Supporting information for

Quantification of dolichyl phosphates using phosphate methylation and reverse-phase liquid chromatography-high resolution mass spectrometry

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Additional experimental information and results on validating the novel approach for MS-based DolP analysis.

Experimental Section

Reagents and chemicals

Potassium hydroxide (KOH), dichloromethane, and TMSD (#362832, 2 M in hexane) were obtained from Sigma-Aldrich. LC-MS grade water, isopropanol, methanol (MeOH), acetonitrile, ammonium acetate, and formic acid were from Fisher Scientific.

Cell culture

For *S. cerevisiae* (strain W303-1B, derived from strain W303 [42]) sample preparations, approximately 5x10^7 cells were cultured in YPD medium and harvested from cultures grown to mid-log phase with an OD600 value of 0.8. Fibroblasts from human patients were obtained with the patients' parents written informed consent. This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty Heidelberg (S-904/2019). The cells were maintained at 37 °C in a humidified atmosphere under 5% CO_2. Patient and control fibroblasts were cultured in Dulbecco's modified Eagle's medium (high glucose; Life Technologies) supplemented with 10% FCS (PAN Biotech, Aidenbach, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin. HeLa or fibroblast cell monolayers with 80 to 90% confluence in a T75 flask were washed twice with PBS and harvested using 2 mL 0.25% (v/v) trypsin-EDTA solution. All cell pellets were shock-frozen in liquid nitrogen and stored at -80 °C until lipid extractions were performed.

Safety considerations for handing TMSD

TMSD must be handled with care, as its inhalation may cause lung damage or central nervous system depression. It was used with the appropriate safety procedures and personal safety protective equipment inside the fume hood. Excess TMSD in the hood atmosphere was neutralized with acetic acid.

Method validation

The methylated DolP standard was reconstituted in methanol, and a dilution series was prepared from 0.05 to 800 pg/µL. For each DolP species, the linearity was assessed by plotting the analyte-to-internal standard peak area against the nominal concentration of individual species calculated based on their mol% distribution in the DolP standard (Table S5, Fig. S6). Calibration curves (n=3) consisting of four to seven calibrator levels were constructed by simple linear regression analysis. For each DolP species, the lower limit of detection (LOD) was chosen as the calibration concentration when a signal-to-noise ratio is at least 5:1.

Validation of the method was assessed at three-level quality control (QC), at low (50), medium (200), and high (500) pg/µL of methylated DolP standard mixture. The intra- and inter-day assays were determined by analyzing the prepared samples on the same day and on three consecutive days. For precision and accuracy evaluations, samples were prepared by spiking the QC levels to the methylated Hela cells extracts (n=3). Accuracy is assessed as an agreement between the back-calculated concentration of individual analyte species in the spiked samples and their nominal concentrations, whereas precision is expressed as the coefficient of variation (%CV) for both intra-day and inter-day analysis values are shown in Table S6. For DolP species extraction recovery (Table S7), samples were prepared as pre-spikes and post-spikes at high and low QC levels in a pure solvent. The samples were extracted as described in the main text experimental section. The post-preparative stability (Table S7) was determined at low and high QC levels. These QC levels were spiked to methylated HeLa cell extracts, kept at 4 ºC for 20 h in an autosampler. The results were compared to conditions using freshly prepared samples with the same nominal concentration.

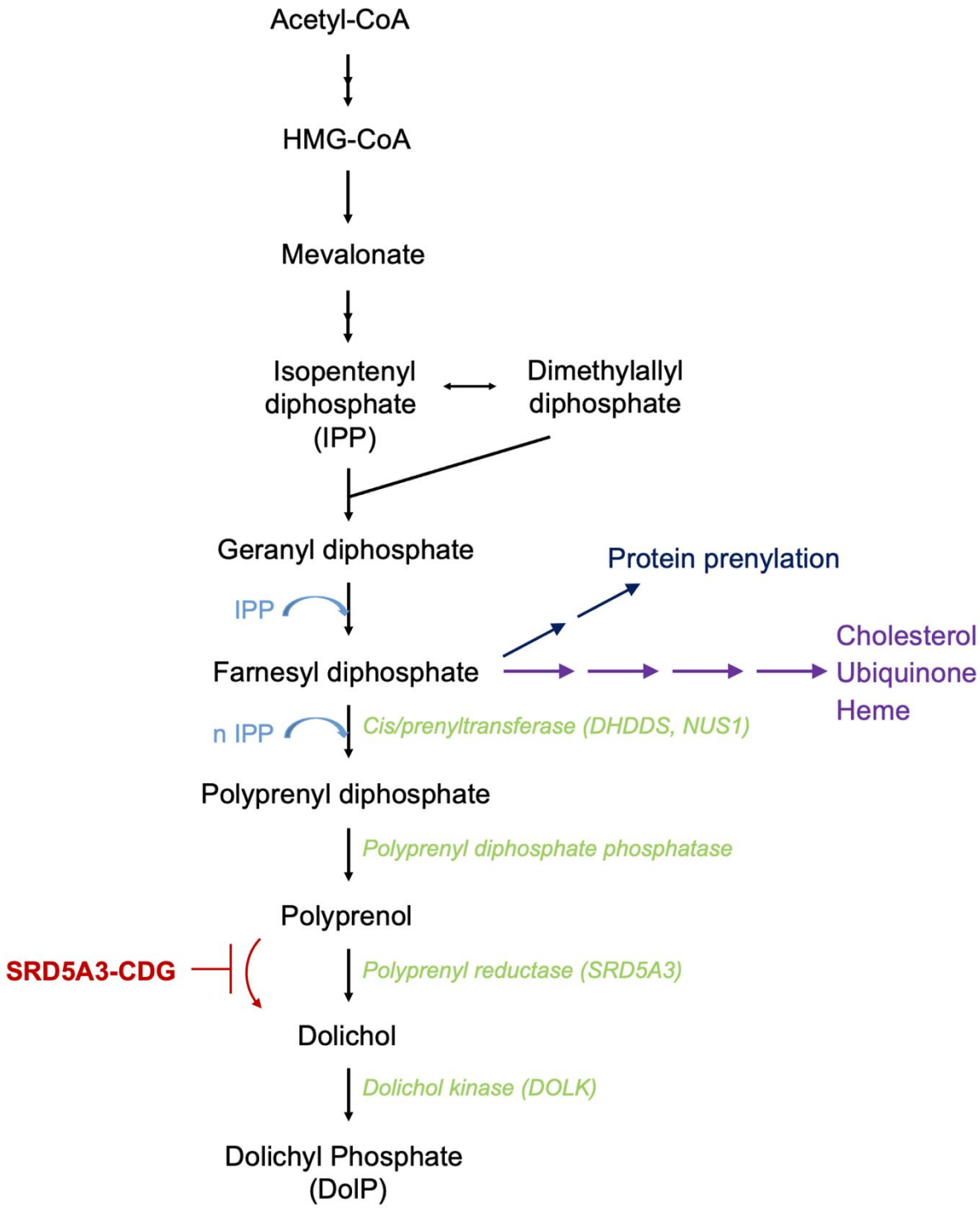

Figure S1. DolP synthesis in mammals.

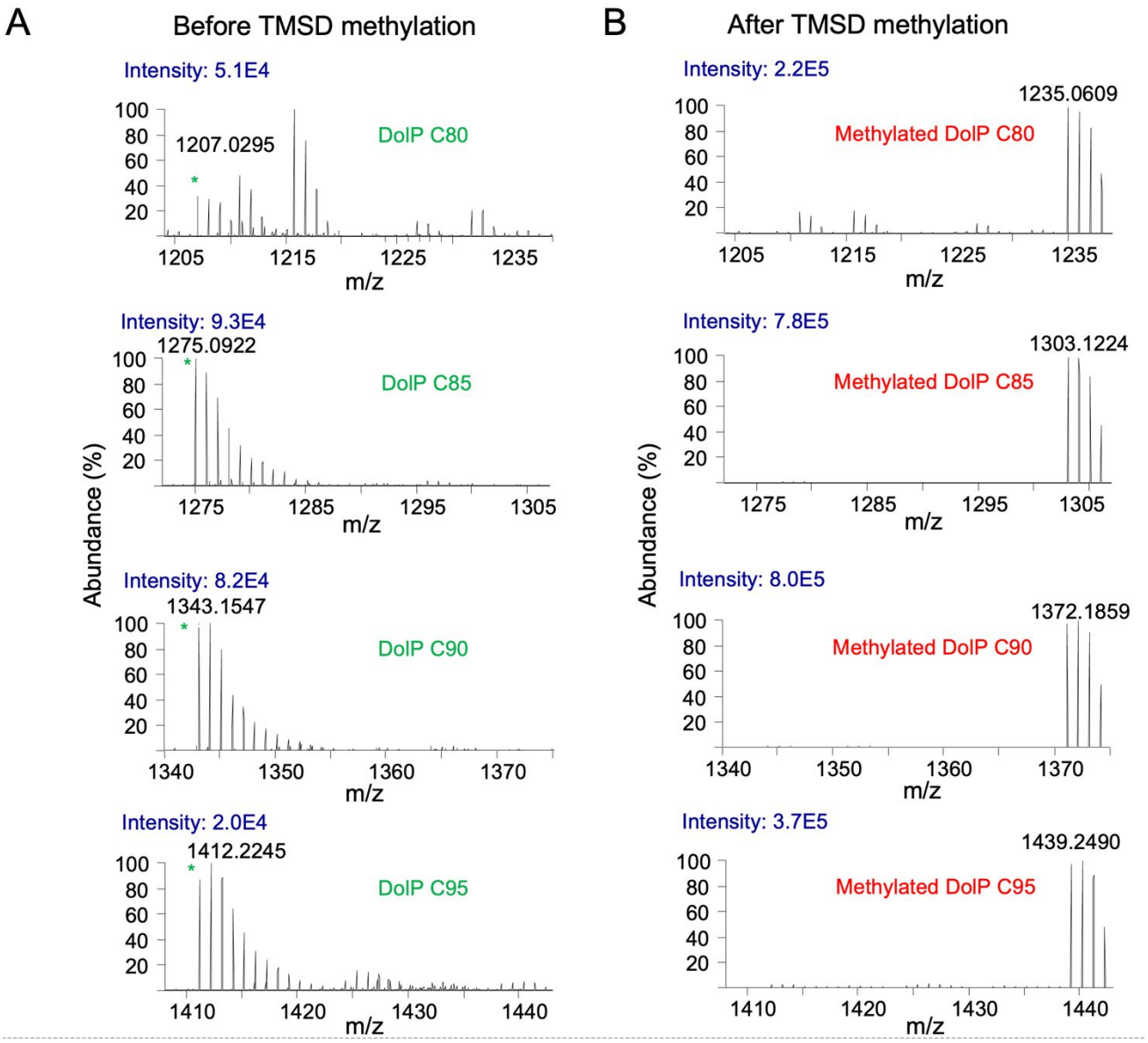

Figure S2. nESI-DIMS analysis of (A) un-methylated and (B) methylated DolP standard. For theoretical m/z values of ammonium adducts of these un-, mono-, di-methylated DolP ions please see Table S2.

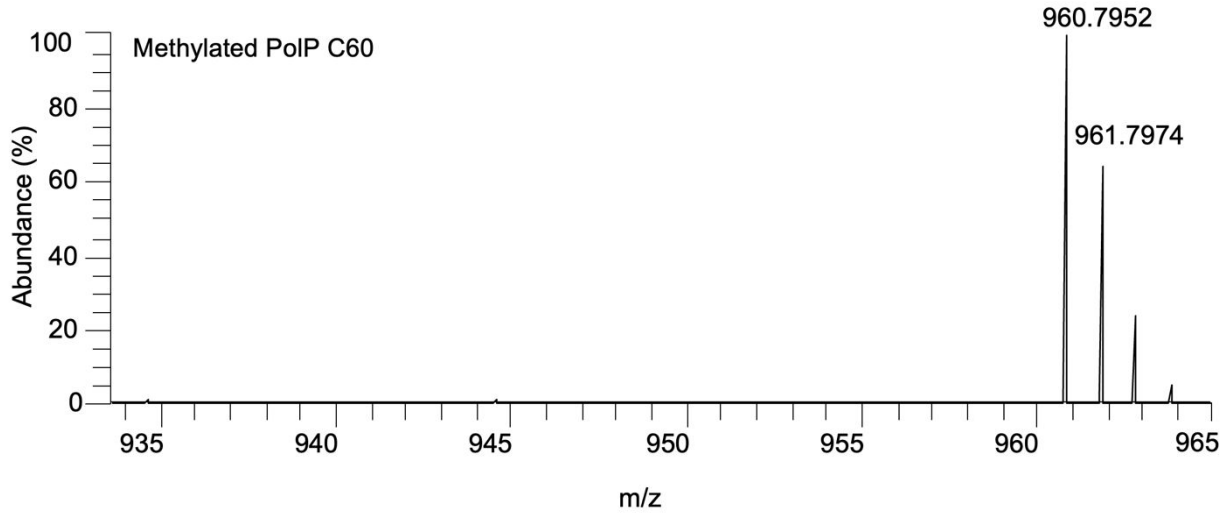

Figure S3. nESI-DIMS analysis of the ammonium adduct of methylated PolP C60 standard.

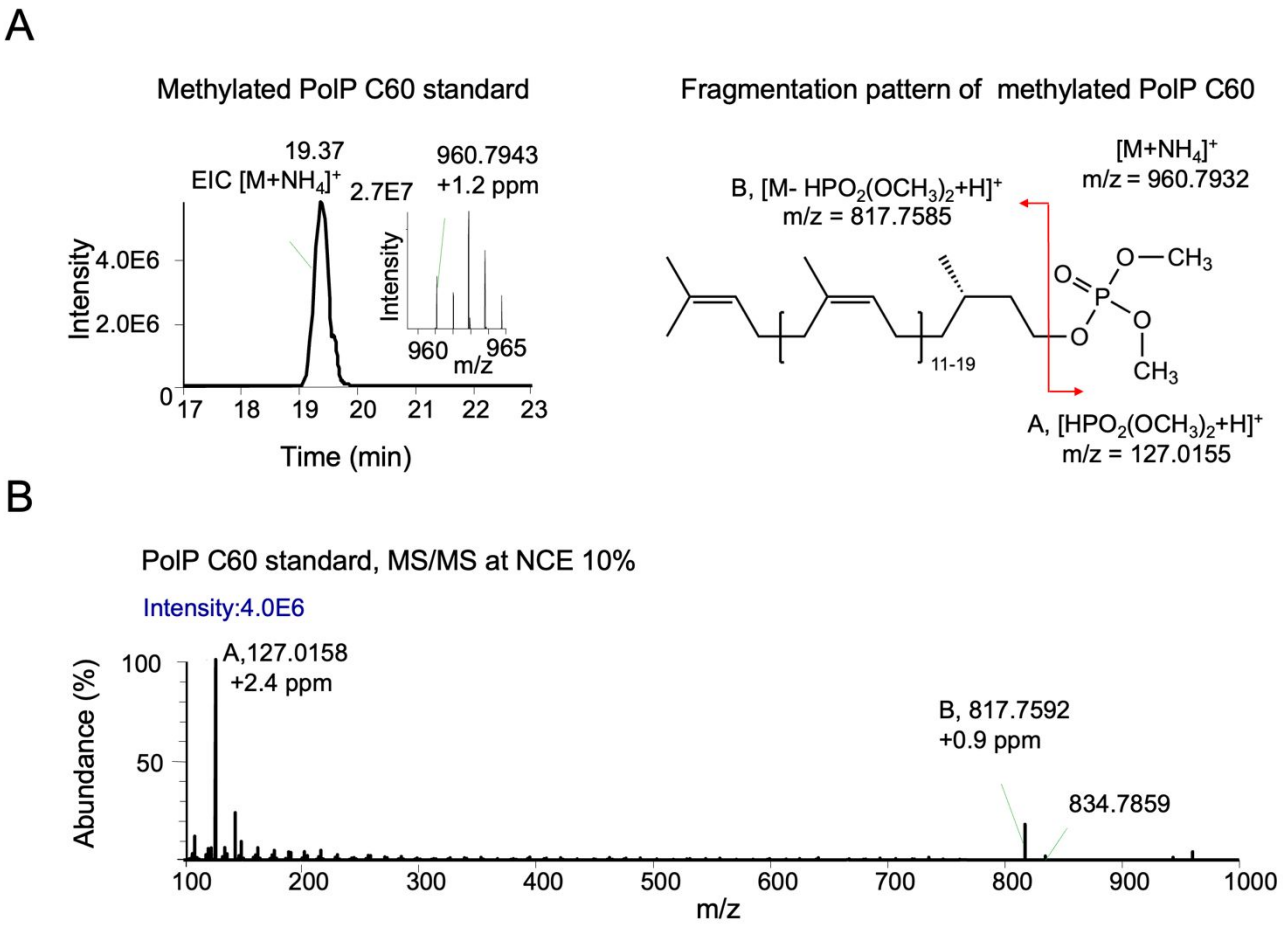

Figure S4. RPLC-MS characteristics of di-methylated PolP C60 standard. **A**, EIC (±5 ppm) of the $[M+NH_4]^+$ ion, with corresponding MS spectrum and theoretical fragmentation pattern. **B**, MS/MS spectrum resulting from low collision-induced dissociation (NCE 10%), showing the di-methylated phosphate head group fragment ion. In B, the letters A and B refer to the illustration of the fragmentation pattern shown in (A).

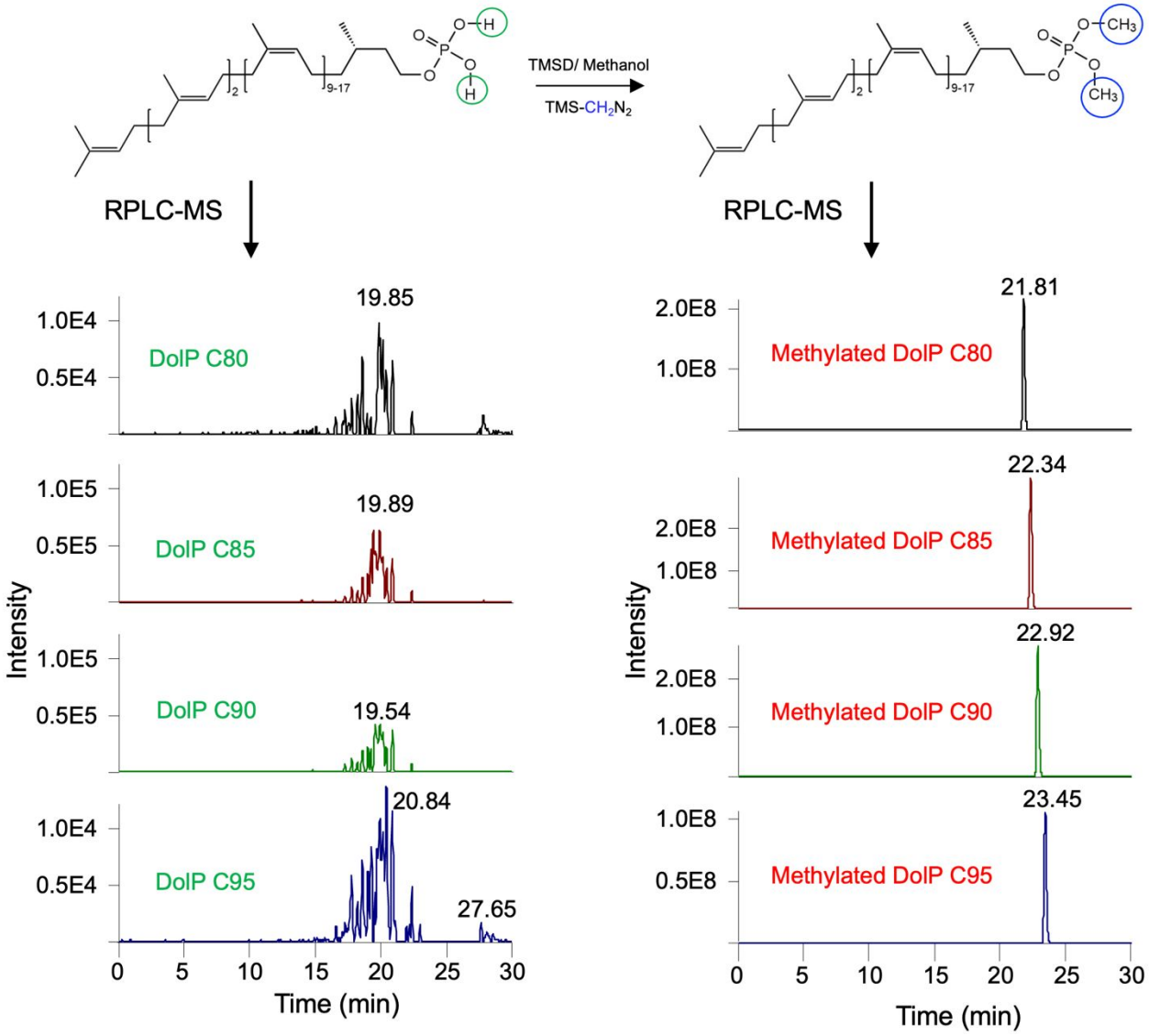

Figure S5. EICs of $[M+NH_4]^+$ ions of the major DolP species in the DolP standard mixture before and after methylation.

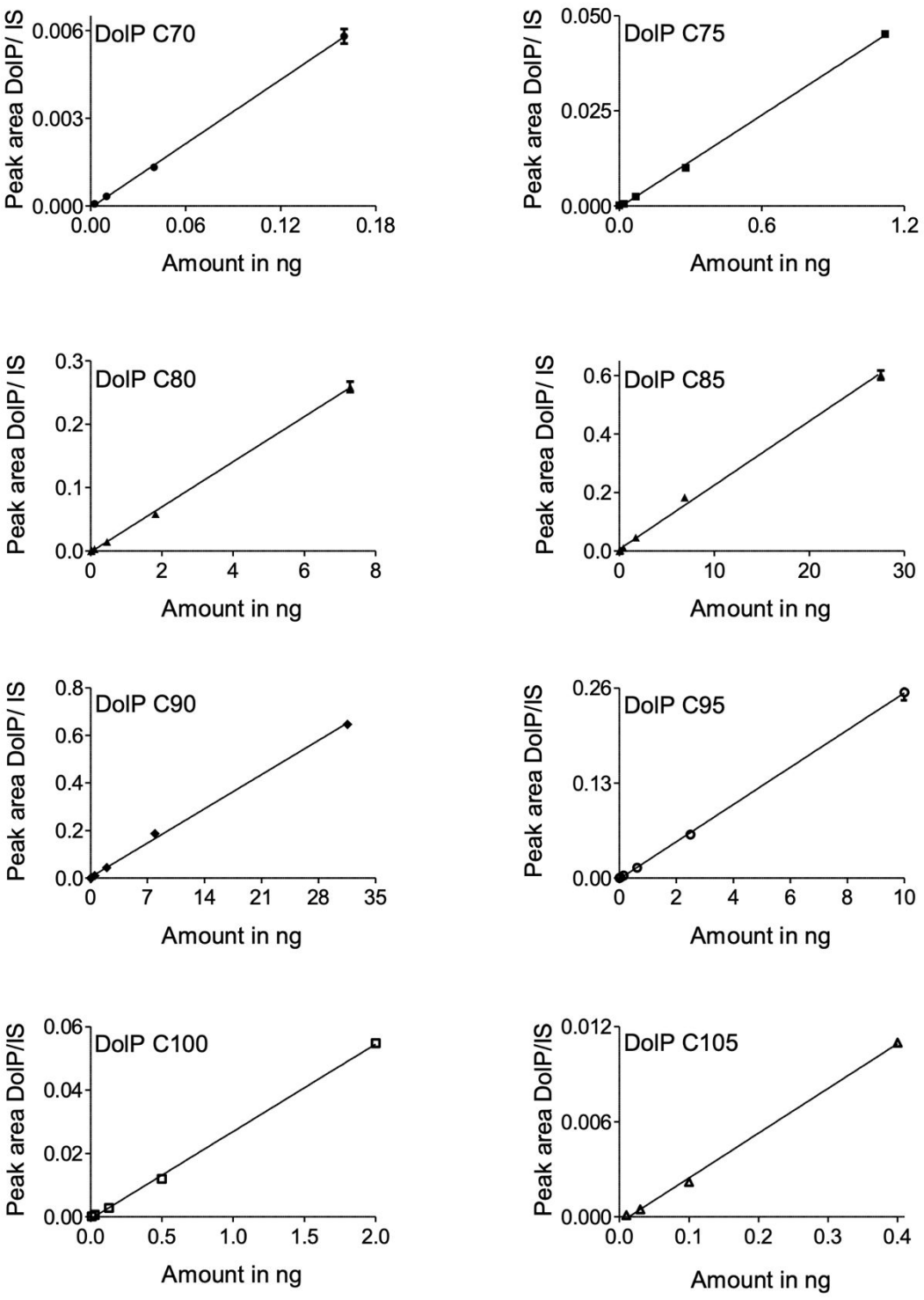

Figure S6. Linearity curves for the RPLC-MS analysis of C70-105 species (on column) in DolP standard mixture.

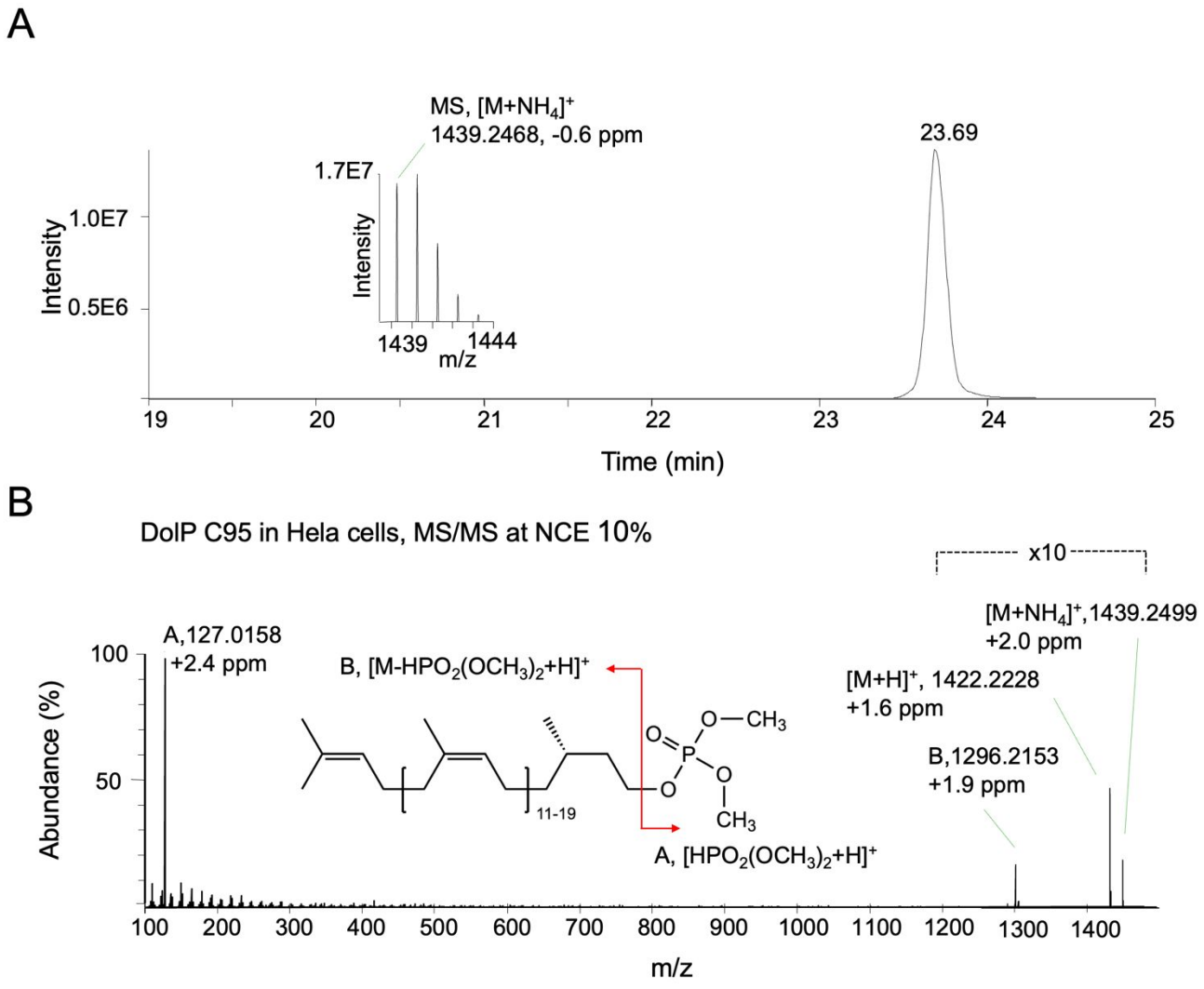

Figure S7. RPLC-MS characteristics of endogenous di-methylated DolP C95 from HeLa cells. **A**, EICs (±5 ppm) of the $[M+NH_4]^+$ ions, with corresponding MS spectrum. **B**, MS/MS spectrum resulting from low collision-induced dissociation (NCE 10%), with fragmentation pattern showing the di-methylated phosphate head group ion and other product ions.

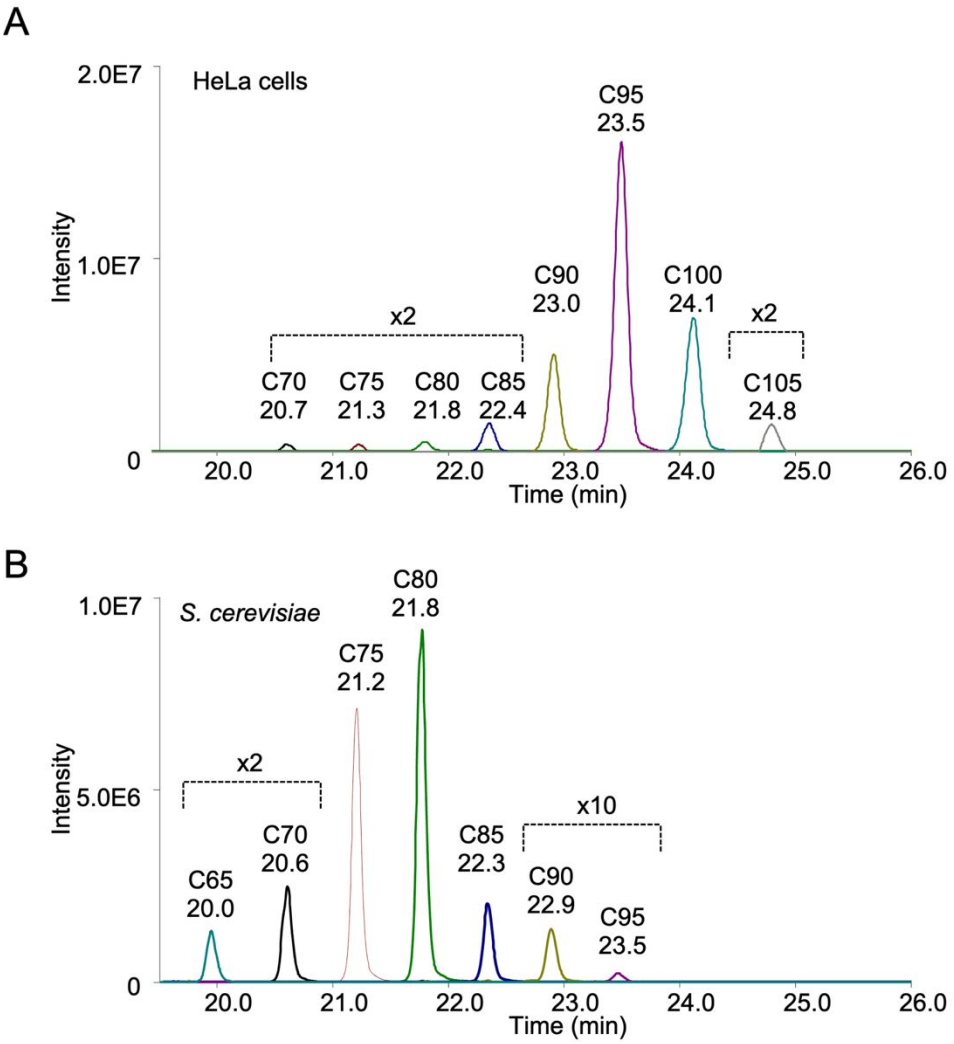

Figure S8. EICs of DolP species from extracts of (A) HeLa cells and (B) *S. cerevisiae* after methylation, analysed by RPLC-MS.

Table S1. DolP and PolP molecular compositions and positive ion mode RPLC-MS characteristics of annotated di-methylated analyte species (as $[M+NH_4]^+$ ions) in this study.

Analyte species	Isoprene units (n)	Molecular formula	Theoretical m/z	Observed m/z	Mass error (ppm)	RT (min)	RRT[a]
PolP C60	12	$C_{62}H_{103}O_4P$	960.7932	960.7924	-0.8	19.1	1.00
DolP C65	13	$C_{67}H_{113}O_4P$	1030.8715	1030.8711	-0.4	20.0	1.05
DolP C70	14	$C_{72}H_{121}O_4P$	1098.9341	1098.9360	1.7	20.6	1.08
DolP C75	15	$C_{77}H_{129}O_4P$	1166.9967	1166.9976	0.8	21.2	1.11
DolP C80	16	$C_{82}H_{137}O_4P$	1235.0593	1235.0613	1.6	21.8	1.14
DolP C85	17	$C_{87}H_{145}O_4P$	1303.1219	1303.1229	0.8	22.3	1.17
DolP C90	18	$C_{92}H_{153}O_4P$	1371.1845	1371.1854	0.7	22.9	1.20
DolP C95	19	$C_{97}H_{161}O_4P$	1439.2471	1439.2476	0.3	23.5	1.23
DolP C100	20	$C_{102}H_{169}O_4P$	1507.3097	1507.3127	2.0	24.1	1.26
DolP C105	21	$C_{107}H_{177}O_4P$	1575.3720	1575.3724	0.3	24.7	1.29

[a] RRT, ratio of the retention of the peak relative to PolP C60

Table S2. Theoretical m/z list of un-methylated (+0 Me), mono-methylated (+1 Me), di-methylated (+2 Me) $[M+NH_4]^+$ ions of C80-C95 species in DolP standard.

DolP species chain length	m/z list of species in DolP standard		
	+0 [a] Me	+1 [a] Me	+2 [a] Me
C80	1207.0280	1221.0436	1235.0593
C85	1275.0906	1289.1062	1303.1219
C90	1343.1532	1357.1688	1371.1845
C95	1411.2158	1425.2314	1439.2471

[a] Number of methylations in DolP

Table S3. Relative methylation efficiency (in %) of C80-C95 species in DolP standard in nESI-DIMS analyses.

DolP species	%[a] of methylated compounds [c]	
chain length	+0[b]Me	+2[b]Me
C80	0 ± 0.34	100 ± 0
C85	0.82 ± 0.46	99.02 ± 0.16
C90	0.33 ± 0.57	99.06 ± 0.2
C95	0 ± 0	100 ± 0

[a]

$$\%\ of\ methylation\ efficiency = \left(1 - \frac{Intensity\ of\ intact\ DolP\ species\ \ after\ methylation}{Intensity\ of\ intact\ DolP\ species\ \ before\ methylation}\right) * 100\%$$

[b] Number of methylations in DolP

[c] Values are mean ± SD, (n = 3)

Table S4. Relative distribution (in %) of C80-C95 species in DolP standard with and without RPLC separation and/or methylation, followed by MS detection.

DolP species chain length	% [a] Distribution of species in DolP standard		
	without methylation	with methylation	
	nESI-DIMS	nESI-DIMS	[b] RPLC-MS
C80	11.1 ± 0.2	11.0 ± 0.3	10.4 ± 0.3
C85	38.4 ± 0.3	36.8 ± 0.5	37.1 ± 1.5
C90	38.3 ± 0.1	39.3 ± 0.3	40.4 ± 1.1
C95	12.2 ± 0.3	12.9 ± 0.5	12.2 ± 0.3

[a] Values represent peak areas of species, normalized to total peak area and are expressed as mean ± SD of n=3 independent measurements.

[b] For detailed distribution of methylated species following RPLC-MS analysis refer to Table S4.

Table S5. Relative composition (in %) of C70-C105 species in methylated DolP standard, calibration curve parameters, instrumental limit of detection (LOD) of the RPLC-MS method for the simultaneous determination of targeted DolP species.

DolP species chain length	% Distribution [a] in DolP standard	Calibration range[a] (pg on column)	LOD[a] (pg on column)	r^2	Calibration Curve equation
C70	0.2 ± 0 [b]	3 - 160	1	0.99	$y = 0.036x - 0.0001$
C75	1.4 ± 0.1 [b]	4 - 1120	1	1.00	$y = 0.041x - 0.0004$
C80	9.1 ± 0.3	7 - 7280	2	1.00	$y = 0.036x - 0.0016$
C85	34.4 ± 1.4	7 - 27520	2	1.00	$y = 0.022x + 0.0060$
C90	39.4 ± 1.0	8 - 31520	2	1.00	$y = 0.021x + 0.0042$
C95	12.5 ± 0.3	10 - 10000	2	1.00	$y = 0.025x - 0.0012$
C100	2.5 ± 0.0 [b]	8 - 2000	2	1.00	$y = 0.028x - 0.0006$
C105	0.5 ± 0.1 [b]	6 - 400	2	1.00	$y = 0.028x - 0.0003$

[a] Amount of different species in the DolP standard, expressed as the ratio of the peak area of each DolP species to the total area of all DolP species, determined in three independent methylation experiments and represent mean ± SD.

[b] Additional DolP species detected in RPLC-MS but not in nESI-DIMS analyses.

Table S6. Results from intra- and inter-day accuracy and precision of RPLC-MS analysis of DolP standard in HeLa cells extracts.

DolP species chain length	QC level (pg/μL)	Accuracy (n=3)		Precision (n=3)		Observed nominal concentration (pmol/10^6 cells) in Hela cells (n=3)	
		Inter-day	Intra-day	Inter-day	Intra-day		
		(Mean ± SD,%)	(Mean ± SD,%)	(CV,%)	(CV,%)	(Mean ± SD, %)	(CV,%)
C70	Low 50	107.2 ± 4.6	104.5 ± 4	3.8	4.3		
	Middle 200	98.5 ± 3.7	96.1 ± 3	3.2	3.8	0.03 ± 0.0	21.4
	High 500	102.7 ± 0.9	102.5 ± 1.1	1.1	0.9		
C75	Low 50	105 ± 1	103.3 ± 2.9	2.8	0.9		
	Middle 200	96.4 ± 4.4	96.6 ± 3.4	3.5	4.6	0.03 ± 0.0	14.8
	High 500	100.1 ± 2.5	101.7 ± 1.9	1.9	2.5		
C80	Low 50	99.2 ± 1.5	99.8 ± 2.8	2.8	1.5		
	Middle 200	95 ± 5.2	96.6 ± 1.8	1.9	5.5	0.05 ± 0.0	2.8
	High 500	99.5 ± 3.2	101.7 ± 2	1.9	3.2		
C85	Low 50	99.8 ± 3.4	98.9 ± 3.7	3.8	3.4		
	Middle 200	96.7 ± 5.9	97.3 ± 1.3	1.3	6.1	0.18 ± 0.0	8.4
	High 500	100.5 ± 0.6	102.2 ± 2.2	2.2	0.6		
C90	Low 50	100.3 ± 3	99.3 ± 4.1	4.1	3.0		
	Middle 200	95.7 ± 4.4	97.4 ± 2.6	2.6	4.6	1.36 ± 0.2	15.9
	High 500	100.1 ± 1.7	104.9 ± 4.3	4.1	1.7		
C95	Low 50	104.5 ± 11.7	105.1 ± 4.1	3.9	11.2		
	Middle 200	98.3 ± 4.4	101.2 ± 4	4.0	4.5	3.56 ± 0.6	17.5
	High 500	98 ± 0.5	106.7 ± 7.6	7.1	0.5		
C100	Low 50	102.2 ± 11.4	101.6 ± 4.8	4.8	11.2		
	Middle 200	100.9 ± 4	103.5 ± 3.6	3.5	4.0	1.28 ± 0.3	19.1
	High 500	99.6 ± 0.6	109.1 ± 8.3	7.6	0.6		
C105	Low 50	110.4 ± 5.5	108.0 ± 7.5	6.7	7.7		
	Middle 200	103.7 ± 3.6	106.2 ± 2.7	3.4	3.4	0.1 ± 0.0	35.4
	High 500	100.7 ± 1.9	110.3 ± 8.3	7.9	1.6		

Table S7. Results from determination of extraction recovery (n=3) in blank solvent and post-preparation stability of RPLC-MS analysis of DolP standard in HeLa cells extracts in autosampler for 20h (n=3).

DolP species chain length	QC level (pg/µL)	Extraction recovery (n=3) (Mean ± SD,%)	(CV,%)	Stability (n=3) (Mean ± SD,%)	(CV,%)
C70	Low (50)	95.5 ± 1.8	1.9	119.6 ± 0.8	0.7
	High (500)	106.4 ± 2.6	2.4	119.3 ± 1.6	1.3
C75	Low (50)	103.6 ± 8.2	7.9	121.7 ± 1.4	1.2
	High (500)	111.2 ± 4.5	4.1	118.5 ± 3.2	2.7
C80	Low (50)	103.9 ± 8.9	8.5	120.5 ± 0.1	0.1
	High (500)	115.3 ± 4.5	3.9	118.8 ± 2.3	2
C85	Low (50)	112.2 ± 8.5	7.6	122.2 ± 1	0.8
	High (500)	123.4 ± 6	4.8	124.9 ± 6.4	5.1
C90	Low (50)	118 ± 9.1	7.7	123.8 ± 2	1.6
	High (500)	125.6 ± 5.9	4.7	118.9 ± 7.3	6.1
C95	Low (50)	123.3 ± 11.9	9.7	123.6 ± 3.2	2.6
	High (500)	123.4 ± 6.2	5	113.8 ± 3.2	2.9
C100	Low (50)	134.6 ± 12.8	9.5	122.3 ± 4.1	3.4
	High (500)	121.6 ± 8	6.6	100.1 ± 2	2
C105	Low (50)	ND[a]	0	92.4 ± 15.9	17.2
	High (500)	95.5 ± 1.8	1.9	99.9 ± 4.8	4.8

ND[a] = Not detected