01	–	22.63 ± 0.03	6.40 ± 0.26	0.72 ± 0.01	14.54 ± 0.02	48.76 ± 0.20	–	–
2	**RT (min)**	25.02 ± 0.00	–	29.09 ± 0.00	28.65 ± 0.01	29.15 ± 0.01	29.88 ± 0.00	31.12 ± 0.00	32.21 ± 0.01	–
	Area (%)	7.42 ± 0.05	–	24.24 ± 0.45	7.12 ± 0.41	0.70 ± 0.01	14.25 ± 0.02	44.36 ± 0.01	0.53 ± 0.01	–
3	**RT (min)**	25.01 ± 0.00	–	29.06 ± 0.00	28.62 ± 0.01	29.14 ± 0.01	29.86 ± 0.00	31.07 ± 0.05	–	–
	Area (%)	6.2 ± 0.00	–	22.82 ± 0.13	6.19 ± 0.00	0.61 ± 0.01	13.41 ± 0.01	51.2 ±0.01	–	–
4	**RT (min)**	25.01 ± 0.00	–	29.05 ± 0.00	28.61 ± 0.01	29.12 ± 0.02	29.86 ± 0.00	31.08 ± 0.00	–	–
	Area (%)	6.15 ± 0.04	–	21.53 ± 0.09	6.43 ± 0.02	1.16 ± 0.35	13.39 ± 0.09	51.10 ± 0.53	–	–
5	**RT (min)**	25.64 ± 0.01	25.63 ± 0.01	29.04 ± 0.02	28.63 ± 0.01	29.15 ± 0.00	29.88 ± 0.001	31.12 ± 0.00	–	29.67± 0.00
	Area (%)	4.70 ± 0.02	4.66 ± 0.06	21.85 ± 0.10	6.72 ± 0.01	0.70 ± 0.00	12.48 ± 0.03	43.89 ± 0.21	–	2.53± 0.01
6	**RT (min)**	25.01 ± 0.001	–	29.04 ± 0.02	28.63 ± 0.01	29.14 ± 0.01	29.87 ± 0.00	31.10 ± 0.01	–	–
	Area (%)	6.23 ± 0.002	–	23.3 ± 0.26	7.3 ± 0.01	0.70 ± 0.05	13.54 ± 0.02	48.3 ± 0.13	–	–
7	**RT (min)**	25.01 ± 0.00	–	29.08 ± 0.01	28.95 ± 0.56	29.13 ±0.02	29.87 ± 0.01	31.12 ± 0.00	–	–
	Area (%)	6.05 ± 0.03	–	22.04 ± 0.04	6.68 ± 0.20	0.64 ± 0.01	13.35 ± 0.13	51.18 ± 0.07	–	–
8	**RT (min)**	25.03 ± 0.00	–	29.10 ± 0.00	28.65 ± 0.01	–	29.87 ± 0.01	31.16 ± 0.01	32.21 ± 0.01	–
	Area (%)	6.0 ± 0.01	–	22.73 ± 0.27	6.37 ± 0.01	–	13.48 ± 0.49	50.36 ± 0.14	0.46 ± 0.01	–
9	**RT (min)**	25.01 ± 0.00	–	29.07 ± 0.05	28.63 ± 0.01	29.17 ± 0.01	29.89 ± 0.00	31.16 ± 0.00	–	–
	Area (%)	6.2 ± 0.00	–	22.87 ± 0.06	6.30 ± 0.03	0.64 ± 0.02	13.56 ± 0.01	50.41 ± 0.17	–	–
10	**RT (min)**	25.02 ± 0.00	–	28.72 ± 0.11	28.63 ± 0.01	29.13 ± 0.02	29.871 ± 0.01	31.12 ± 0.00	–	–
	Area (%)	6.15 ± 0.04	–	22.65 ± 0.02	6.43 ± 0.01	0.67 ± 0.00	13.57 ± 0.01	50.29 ± 0.11	–	–

PA: Palmitic acid, **LA:** Linoleic acid, **PAM:** Palmitoleic acid, **LAA:** Linolenic acid, **SA:** Stearic acid, **GLA:** Gamma linolenic acid, **CHA:** Cis Heptadecanoic acid, **TEA:** Trans-9-Elaidic acid, **OA:** Oleic acid

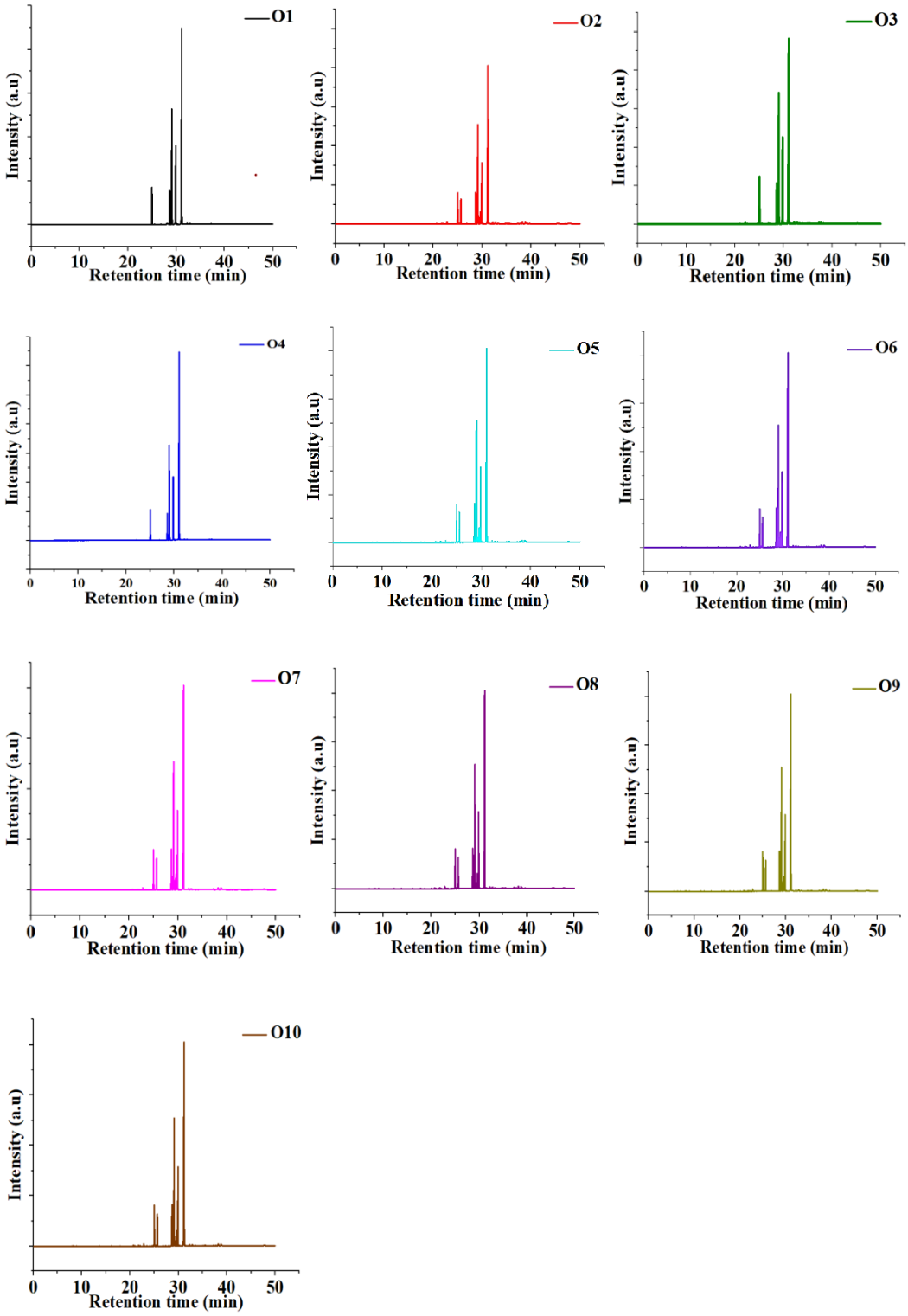

Figure S2. GC-FID chromatograms for oil extracted from ten extraction cycles using hexane

Chromatograms of solvent (hexane) recovered from different Soxhlet extraction cycles were obtained for confirming the quality of hexane and if any degradation of impurities present. The chromatogram of pure hexane showed a peak at 2.09 min with an area percentage of 99.64 ± 0.02%. The GC-FID analysis of hexane recovered from the 10 extraction cycles showed peaks at 2.09 min with 99.15 ± 0.01

to 99.76 ± 0.02% area percentage with no additional peaks (**Figure S3**). This confirms that the recovered hexane after the extraction cycles did not degrade, and hence that the solvent was free of impurities. The resulting chromatograms are presented in **Figure S3.**

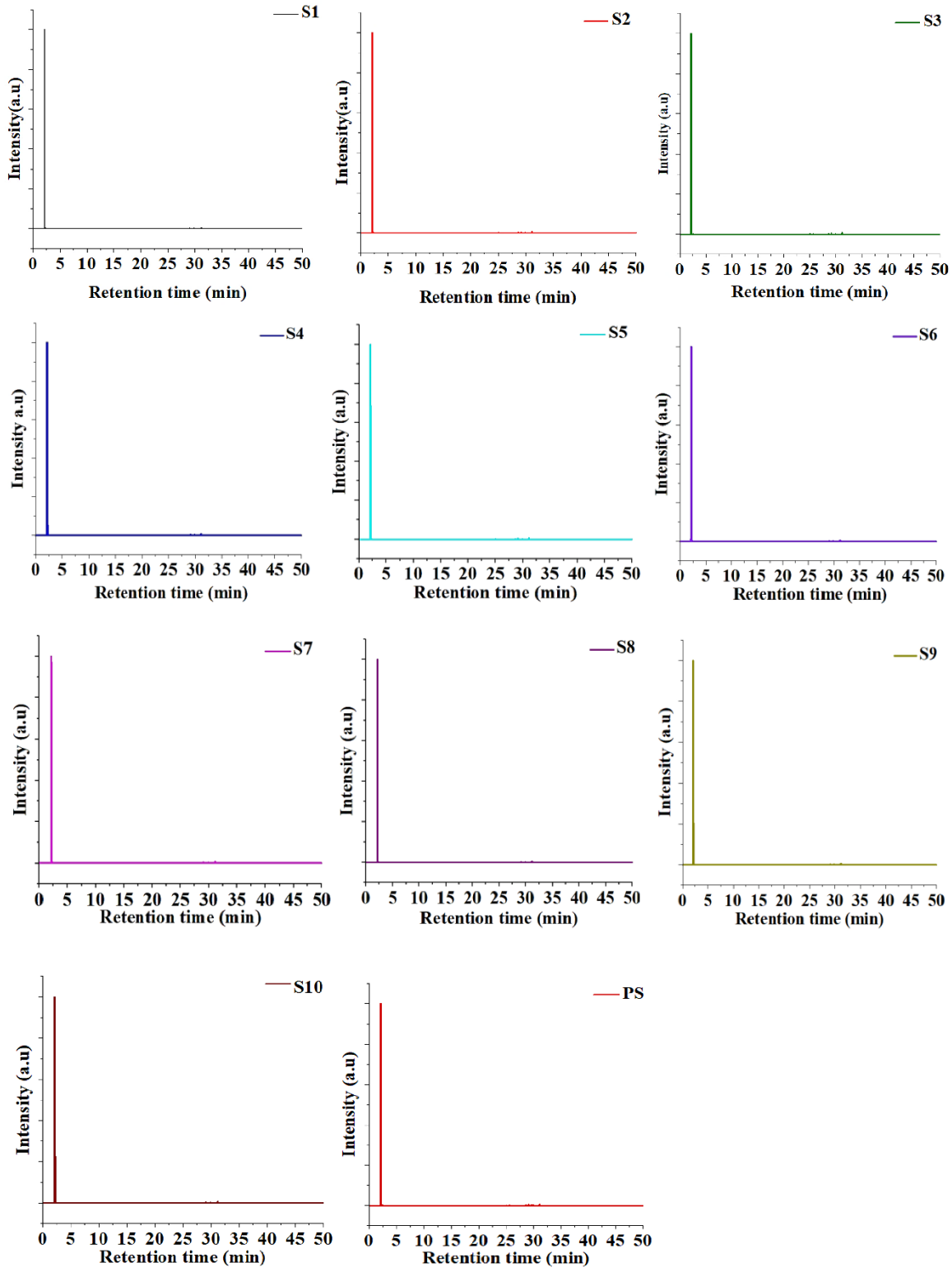

Figure S3. GC-FID chromatograms for recovered hexane from ten extraction cycles (S: hexane, PS: Pure hexane)

3. FT-IR Analysis

The FT-IR spectra of flax oil showed characteristic absorption bands at 3012, 2925, 2854, 1743, 1461, 1376, 1238, 1162, 1099 and 723 cm^{-1}, indicating functional groups as mentioned in **Table S10**. These bands represent =C-H Stretching(olefinic), asymmetric and symmetric stretching of -CH_2 groups, C=O stretching, CH_2, bending, CH_3 symmetric bending, C-O-C and C-O stretching vibrations. The FT-IR spectra of the oils extracted from each Soxhlet extraction cycle remained consistent with respect to the peak positions and functional groups observed (**Figure S4**). This confirms that the chemical structure of the oil did not change during repeated extraction cycles.

Table S10: Functional groups with type of vibrations for FT-IR spectra of flax oil

Band frequency (cm^{-1})	**Functional group**	**Type of vibrations**
3012	Olefinic	=C-H Stretching
2925	Aliphatic	asymmetrical stretching of the methylene group (-CH_2)
2854	Aliphatic	symmetrical stretching of the methylene group (-CH_2)
1743	Ester	C=O stretching (carbonyl group)
1461	Alkane (-CH_2)	Bending vibration of CH_2
1376	Alkyl group (-CH_3)	Symmetric bending of CH_3 group
1238	Ester/Ether	C-O-C stretching vibration
1162	Ester	C-O stretching (ester linkage)
1099	Ester/Ether	C-O stretching
723	Alkane	C-H rocking bond vibrations

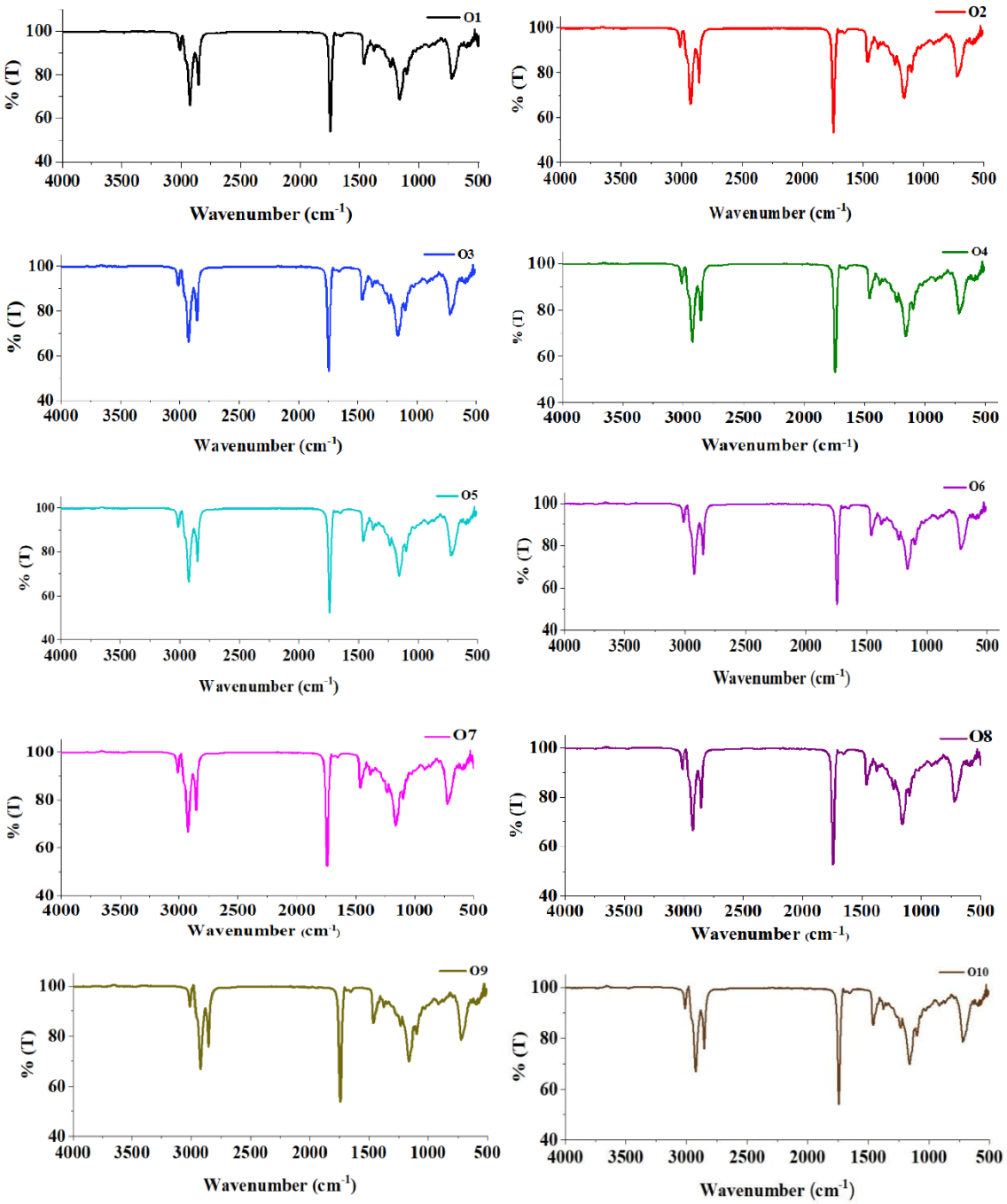

Figure S4. FT-IR spectra of flax oil extracted using hexane as a solvent across ten Soxhlet extraction cycles

The FT-IR spectra of the recovered hexane showed characteristic peaks at 2960, 2923, 2854, 1461, 1377, and 724 cm^{-1}. The spectra remained largely unchanged across different extraction cycles, which indicates that hexane retained the chemical structure. As shown in Figure S5, minor variations in the peak intensity were observed from the fourth extraction cycle onwards. This change in intensity may be attributed to the presence of trace co-extracted compounds. Also, no additional peaks corresponding to new functional groups were detected, confirming the absence of significant chemical changes in the recovered hexane. Although minor changes in peak intensity were observed in the recovered hexane,

these changes did not affect the solvent's performance in terms of oil yield and oil quality, as indicated by consistent results for oil yield and the oil's physicochemical properties across successive extraction cycles presented in the main manuscript.

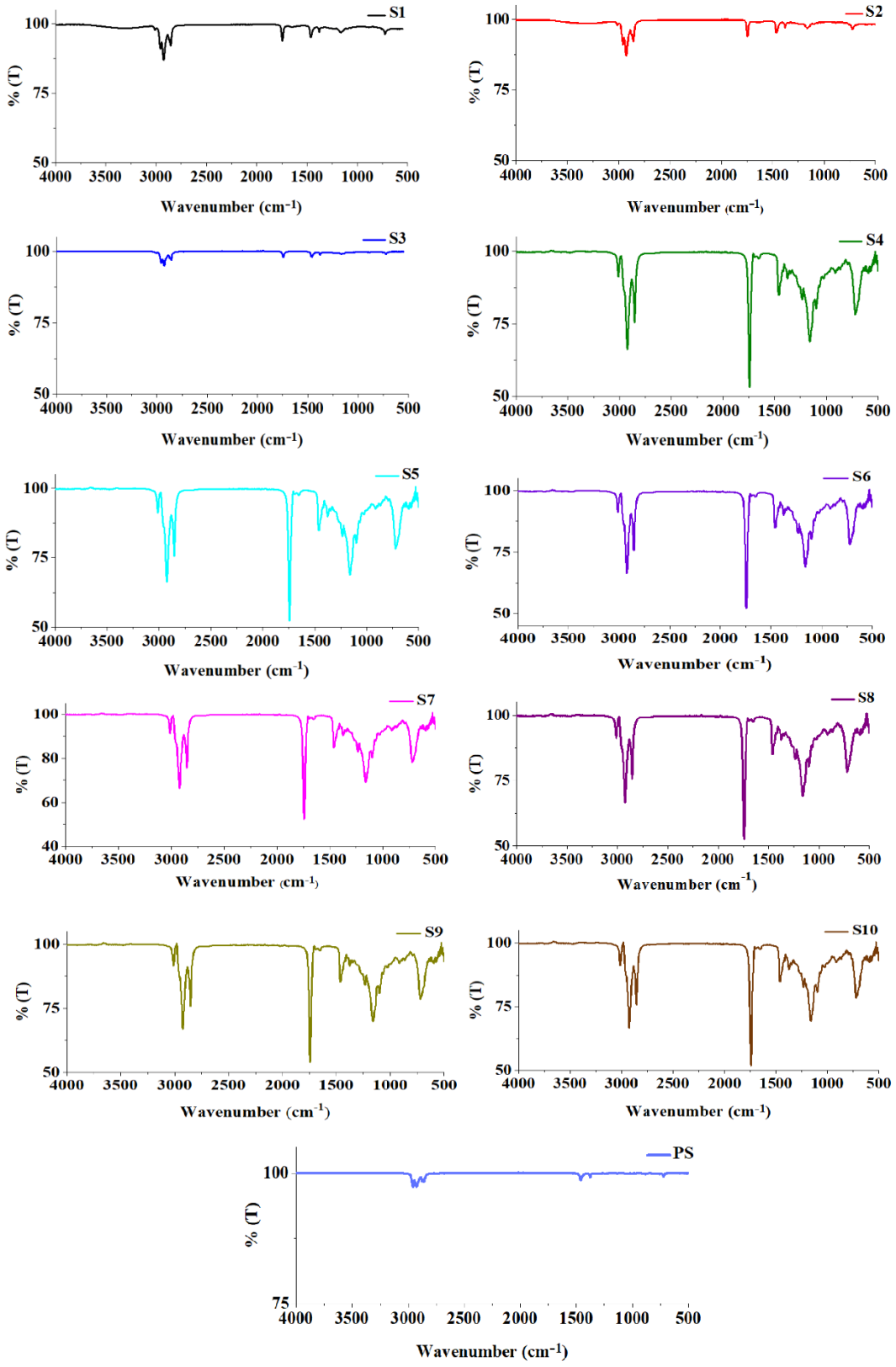

Figure S5. FT-IR spectra of recovered hexane from 10 successive Soxhlet extraction cycles (S: hexane, PS: Pure hexane).