SUPPLEMENTARY DATA

Development and validation of a novel UPLC-ELSD method for the assessment of lipid composition of nanomedicine formulation.

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Table S1: Theoretical concentration of the nanoparticle components (F50) along the process of quantification: from the formulation step to the analysis by UPLC-ELSD.

Lipid	Formulated[a] conc. (mg/mL)	Solubilized[b] Conc. (µg/mL)	Analysis[c] Conc. (µg/mL)
Soybean (SB)	**11.33**	**2833.3**	**113.3**
Suppocire (SC)	**34.00**	**8500.0**	**340.0**
Myrj-S40 (S40)	**46.00**	**11500.0**	**460.0**
PEG-OH	9.88	2468	98.8
PEG-C16	11.34	2837	113.4
PEG-C18	11.57	2894	115.7
Lipoid-S75 (S75)	**8.67**	**2166.7**	**216.7**
PC	6.07	1516.7	151.6
PE	0.87	216.7	21.7

[a]Concentration calculated after dialysis for a 50 nm formulation (F50) (total lipids at a theoretical concentration of 100 mg/mL). Concentration of PEG components were calculated by considering they represent 71.3% (w/w) of S40, respectively composed of 30.1, 34.6 and 35.3% (w/w) of PEG-OH, PEG-C16 and PEG-C18. Concentration of PC and PE were calculated by considering S75 is composed of PC and PE at 70 and 10% (w/w), respectively.

[b]Concentration calculated after solubilizing a freeze-drying powder at a theoretical concentration of 25 mg/mL of total lipids only (amount of PBS was not considered).

[c]Expected concentration of the samples prepared for the analysis by UPLC-ELSD. For the analysis of SB, SC and S40 (QMC18), the concentration (theoretical value) is 1 mg/mL of total lipids. For the HILIC mode (QMHILIC), the theoretical concentration is equal to 2.5 mg/mL of total lipids.

Table S2: Initial amount (mg) of lipid components incorporated in the formulation process of 50 nm (F50), 80 nm (F80) and 120 nm (F120) nanoparticles and final volume (V_{total}) of the suspensions before dialysis (200 mg/mL of lipids).

	F50		**F80**		**F120**	
	Initial quantities (mg)	**[a]Formulated conc. (mg/mL)**	**Initial quantities (mg)**	**Formulated conc. (mg/mL)**	**Initial quantities (mg)**	**Formulated conc. (mg/mL)**
SB (mg)	85	11.33	102.5	13.49	150	17.44
SC (mg)	255	34.00	307.5	40.46	450	52.33
S75 (mg)	65	8.67	50	6.58	45	5.23
S40 (mg)	345	46.00	300	39.47	215	25.00
Total (mg)	750	-	760	-	860	-
V_{total} (µL)	3750	-	3800	-	4300	-
[b][Lipids] for SPE solution (mg/mL)	25.0		32.9		41.4	
[c]Vol. for SB/SC analysis (µL)	200.0		127.6		78.5	
[d]Vol. for S40 analysis (µL)	200.0		200.0		222.2	

[a]Concentration calculated after dialysis (total lipid excipients at a theoretical concentration of 100 mg/ml);

[b]Solution prepared for SPE in order to reach the analysis/target concentration of 216.7 µg/mL for S75;

[c]Volume of the solution prepared for SPE and added to a 5 mL volumetric flask in order to reach the analysis/target concentration of 113.3 and 340.0 µg/mL for SB and SC, respectively;

[d]Volume of the solution prepared for SPE and added to a 5 mL volumetric flask in order to reach the analysis/target concentration of 460 µg/mL for S40.

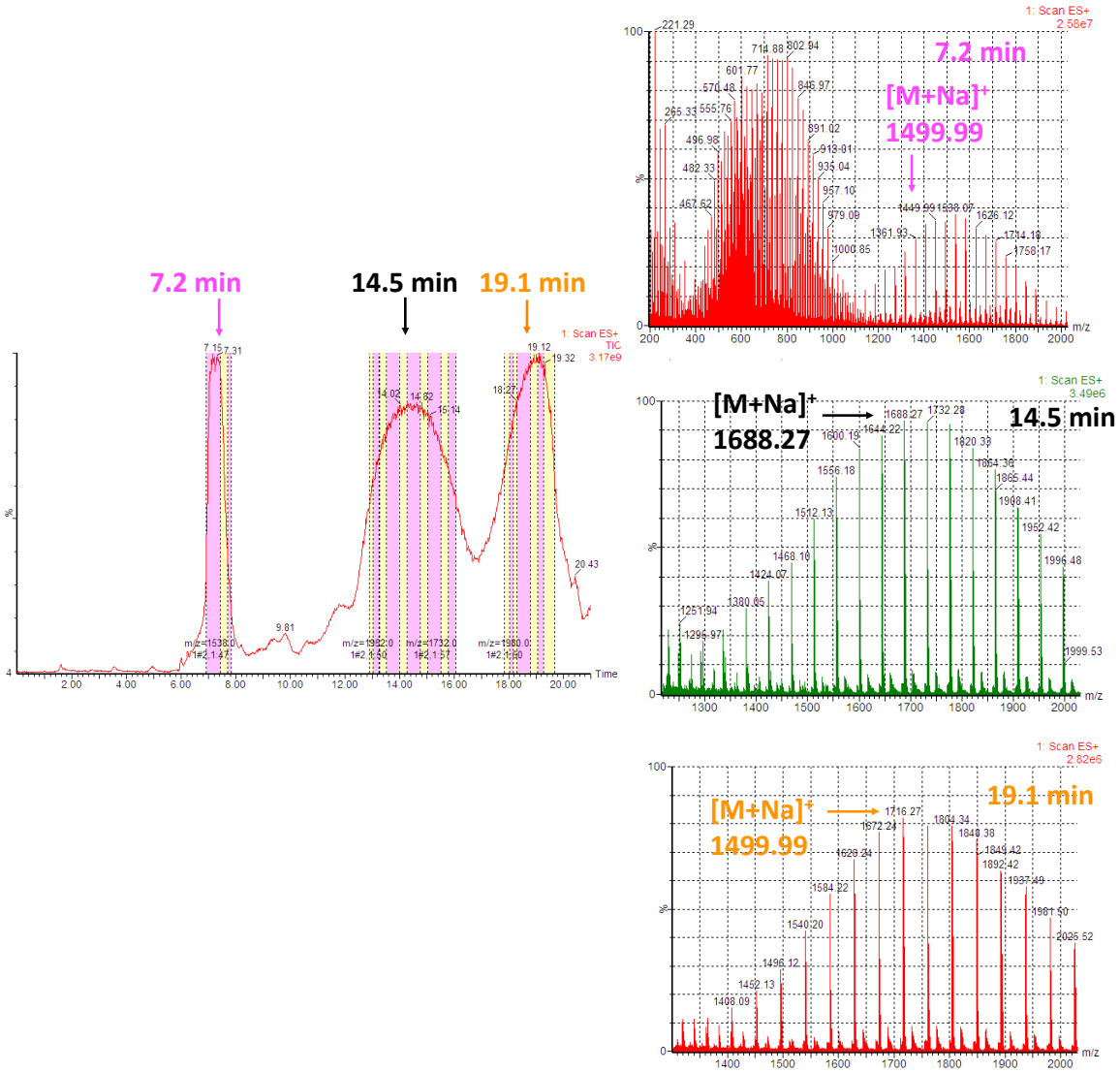

Figure S1: Typical chromatogram obtained for the isolation of S40 components by preparative HPLC and corresponding mass spectra.

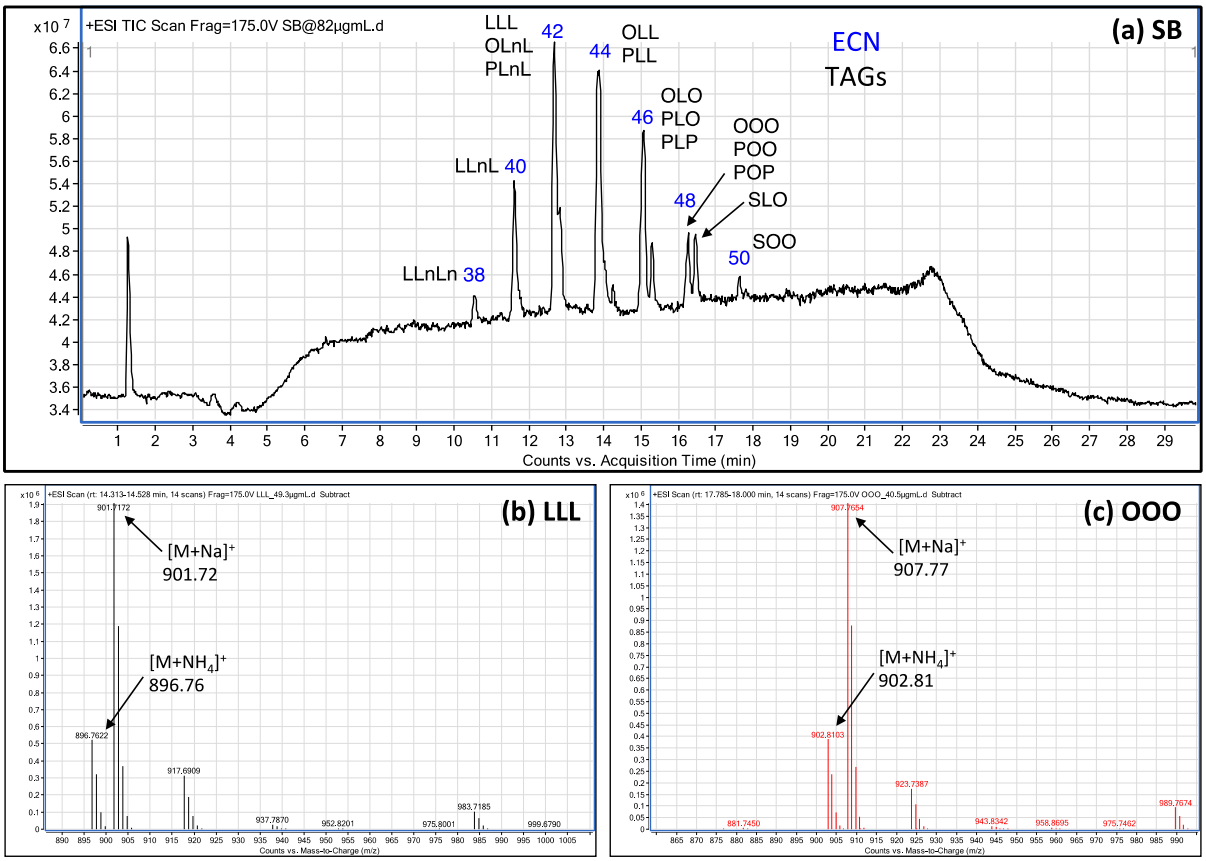

Figure S2: Soybean (SB) analysis by NARP-UPLC coupled with a time-of-flight (TOF) mass spectrometer (MS). (a) Total ion current chromatogram (TIC) for SB prepared at 82 µg/mL in a mixture of IPA and MeOH (1:1, v/v). ESI-MS spectra obtained using standards of (b) LLL and (b) OOO. ECN (in blue) corresponds to the equivalent carbon numbers, ECN=CN-2DB, where CN is the number of carbon atoms in all acyls and DB is the number of double bonds. Abbreviations of fatty acids on TAGs: Ln, Linolenic acid (ECN=12), L, linoleic acid (ECN=14), O, Oleic acid (ECN=16), P, palmitic acid (ECN=16) and S stearic acid (ECN=18). Analysis conditions are in the Experimental Section in the text. LLnLn: Dilinolenoyl-linoleoyl-glycerol, LLnL: Dilinoleoyl-linolenoyl-glycerol, LLL: Trilinolein, OLnL: Dilinolenoyl-oleoyl-glycerol, PLnL: Palmitoyl-linolenoyl-linoleoyl glycerol, OLL: Dilinoleoyl-oleoyl-glycerol, PLL: Dilinoleoyl-palmitoyl glycerol, OLO: oleoyl-linoleoyl-glycerol, PLO: Palmitoyl-linoleoyl-oleoyl-glycerol, PLP: Dipalmitoyl-linoleoyl-glycerol, OOO: Triolein, POO: Dioleoyl-palmitoyl-glycerol, POP: Dipalmitoyl-oleoyl-glyercol, SLO: Stearyl-linoleoyl-oleoyl-glycerol, SOO: Dioleoyl-stearyl-glycerol. Positional isomers (e.g. 1,2-OO-3-L vs. 1,3-OO-2-L or 1-O-2-L vs. 1-L-2-O) are not distinguished.

Table S3: Peak identification of SB from NARP-UHPLC-TOF/MS.

Retention time (min)	Molecular formula	Molecular species	Exact mass (neutral)	Abbreviations	ECN	$[M+Na]^+$ experimental	$[M+Na]^+$ theoretical	$[M+NH_4]^+$ experimental	$[M+NH_4]^+$ theoretical
10.5 - 10.6	$C_{57}H_{94}O_6$	C18:2/C18:3/C18:3	874.71	LLnLn	38	897.71	897.69	892.75	892.74
11.5 - 11.8	$C_{57}H_{96}O_6$	C18:2/C18:2/C18:3	876.72	LLnL	40	899.72	899.71	894.77	894.75
12.6 - 12.9	$C_{57}H_{98}O_6$	C18:2/C18:2/C18:2	878.74	LLL	42	901.74	901.73	896.77	896.77
	$C_{57}H_{98}O_6$	C18:1/C18:2/C18:3	878.74	OLnL	42	901.74	901.73	896.77	896.77
	$C_{55}H_{96}O_6$	C16:0/C18:2/C18:3	852.72	PLnL	42	875.72	875.71	870.77	870.75
13.8 - 14.3	$C_{57}H_{100}O_6$	C18:1/C18:2/C18:2	880.73	OLL	44	903.75	903.74	898.79	898.79
	$C_{55}H_{98}O_6$	C16:0/C18:2/C18:2	854.74	PLL	44	877.74	877.73	872.78	872.77
14.9 - 15.3	$C_{57}H_{102}O_6$	C18:1/C18:1/C18:2	882.77	OLO	46	905.77	905.77	900.81	900.80
	$C_{55}H_{100}O_6$	C16:0/C18:1/C18:2	856.75	PLO	46	879.75	879.74	874.80	874.79
	$C_{53}H_{98}O_6$	C16:0/C16:0/C18:2	830.74	PLP	46	853.74	853.73	848.78	848.77
16.2 - 16.3	$C_{55}H_{102}O_6$	C18:1/C18:1/C18:1	884.78	OOO	48	907.79	907.77	902.83	902.82
	$C_{55}H_{102}O_6$	C16:0/C18:1/C18:1	858.77	POO	48	881.77	881.77	876.81	876.80
	$C_{53}H_{100}O_6$	C16:0/C16:0/C18:1	832.73	POP	48	855.75	855.74	850.80	850.79
16.4 - 16.5	$C_{57}H_{104}O_6$	C18:0/C18:1/C18:2	884.78	SLO	48	907.79	907.77	902.83	902.82
17.6 - 17.7	$C_{57}H_{106}O_6$	C18:0/C18:1/C18:1	886.79	SOO	50	909.8	909.79	904.84	904.83

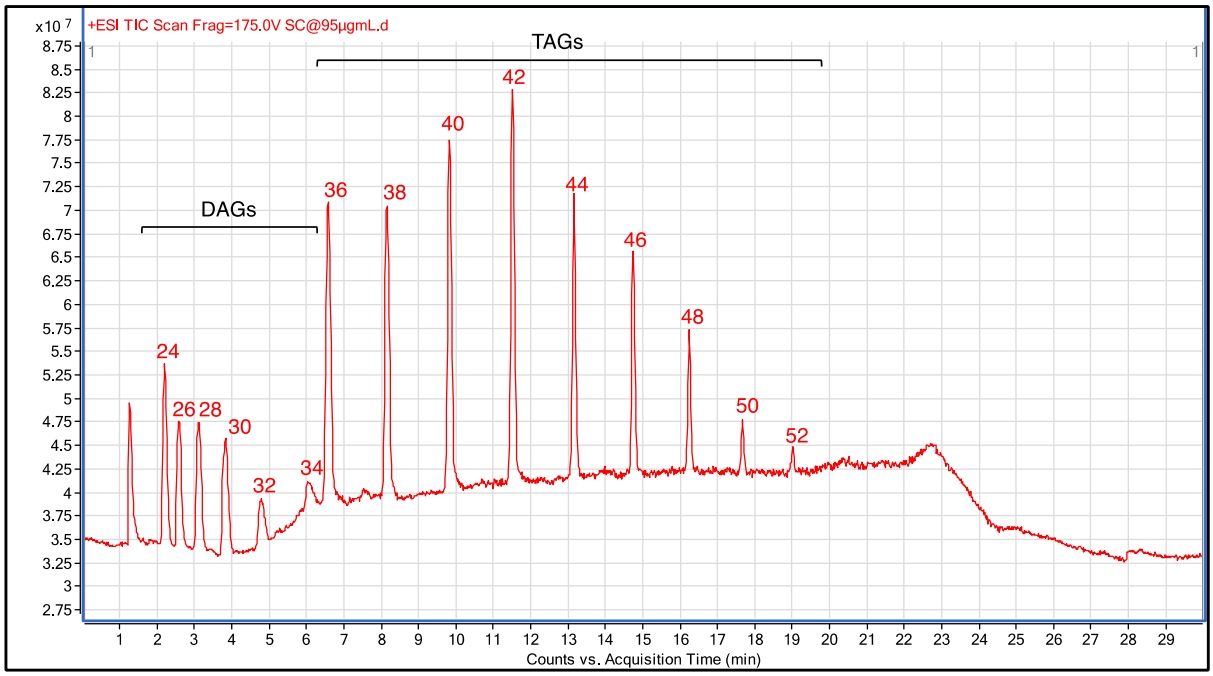

Figure S3: Suppocire (SC) analysis by NARP-UPLC coupled with a time-of-flight (TOF) mass spectrometer (MS). Total ion current chromatogram (TIC) for SC prepared at 95 µg/mL in a mixture of IPA and MeOH (1:1, v/v). Analysis conditions are presented in the Experimental Section in the text.

Table S4: Peak identification of SC from NARP-UHPLC-TOF/MS.

Retention time (min)	Molecular formula	DAGs/TAGs	Exact mass (neutral)	ECN	$[M+Na]^+$ experimental	$[M+Na]^+$ theoretical	$[M+NH_4]^+$ experimental	$[M+NH_4]^+$ theoretical
2.2	$C_{27}H_{52}O_5$	DAGs	456.38	24	479.38	479.37	474.42	474.42
2.6	$C_{29}H_{56}O_5$		484.41	26	507.41	507.40	502.45	502.45
3.1	$C_{31}H_{60}O_5$		512.44	28	535.44	535.43	530.48	530.48
3.7	$C_{33}H_{64}O_5$		540.48	30	563.47	563.46	558.51	558.51
4.7	$C_{35}H_{68}O_5$		568.51	32	591.51	591.50	586.55	586.54
6.1	$C_{37}H_{72}O_5$		596.54	34	619.54	619.53	614.58	614.57
6.6	$C_{39}H_{74}O_6$	TAGs	638.55	36	661.55	661.54	656.59	656.58
8.2	$C_{41}H_{78}O_6$		666.58	38	689.58	689.57	684.62	684.61
9.8	$C_{43}H_{82}O_6$		694.61	40	717.61	717.60	712.66	712.65
11.5	$C_{45}H_{86}O_6$		722.64	42	745.64	745.63	740.69	740.68
13.2	$C_{47}H_{90}O_6$		750.67	44	773.67	773.66	768.72	768.71
14.7	$C_{49}H_{94}O_6$		778.71	46	801.71	801.69	796.75	796.74
16.3	$C_{51}H_{98}O_6$		806.74	48	829.74	829.73	824.78	824.77
17.6	$C_{53}H_{102}O_6$		834.77	50	857.77	857.76	852.81	852.80
18.9	$C_{55}H_{106}O_6$		862.80	52	885.80	885.79	880.84	880.83

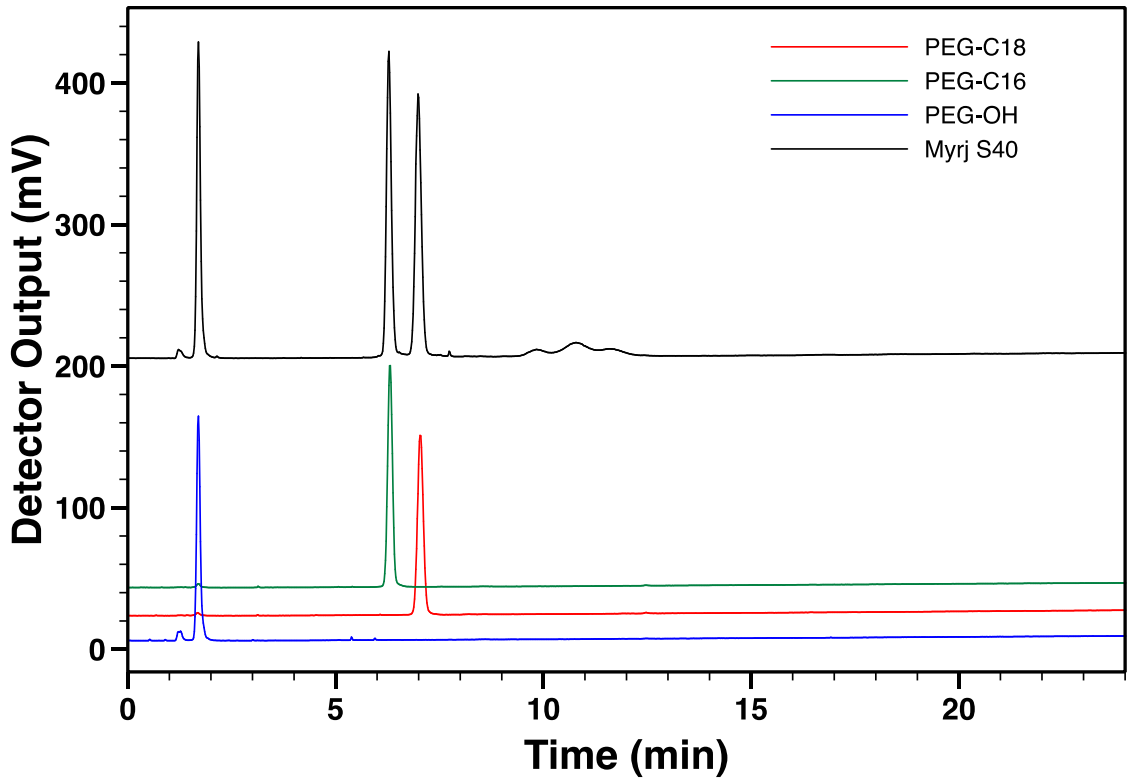

Figure S4: Typical UPLC-ELSD chromatograms using QMC18 method of the PEG standards isolated by preparative HPLC separation. PEG standards (PEG-OH, PEG-C16 and PEG-C18) and Myrj™ S40 were respectively at 150 µg/mL and 569 µg/mL in a mixture of $CHCl_3$ and MeOH (2:1, v/v).

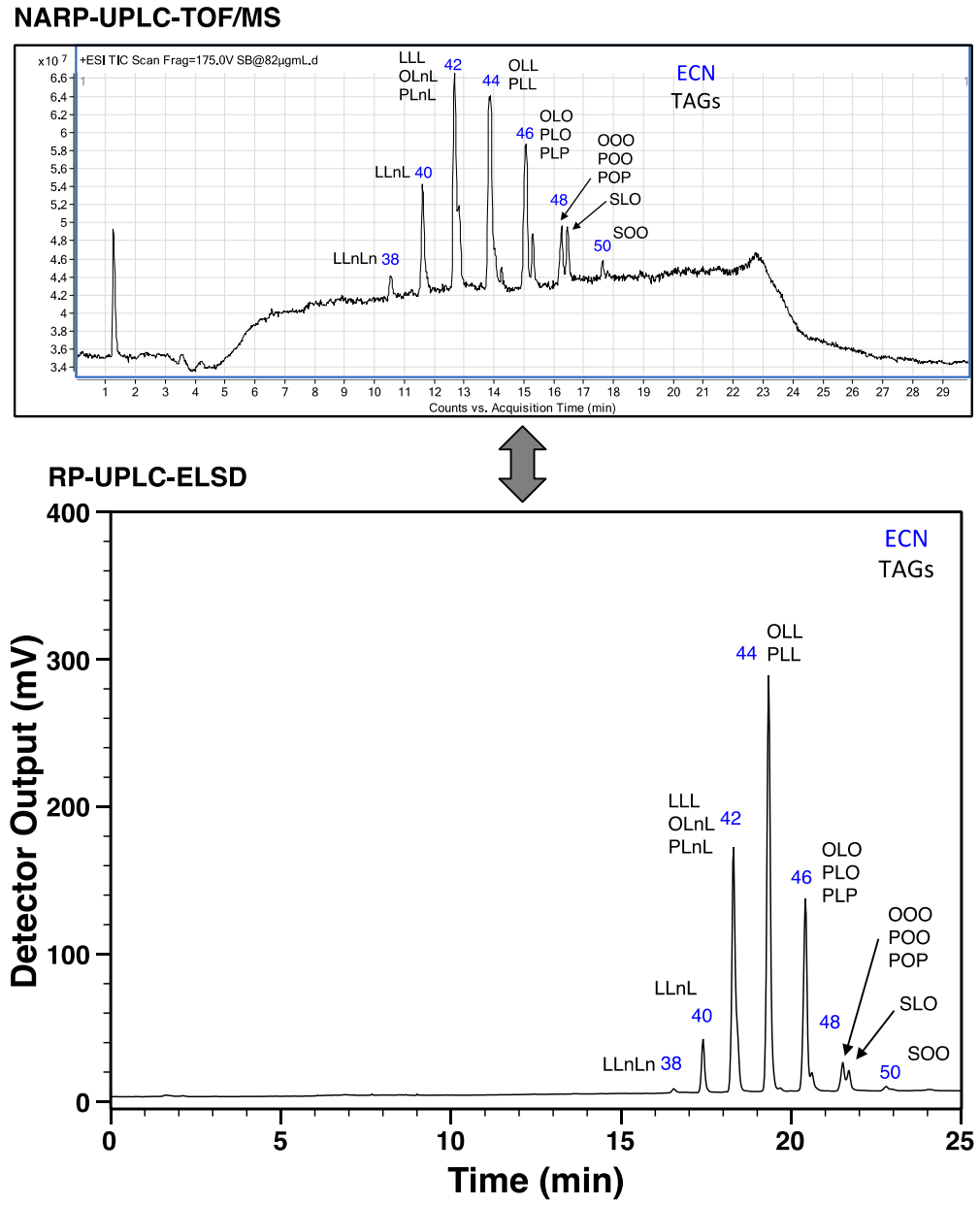

Figure S5: Correlation between the patterns of SB obtained using NARP-UPLC-TOF/MS and RP-UPLC-ELSD.

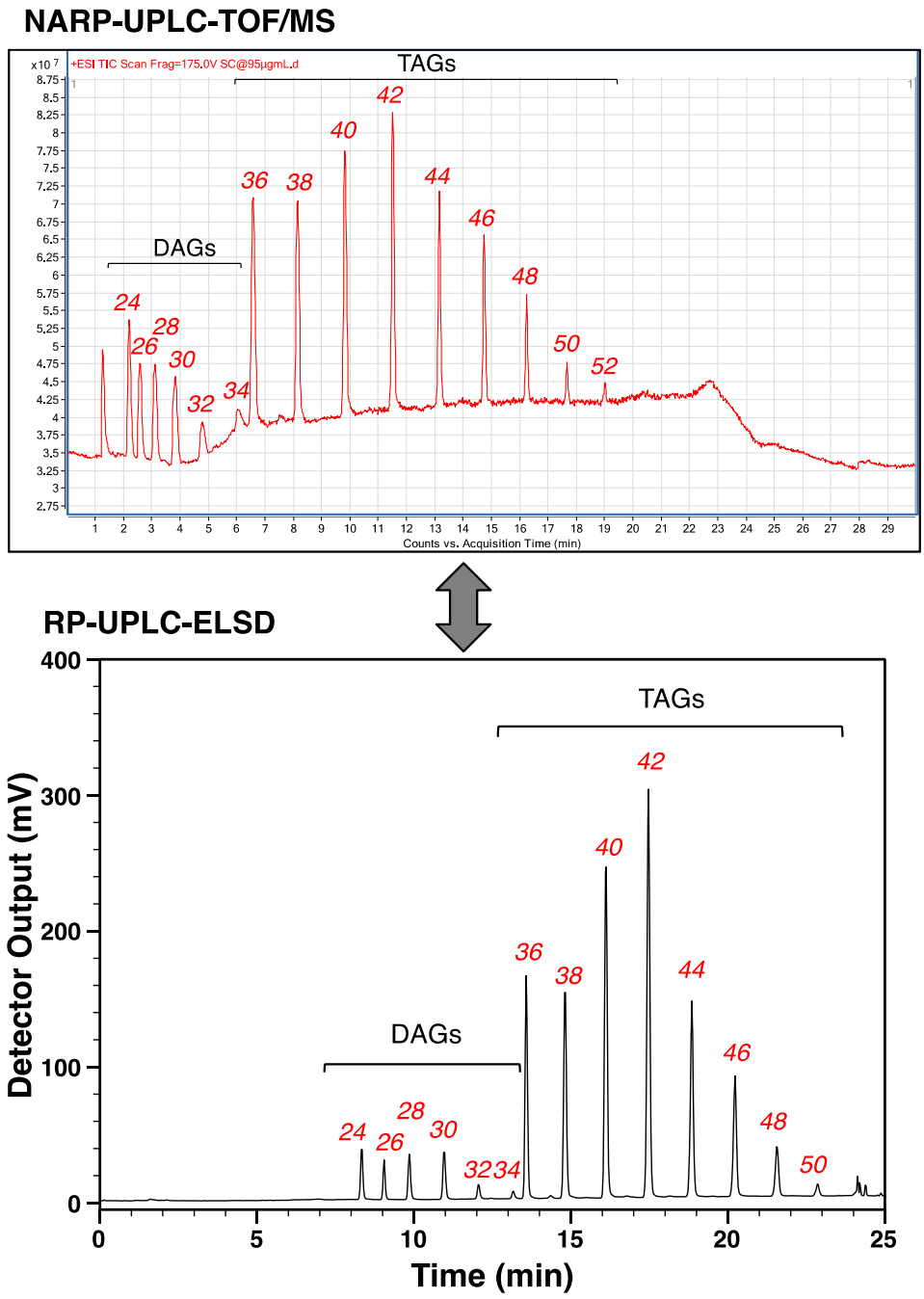

Figure S6: Correlation between the patterns of SC obtained using NARP-UPLC-TOF/MS and RP-UPLC-ELSD.

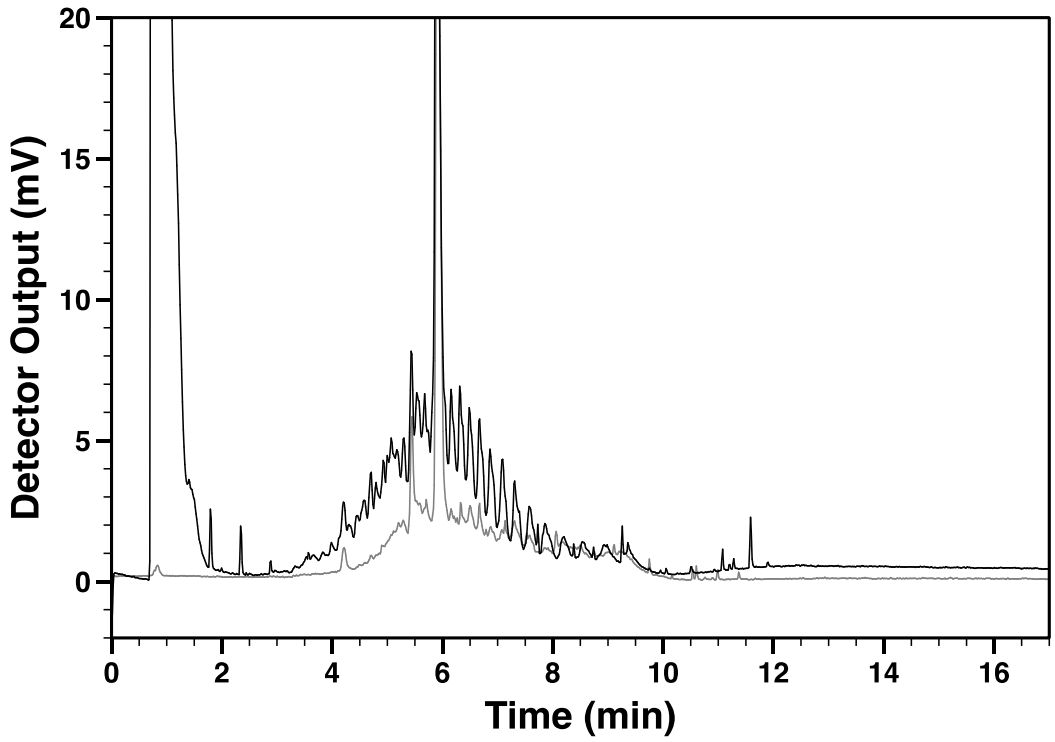

Figure S7: HILIC-UPLC-ELSD chromatograms obtained using QMHILIC method with (gray line) or without SPE (black line). The concentration of Soy PC and Soy PE standards correspond to 60.6 and 8.7 µg/mL (40% of the target concentration), respectively. SB, SC and S40 as crude excipients were spiked into the samples at the theoretical concentration calculated for the 50-nm lipid suspension. The sample without SPE step was diluted by 10-fold using a mixture of $CHCl_3$, MeOH and H_2O (3:5:2, v/v/v) prior to analysis.

Table S5: Removal efficacy (Eff %) of the SPE method.

%/[target]	**40%**			**100%**			**120%**		
Components	Conc. before SPE (µg/mL)	Conc. after SPE (µg/mL)	Eff. (%)	Conc. before SPE (µg/mL)	Conc. after SPE (µg/mL)	Eff. (%)	Conc. before SPE (µg/mL)	Conc. after SPE (µg/mL)	Eff. (%)
SB	79	0	**100**	82	0	**100**	80	0	**100**
SC	299	0	**100**	313	0	**100**	306	0	**100**
PEG-OH	Not quantifiable			Not quantifiable			Not quantifiable		
PEG-C16	110	29	**74**	113	32	**71**	111	28	**74**
PEG-C18	113	29	**75**	116	32	**73**	114	28	**76**

Table S6: Recovery obtained for phospholipids using QMHLIC evaluated by comparing two calibrations curves performed, with or without SPE, *i.e.* with or without adding the other excipients. Concentrations were calculated using the calibration curve performed without applying the SPE process.

% PC or %PE/[Target]	Recovery Soy PC (%)	Recovery Soy PE (%)
120	99.1	101.6
100	104.7	103.2
80	105.7	111.3
60	105.5	112.4
40	107.3	118.4

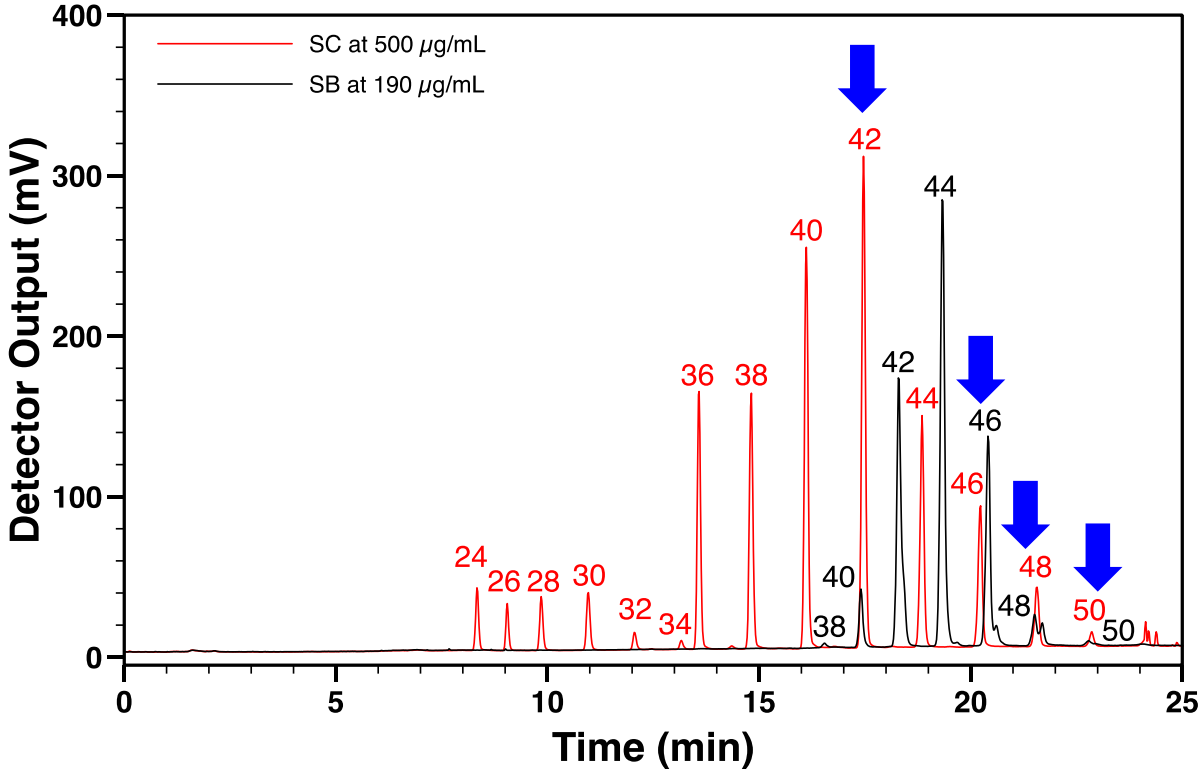

Figure S8: RP-UPLC-ELSD chromatograms of SB at 190 µg/mL and SC at 500 µg/mL. Samples were prepared in a mixture of $CHCl_3$ and MeOH (2:1, v/v). Each peak is labelled with the Equivalent Carbon Number (ECN) defined as total carbon number – 2 x (number of double bonds in fatty acids). Blue arrows: peak overlap.

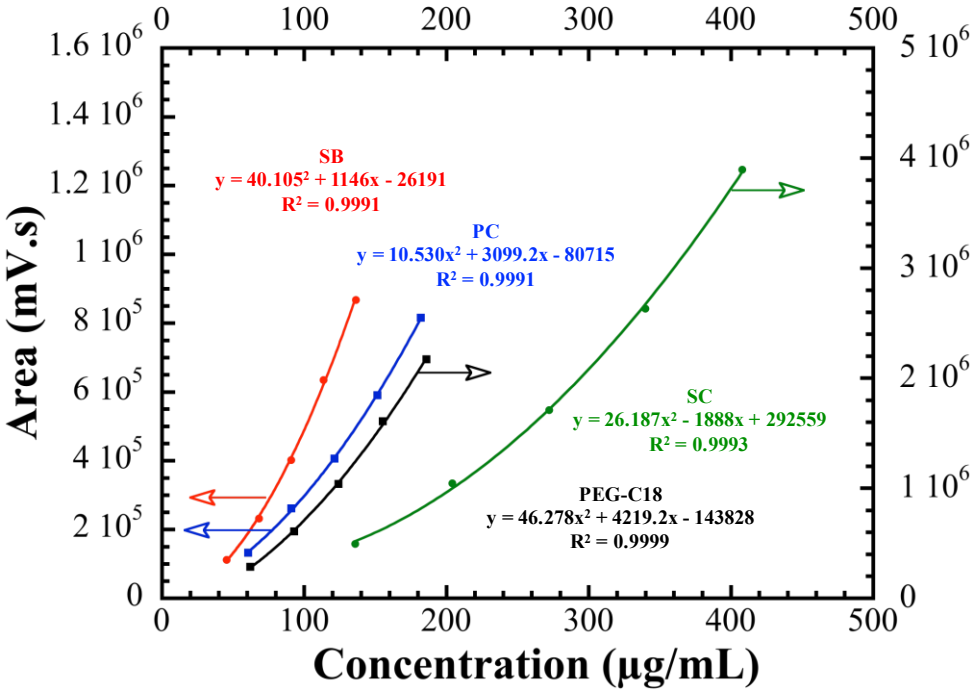

Figure S9: Calibration curves for SB, SC, PC and PEG-C18. Scale for SB and PC calibration curves is on the left, scale for SC and PEG-C18 is on the right.

Table S7: Linear regression analyses of log transformed analyte concentration and log transformed ELSD response.

Coumpound	Target conc. (µg/mL)	Calibration range (µg/mL)	Equation	R^2
SB	133.3	45.4 - 136.1	y =1.878x + 1.933	0.9995
SC	340.0	135.8 - 407.9	y = 1.853x + 1.736	0.9988
PEG-OH	100.0	62.0 - 186.1	y = 1.730x + 2.385	0.9999
PEG-C16	115.0	62.0 - 186.1	y = 1.833x + 2.194	0.9995
PEG-C18	115.0	62.0 - 186.1	y = 1.861x + 2.120	0.9995
Soy PE	21.7	8.7 - 26.0	y = 1.471x + 2.165	0.9979
Soy PC	151.6	60.6 - 182.0	y = 1.639x + 2.202	0.9996

Table S8: Evolution of the lipid excipients quantities along the formulation process.

Lipid/Process	Before US		After US		After filtration 5 µm		After dialysis		After filtration 0.22 µm	
	(%)	%RSD	(%)	%RSD	(%)	%RSD	(%)	%RSD	(%)	%RSD
SB (%)	100.0%	0.8%	99.8%	0.6%	76.1%	1.2%	69.8%	1.8%	71.9%	0.7%
SC (%)	100.0%	0.9%	99.8%	0.4%	78.0%	0.8%	70.1%	0.8%	73.2%	1.6%
PEG-OH (%)	100.0%	2.6%	94.9%	1.4%	73.6%	1.5%	3.6%	0.9%	4.9%	3.3%
PEG-C16 (%)	100.0%	0.7%	95.2%	0.4%	76.1%	2.2%	71.3%	1.2%	74.9%	2.4%
PEG-C18 (%)	100.0%	1.3%	98.7%	1.3%	76.4%	1.6%	69.6%	1.9%	74.7%	1.1%
PE (%)	100.0%	0.7%	85.9%	3.8%	64.1%	4.9%	52.1%	6.2%	52.6%	3.1%
PC (%)	100.0%	2.3%	98.6%	1.1%	73.1%	0.4%	65.9%	1.2%	69.8%	1.0%

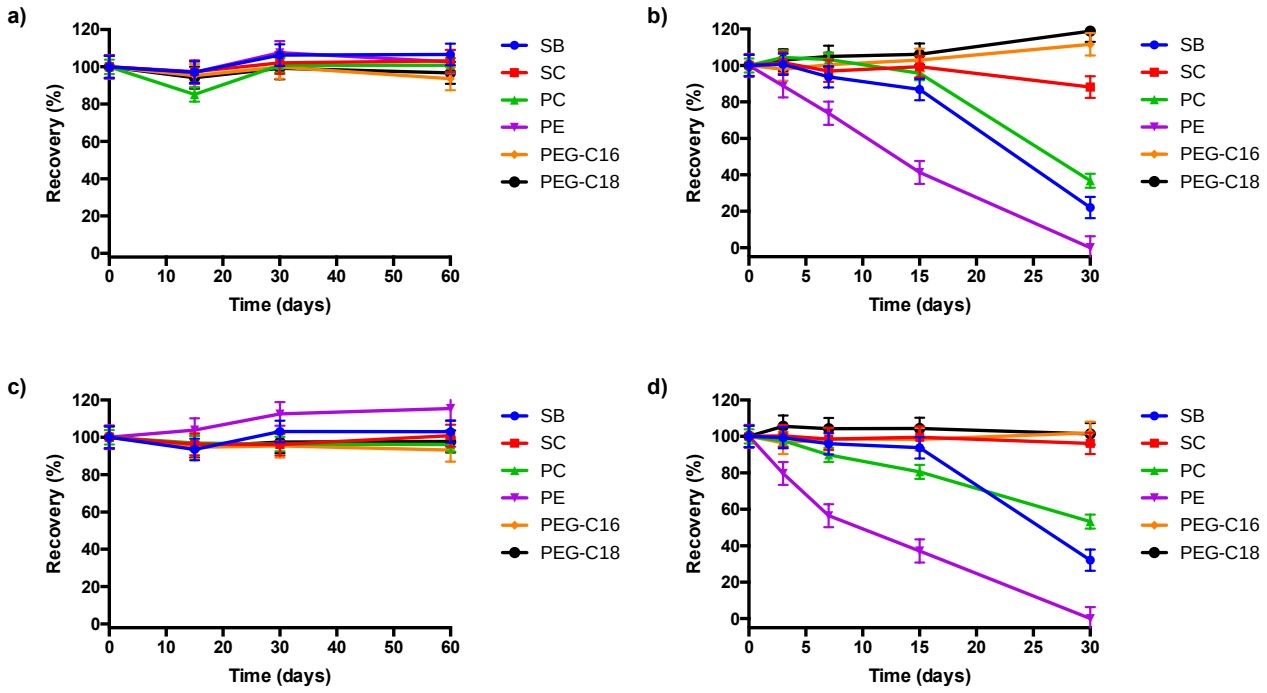

Figure S10: Stability evaluation (lipid composition) of 80 nm (F80) and 120 nm (F120) nanoparticle suspensions stored at 4°C or 60°C. a) F80 4°C, b) F80 60°C, c) F120 4°C and d) F120 60°C.

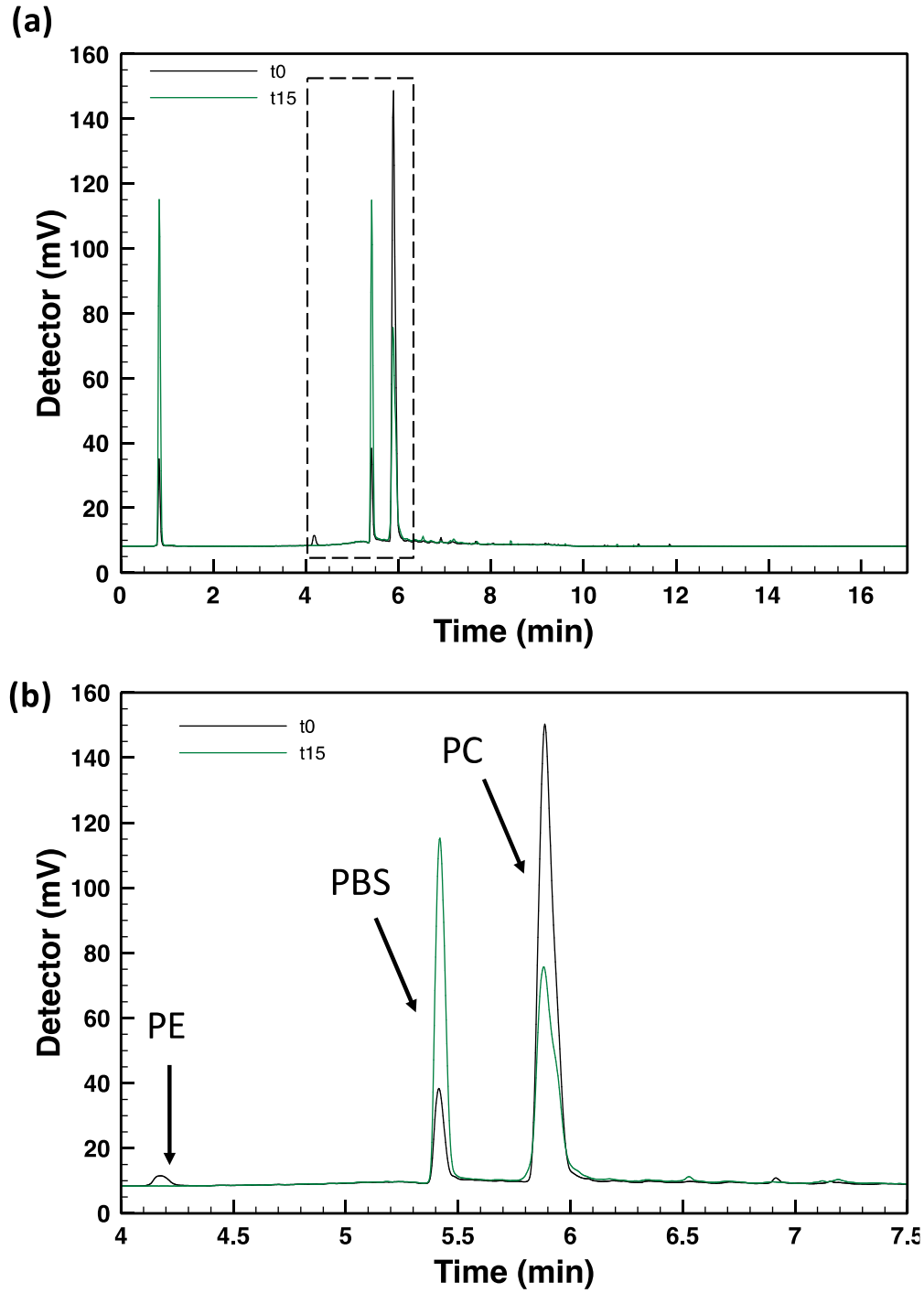

Figure S11: HILIC-UPLC-ESLD chromatograms of lipid excipients extracted from a 50 nm formulation (F50) stored at 60°C for t = 0 and t = 15 days and analyzed using QMHILIC method. (b) Zoom of (a) within the area of interest (dashed rectangle).

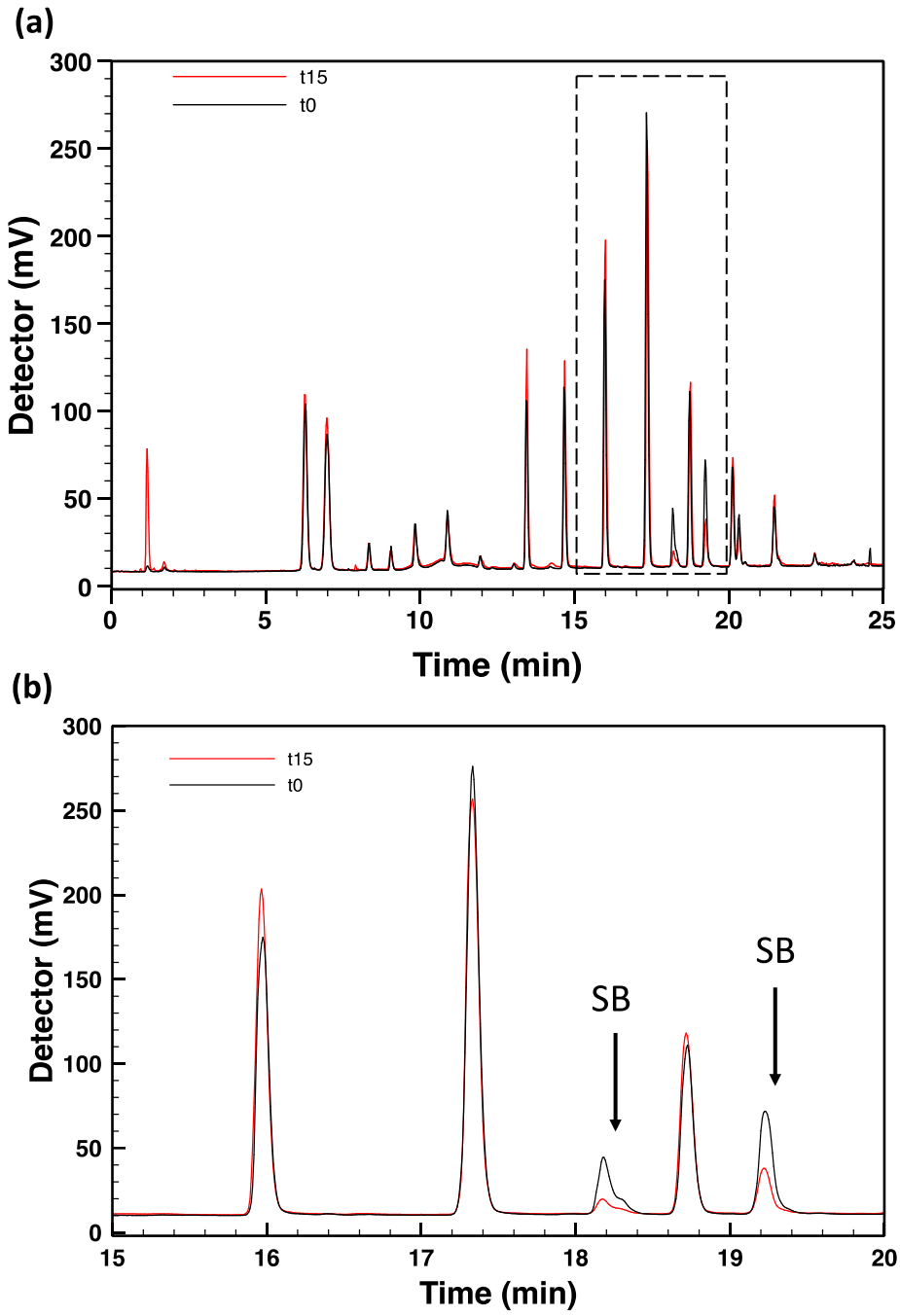

Figure S12: RP-UPLC-ELSD chromatograms of lipid excipients from a 50 nm formulation (F50) stored at 60°C for t = 0 and t = 15 days and analyzed using QMC18 method. (b) Zoom of (a) within the area of interest (dashed rectangle).