Supplementary Material

Analytical and preparative separation and isolation of functionalized fullerenes by conventional HPLC stationary phases: Method development and column screening

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1. Reagents and Materials

All chemicals, including C_{60} and C_{70} were purchased from Sigma Aldrich and used as received. Acetonitrile and toluene was purchased from VWR Chemicals as HPLC Grade. The columns used are listed in Table S 1. The analytical columns were used with a Gemini C18 pre-guard cartridge (2.0 x 4.0 mm, Phenomenex, USA), the preparative column was used with ACE 5 C18 (10.0 x 10.0, Advanced Chromatography Technologies Ltd, UK) pre-guard cartridge.

Table S 1 The coding and properties of the columns used in this study

Column Code	Brand name	Inner Dimensions length x radius	Particle size	Surface area (m^2/g)	Carbon Load (%)	Column packing	End-capping
Col 1[a]	Inertsil ODS 4	250 x 4.6 mm	5.0 µm	450	11	C_{18}	TMS
Col 2[b]	Synergi Hydro-RP	100 x 4.6 mm	4.0 µm	400	19	C_{18}	Hydrophilic
Col 3[b]	Synergi Max-RP	150 x 4.6 mm	4.0 µm	400	17	C_{12}	TMS
Col 4[b]	Gemini NX	100 x 3.0 mm	3.0 µm	375	14	C_{18}	TMS
Col 5[b]	Kinetex Biphenyl	100 x 4.6 mm	2.6 µm	200	11	C_{12} (Biphenyl)	TMS
Col 6[b]	Kinetex Phenyl-Hexyl	150 x 4.6 mm	5.0 µm	200	11	C_{12} (Phenyl-hexyl)	TMS
Col 7[c]	ACE 5	150 x 21.0 mm	5.0 µm	300	15.5	C_{18}	TMS

[a] from GL Sciences, Japan, [b] from Phenomenex, USA, [c] from Advanced Chromatography Technologies Ltd, UK

In order to describe the quality of the peaks and the efficiency of the separation for all analytical columns, retention times (Rt), total analysis time (run time), capacity factors (k') and resolution (Rs) were evaluated and summarized in Table S 2. The capacity factor (k') is a measure that gives the molecules retention compared to the un-retained component or solvent, typically preferred to be higher than 0.8. Resolution (Rs) is a measure to describe the separation between two adjacent peaks, typically preferred to be higher than 2.0.

2. Instrumentation

All analyses were carried out using a Gilson HPLC system consisting of a Gilson 331 Pump, Gilson 156 Dual UV/VIS detector, Gilson 215 Nebula Liquid Handler and fraction collector, Gilson 819 Injection Module and Gilson 506C System Interface, using Gilson Unipoint Software (Version 5.11, USA). The HPLC separations were performed using isocratic flow rate and the columns were kept at room temperature (between 20°C and 23°C). Detection was carried out at 285 nm and 350 nm simultaneously. In order to monitor the mobile phase effects, four different mobile phases; toluene, 45% (v/v) acetonitrile in toluene, 50% (v/v) acetonitrile in toluene and 55% (v/v) acetonitrile in toluene were used. The flow rate was adjusted between 0.4 mL/min – 1 mL/min based on the backpressure of the columns. Injection volumes were between 30-50 µL for analytical separation, and 4000-5000 µL for preparative separation. Preparative separations were conducted by applying the corresponding analytical methodology but increasing the injection volume and employing two flow rates; starting at 13 mL/min and, after the elution of monoadduct, increased to 16 mL/min for faster elution of unreacted C_{60}. All the injections were made within 30 minutes of the sample preparation.

NMR spectra of reaction mixtures and isolated monoadducts were recorded on a Varian 500 MHz and Agilent 400 MHz NMR Spectrometers. Mass spectrometry of reaction mixtures and isolated monoadducts was carried out using a Bruker Autoflex 2 MALDI-TOF spectrometer in positive and

negative ion mode and *trans*-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB) was used as matrix. Matrix solution was prepared by dissolving 10 mg of DCTB in 1 mL of chloroform. The analytes to be measured were then dissolved in chloroform and mixed with a small amount of matrix solution and loaded on the target plate.

3. Sample preparation for HPLC analysis

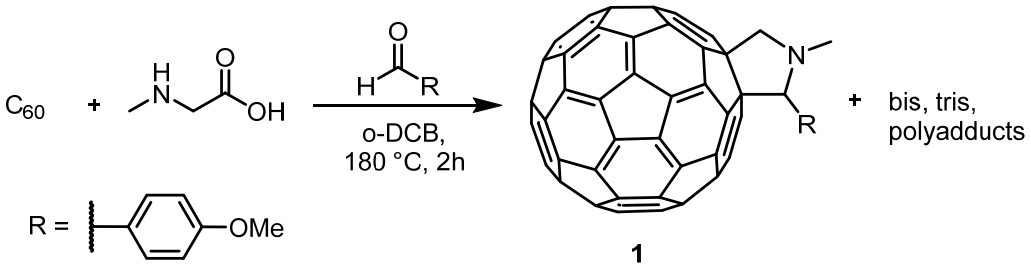
3.1. C_{60} + C_{70} mixture solution that was prepared from C_{60} and C_{70}

3.1.1. C_{60} solution*:* 15 mg of C_{60} was weighed to a 10 mL volumetric flask and dissolved using toluene by sonication and marked up to its volume by toluene. Then 4 mL of this stock solution was pipetted into a 10 mL volumetric flask and diluted to its volume by 50% (v/v) toluene in acetonitrile or toluene depending on the mobile phase. The solution is then mixed by shaking thoroughly and filtered through 0.45 µm PVDC filter into an HPLC vial.

3.1.2. C_{70} solution*:* 7 mg of C_{70} was weighed to a 5 mL volumetric flask and dissolved using toluene by sonication and marked up to its volume by toluene. Then 2 mL of this stock solution was pipetted into a 10 mL volumetric flask and diluted to its volume by 50% (v/v) toluene in acetonitrile or toluene depending on the mobile phase. The solution is then mixed by shaking thoroughly and filtered through 0.45 µm PVDC filter into an HPLC vial.

3.1.3. C_{60} + C_{70} mixture solution*:* 2 mL of C_{60} solution and 2 mL of C_{70} solution were pipetted into a 10 mL volumetric flask and diluted to its volume using 50% (v/v) toluene in acetonitrile. The solution is then mixed by shaking thoroughly and filtered through 0.45 µm PVDC filter into an HPLC vial.

3.2. Reaction Product Mixture 1 (RPM1)



Scheme S 1 Pyrrolidination/1,3-dipolar cycloaddition reaction of fullerene C_{60} with 4-anisaldehyde and sarcosine. Before HPLC sample preparation, all reagents were washed away by extraction, having only unreacted $C_{60,}$ product **1**, bis, tris and higher adducts in the RPM1.

As reported by Prato et.al.[1] and applied in-house conditions; in a microwave vial, C60 (1 equivalent, 50.0 mg), sarcosine (2 equivalent, 12.5 mg) was weighed and a cross-shaped magnetic stirrer was placed. Then o-DCB (3 mL, anhydrous) was added to dissolve (**Scheme S 1**). After stirring for approximately 5 min, p-anisaldehyde (5 equivalents, 42.5 µL) was added to the mixture. Then the cap of the vial was sealed and N_2 gas was purged in. The vial was placed in an oil bath and heated to 180 °C while stirring for 2 h. Then the mixture was cooled down to room temperature, and the solvent was evaporated under vacuum. The remaining crude was then dissolved with DCM and washed 2 times with K_2CO_3(aq) (10%, 20 mL). The organic phase was dried over $MgSO_4$ and the solvent was removed yielding a brown residue (RPM1) containing only fullerene adducts and very low amount of unreacted fullerene. Product identity is verified by ^{1}H NMR (**Fig. S 1**) and mass spectroscopy (**Fig. S 2**).

For analytical HPLC sample preparation, approximately 15 mg of RPM1 was weighed to a 10 mL volumetric flask and dissolved using toluene by sonication and marked up to its volume by toluene. Then 4 mL of this solution was pipetted into a 10 mL volumetric flask and diluted to its volume by 50% (v/v) toluene in acetonitrile or toluene depending on the mobile phase. The solution is then mixed by shaking thoroughly and filtered through 0.45 µm PVDC filter into an HPLC vial.

For preparative HPLC sample preparation, approximately 12 mg of RPM1 was weighed to a 10 mL volumetric flask and dissolved using 6 mL of toluene by sonication. Then the solution is diluted to its volume with acetonitrile, mixed by shaking thoroughly and sonicated for 10 minutes. The solution is then centrifuged for 5 minutes at 3000 rpm.

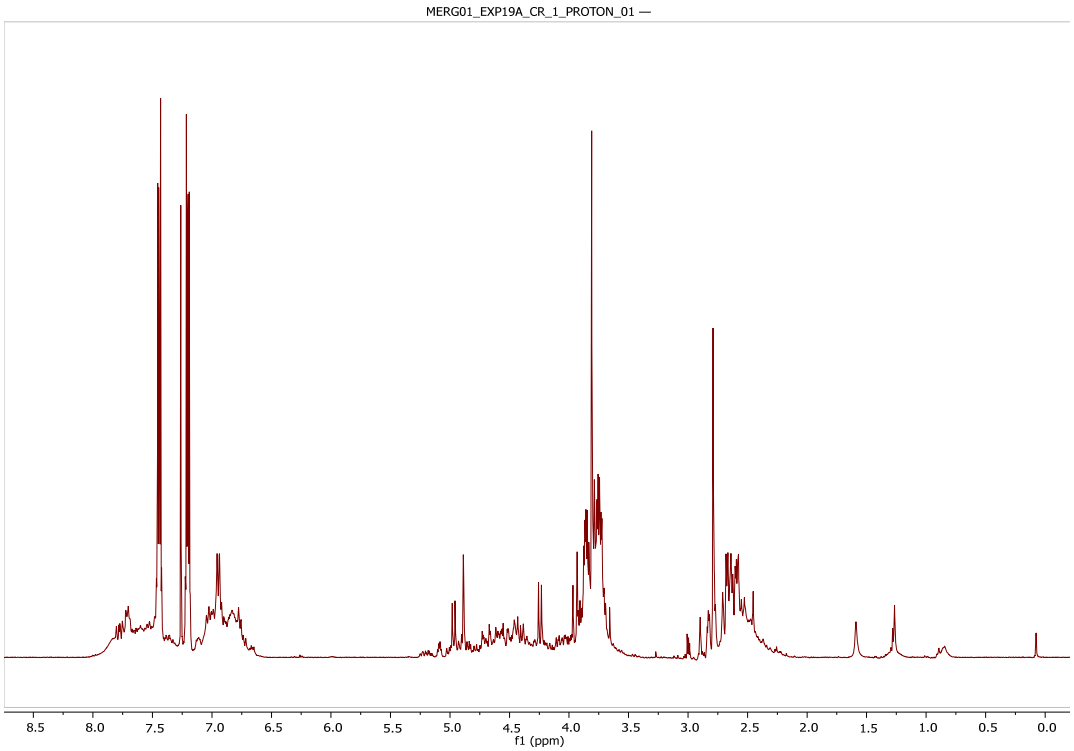


Fig. S 1 ^{1}H NMR Spectrum of RPM1 in $CDCl_3$:CS_2 (1:5, v/v)

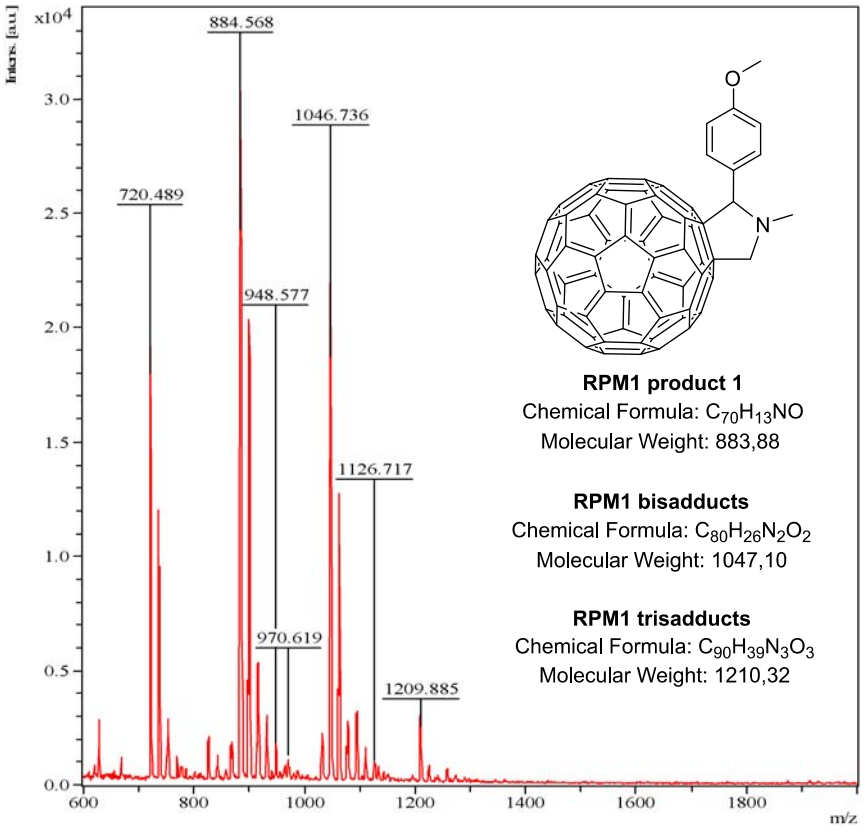
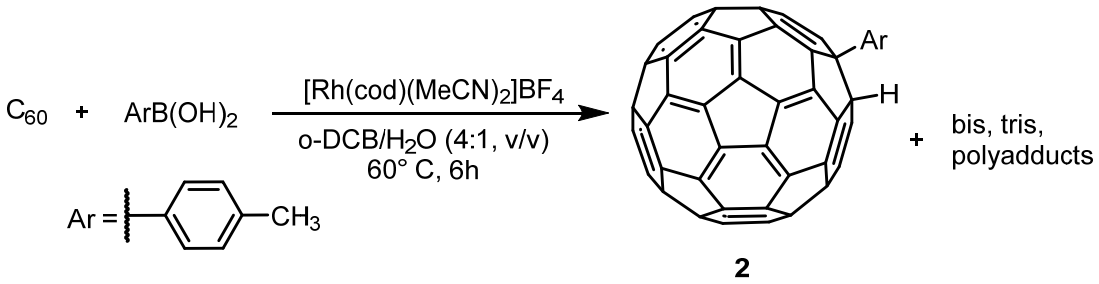


Fig. S 2 MALDI-MS Spectrum of RPM1

3.3. Reaction Product Mixture 2 (RPM2)



Scheme S 2 Hydroarylation reaction of C_{60} with p-tolylboronic acid using a Rh catalyst. Before HPLC sample preparation, all reagents were washed away by extraction, having only unreacted C_{60}, **2** and higher adducts in the RPM2

As reported by Itami et.al.[2] and applied in-house conditions; in a microwave vial, C_{60} (1 equivalent, 50.4 mg), p-tolylboronic acid (1.2 equivalent, 11.3 mg) and $[Rh(cod)(MeCN)_2]BF_4$ (0.1 equivalent, 2.7 mg) were weighed and a cross-shaped magnetic stirrer was placed (**Scheme S 2**). After closing the seal of the vial, air was vacuumed and then N_2 gas was bubbled in and this cycle was repeated 3 times. After creating the final N_2 atmosphere in the vial, 1,2-dichlorobenzene (o-DCB) (8.4 mL, anhydrous), and H_2O (2.1 mL) were added. The sealed reaction mixture in vial was then heated to 60 °C for 6 hours. The reaction mixture was cooled down and passed through 20 g of silica using toluene as an eluent to remove the catalyst and unreacted p-tolyboronic acid. The solvent was then evaporated under vacuum yielding the mixture containing only the unreacted fullerenes and fullerene adducts. Product identity is verified by ^{1}H NMR (**Fig. S 3**) and mass spectroscopy (**Fig. S 4**). In order to verify if it is in accordance to the reported value, the yield was calculated by quantitative analysis of ^{1}H NMR spectrum of RPM2 by using 1 mg (0.005 mmol) dimethyl terephthalate (DMT) as internal standard.

For analytical HPLC sample preparation, approximately 15 mg of RPM2 was weighed to a 10 mL volumetric flask and dissolved using toluene by sonication and marked up to its volume by toluene. Then 4 mL of this solution was pipetted into a 10 mL volumetric flask and diluted to its volume by 50% (v/v) toluene in acetonitrile or toluene depending on the mobile phase. The solution is then mixed by shaking thoroughly and filtered through 0.45 µm PVDC filter into an HPLC vial.

For preparative HPLC sample preparation, approximately 12 mg of RPM2 was weighed to a 10 mL volumetric flask and dissolved using 6 mL of toluene by sonication. Then the solution is diluted to its volume with acetonitrile, mixed by shaking thoroughly and sonicated for 10 minutes. The solution is then centrifuged for 5 minutes at 3000 rpm

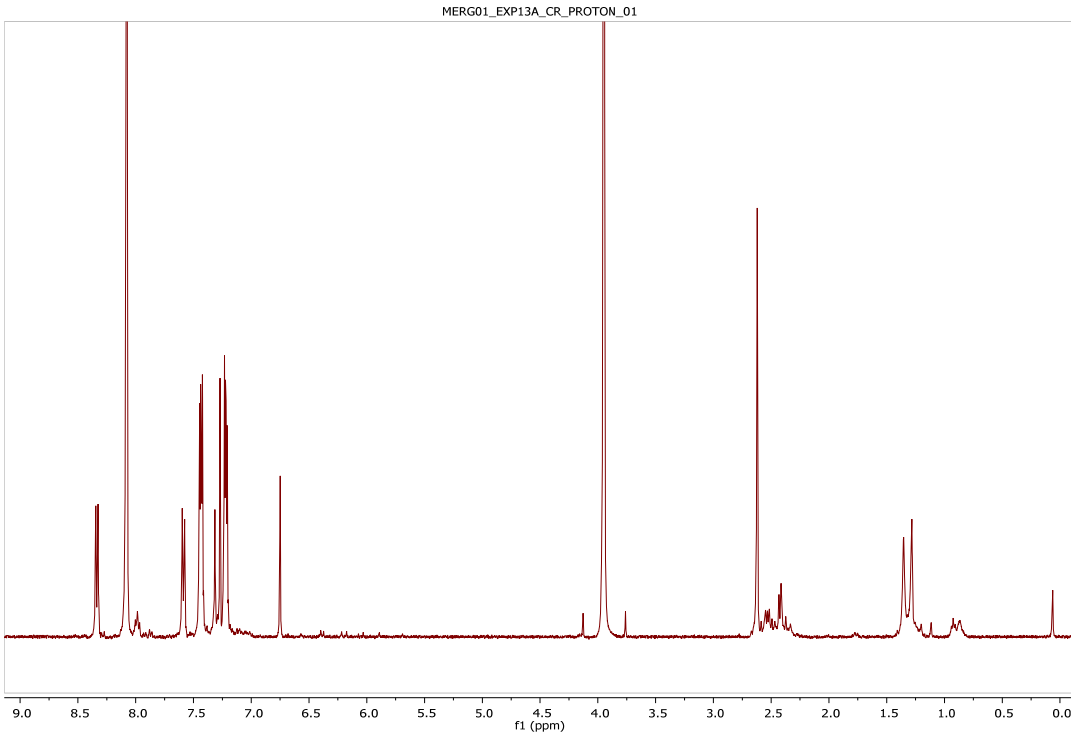


Fig. S 3 ^{1}H NMR Spectrum of RPM2 with added 1.0 mg (0.005 mmol) DMT as internal standard in $CDCl_3$:CS_2 (1:5, v/v)

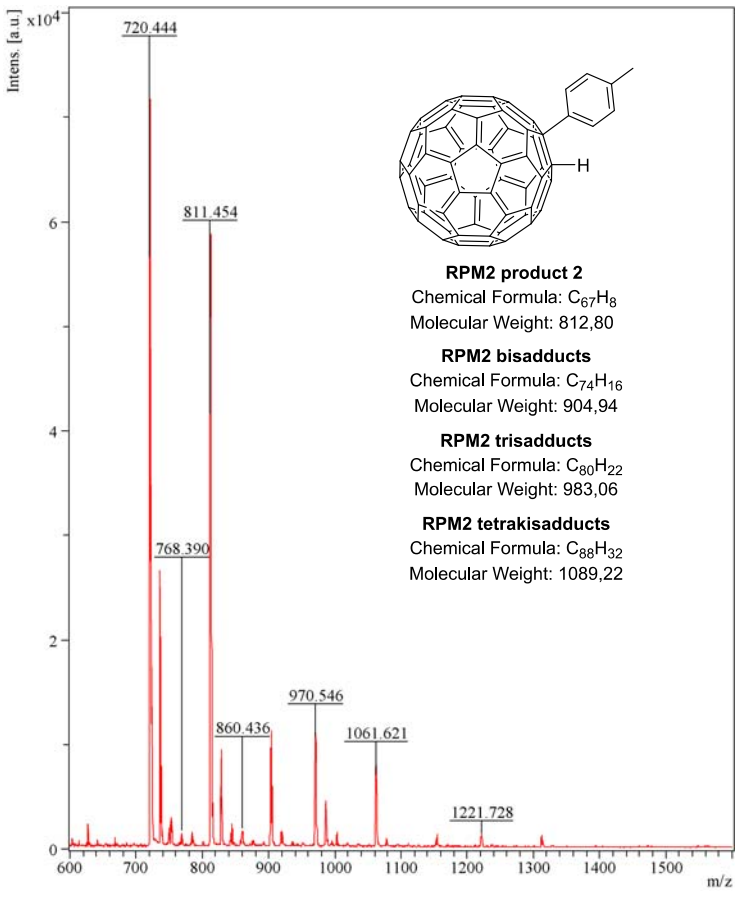


Fig. S 4 MALDI-MS Spectrum of RPM2

4. HPLC Analyses of C_{60} + C_{70} mixture, RPM1 and RPM2

4.1. HPLC Analyses of C_{60} + C_{70} mixture

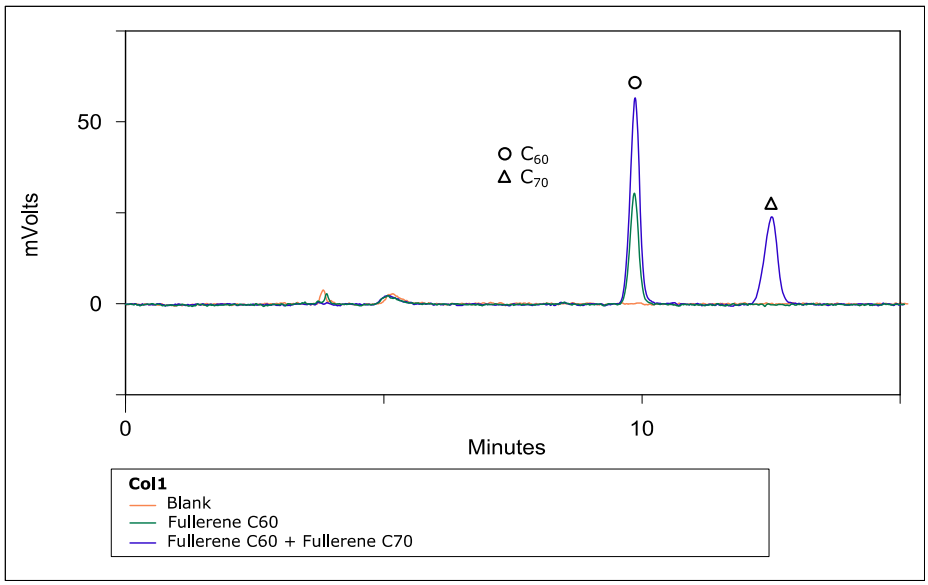


Fig. S 5 Blank, fullerene C_{60} and fullerene C_{60} + C_{70} mixture chromatograms obtained by eluting with 1 ml/min of 45% (v/v) acetonitrile in toluene at 285 nm UV detection.

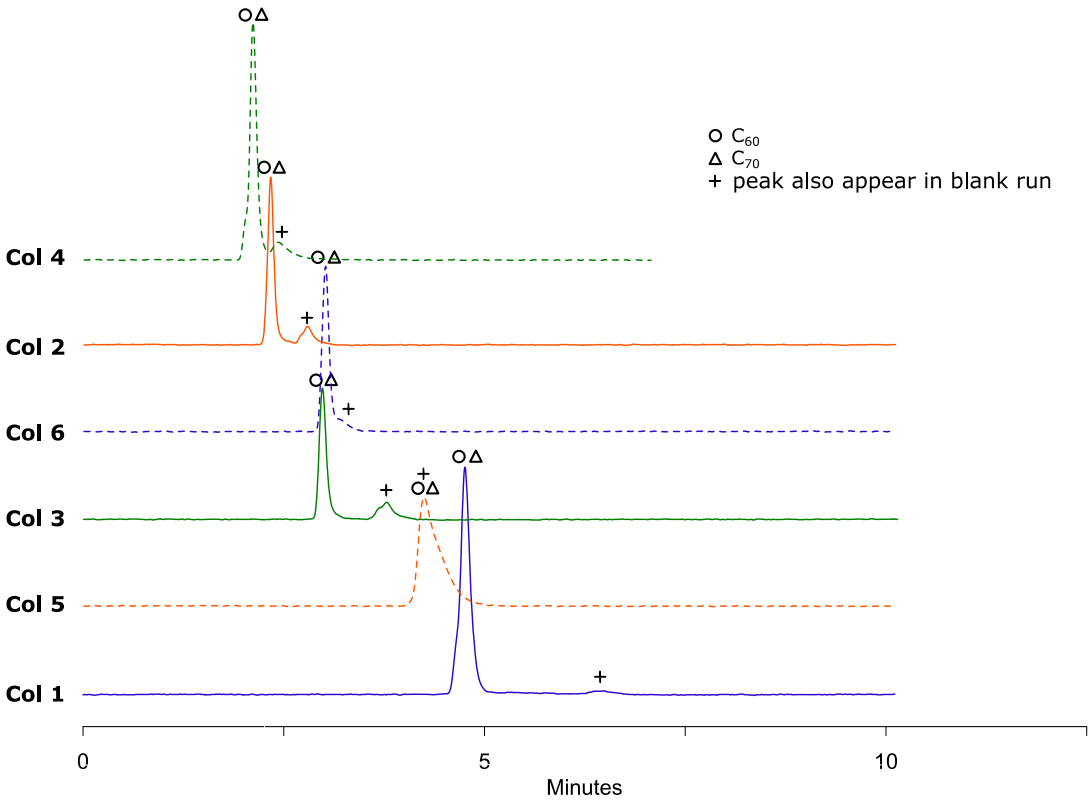


Fig. S 6 C_{60}+ C_{70} mixture chromatograms in Toluene at 285 nm detection showed no separation

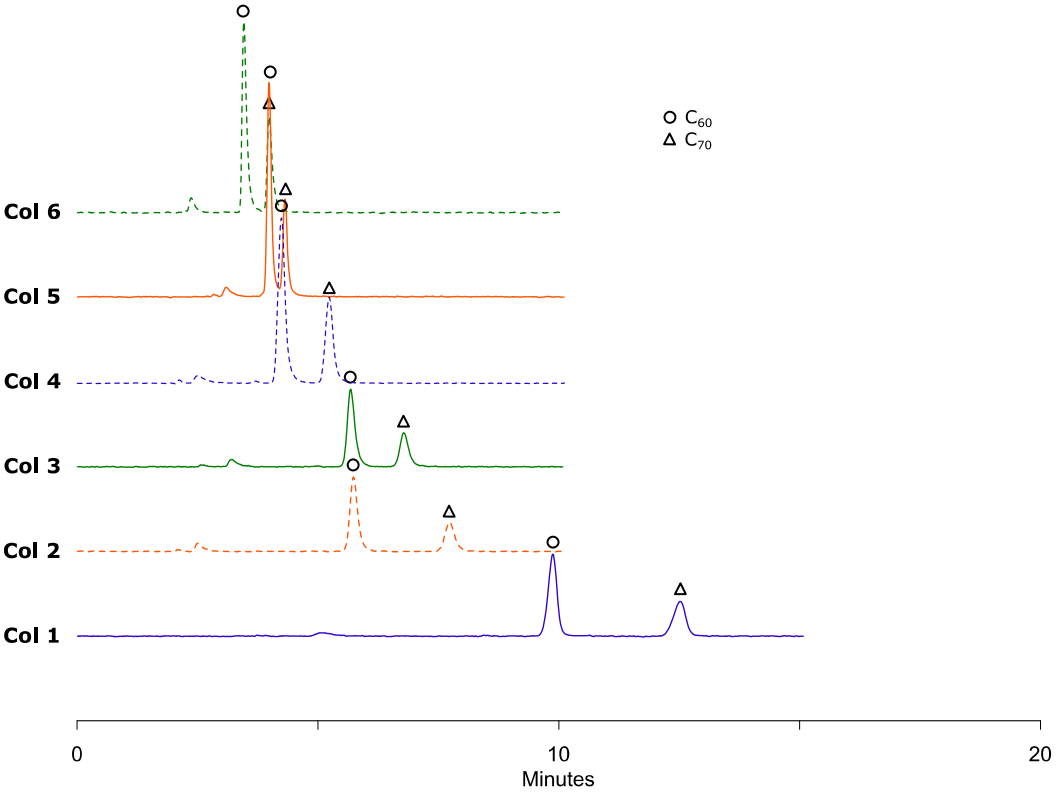


Fig. S 7 C_{60} +C_{70} mixture chromatograms obtained by eluting with 45% (v/v) acetonitrile in toluene at 285 nm UV detection where all columns showed good separation.

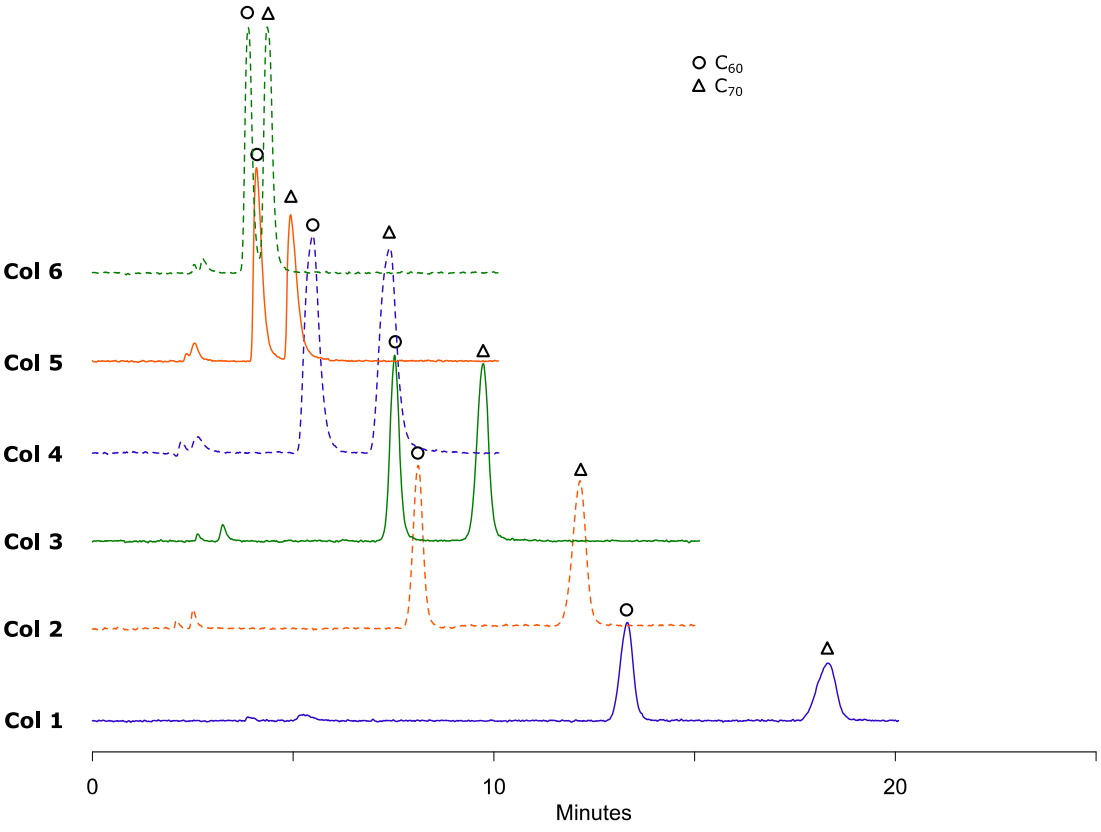


Fig. S 8 C_{60} +C_{70} mixture chromatograms obtained by eluting with 50% (v/v) acetonitrile in toluene at 285 nm UV detection where all columns showed good separation.

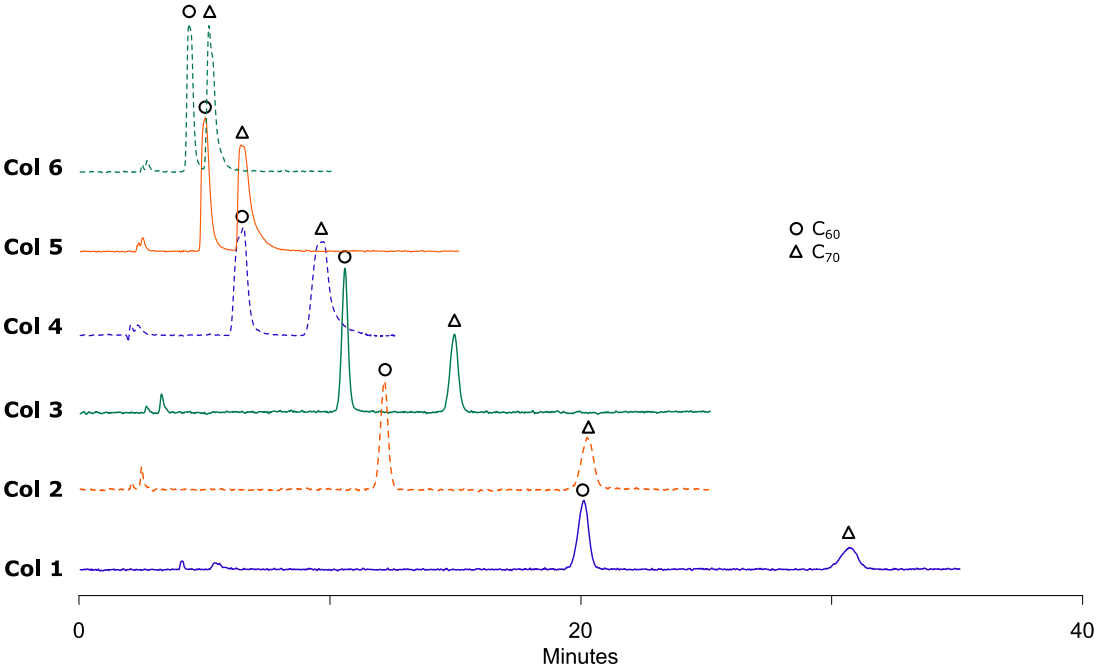


Fig. S 9 C_{60} +C_{70} mixture chromatograms obtained by eluting with 55% (v/v) acetonitrile in toluene at 285 nm UV detection where all columns showed good separation.

4.2. HPLC analyses of RPM1

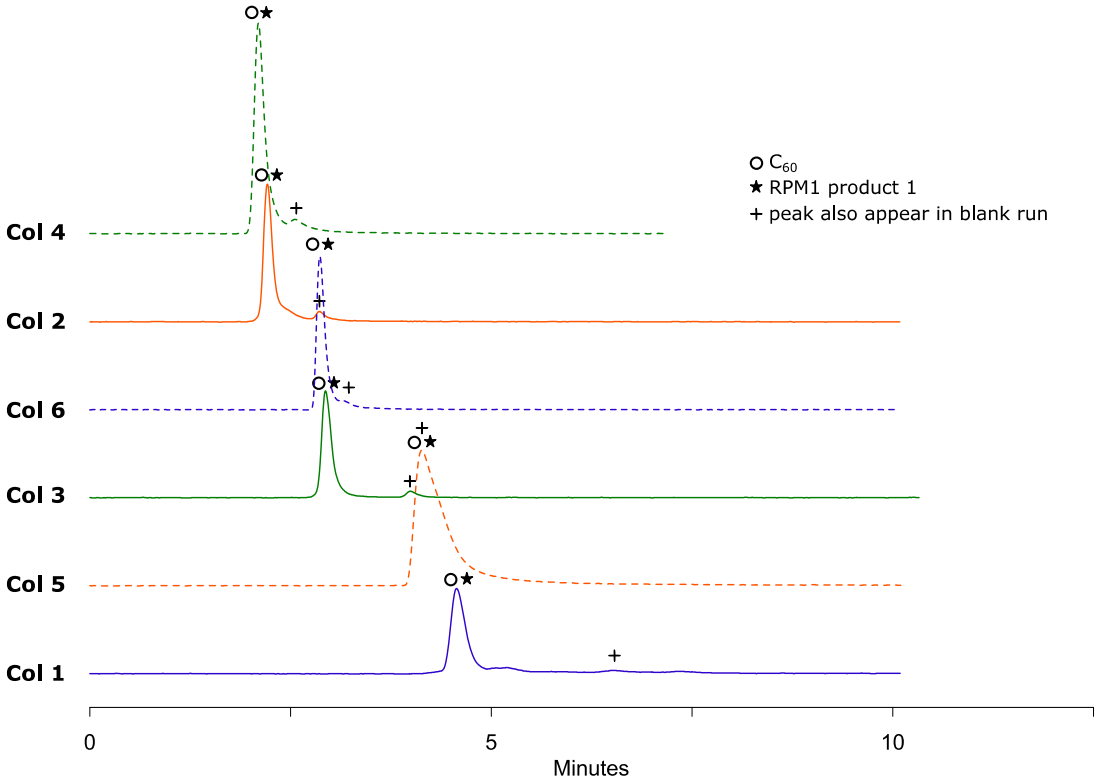


Fig. S 10 RPM1 chromatograms in toluene at 285 nm UV detection where all columns show no separation.

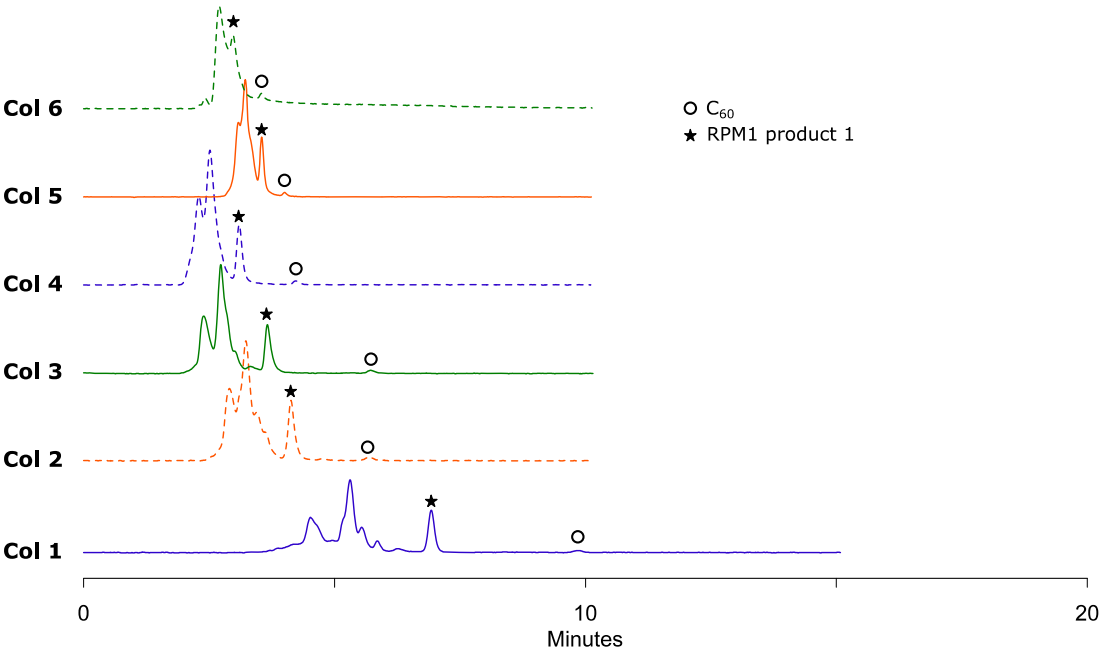


Fig. S 11 RPM1 chromatograms obtained by eluting with 45% (v/v) acetonitrile in toluene at 285 nm UV detection where Col 1, Col 2 and Col 3 show very good separation, Col 4 shows moderate separation and Col 5 and Col 6 does not separate RPM1 product **1** from bis, tris and polyadducts.

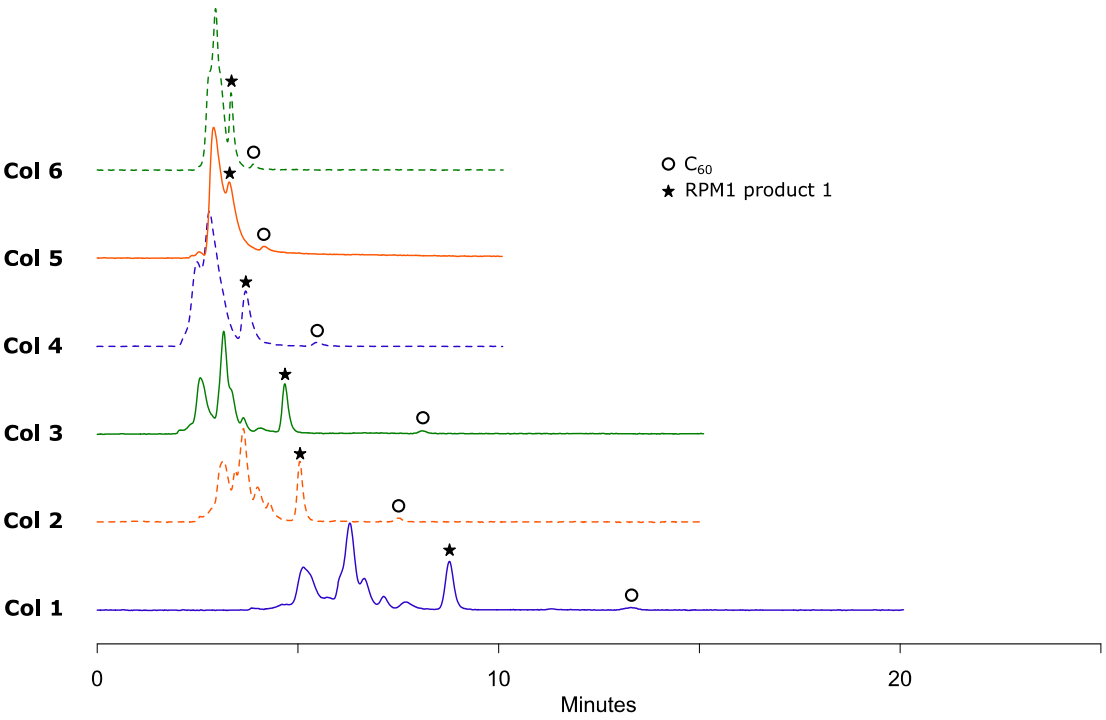


Fig. S 12 RPM1 chromatograms obtained by eluting with 50% (v/v) acetonitrile in toluene at 285 nm UV detection where Col 1, Col 2 and Col 3 show very good separation, Col 4 shows moderate separation and Col 5 and Col 6 does not separate RPM1 product 1 from bis, tris and polyadducts.

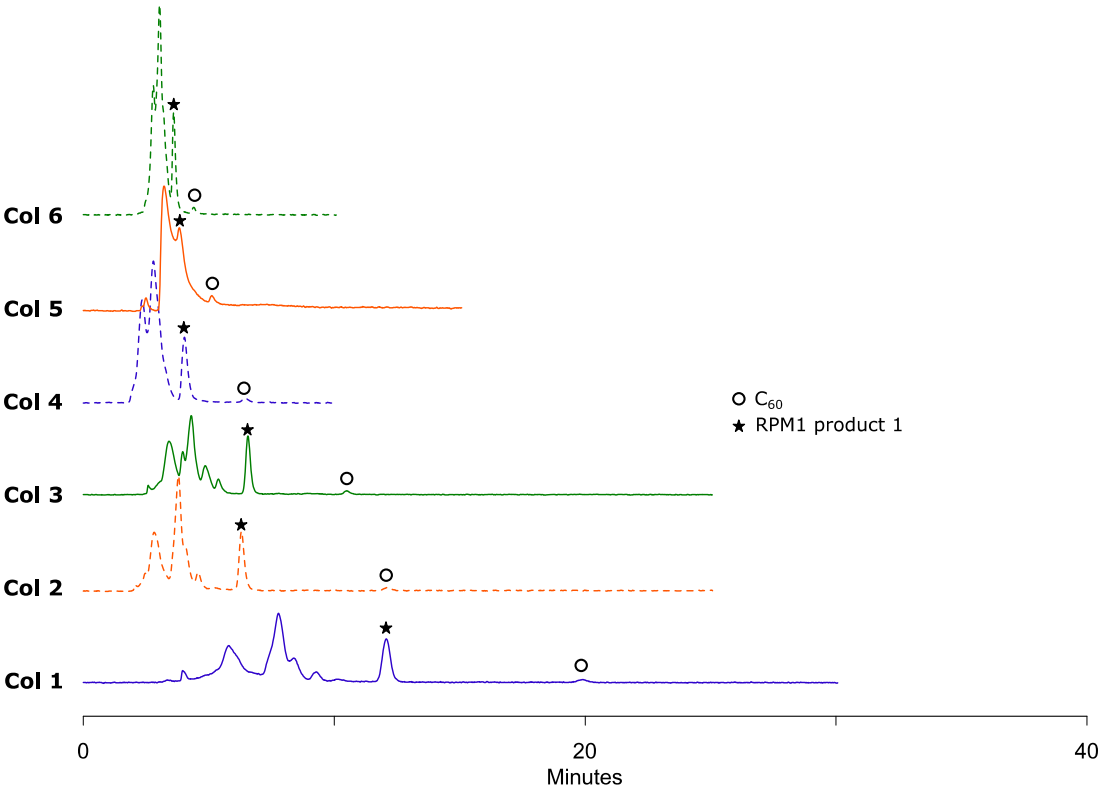


Fig. S 13 RPM1 chromatograms obtained by eluting with 55% (v/v) acetonitrile in toluene at 285 nm UV detection where Col 1, Col 2 and Col 3 show very good separation, Col 4 shows moderate separation and Col 5 and Col 6 does not separate RPM1 product 1 from bis, tris and polyadducts.

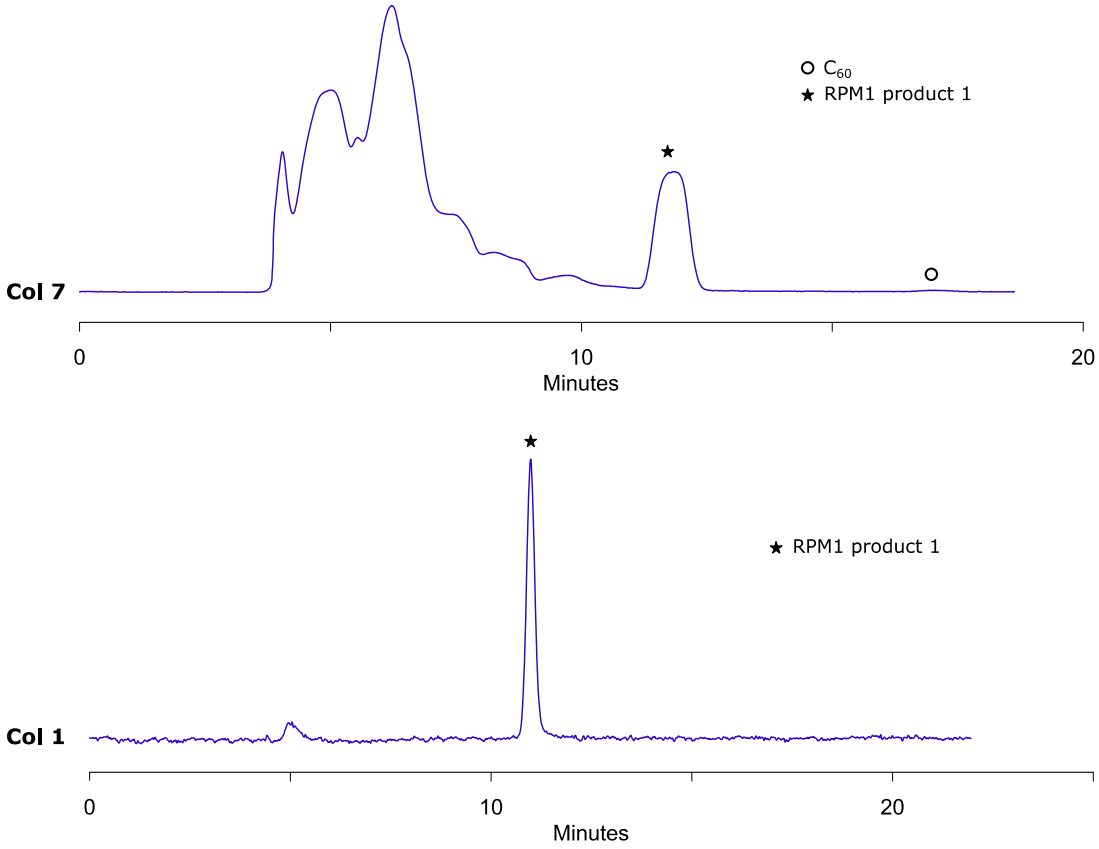


Fig. S 14 Upper trace: Preparative RPM1 chromatogram obtained by Col 7 eluting with 55% (v/v) acetonitrile in toluene at 285 nm UV detection that shows very effective separation of RPM1 product **1** from higher adducts and unreacted C_{60}. Lower trace: Analytical chromatogram obtained by injecting collected RPM1 product **1** fraction from preparative RPM1 injection by Col 1 eluting with 55% (v/v) acetonitrile in toluene at 285 nm UV detection that shows RPM1 product **1** is well separated from other peaks and isolated pure.

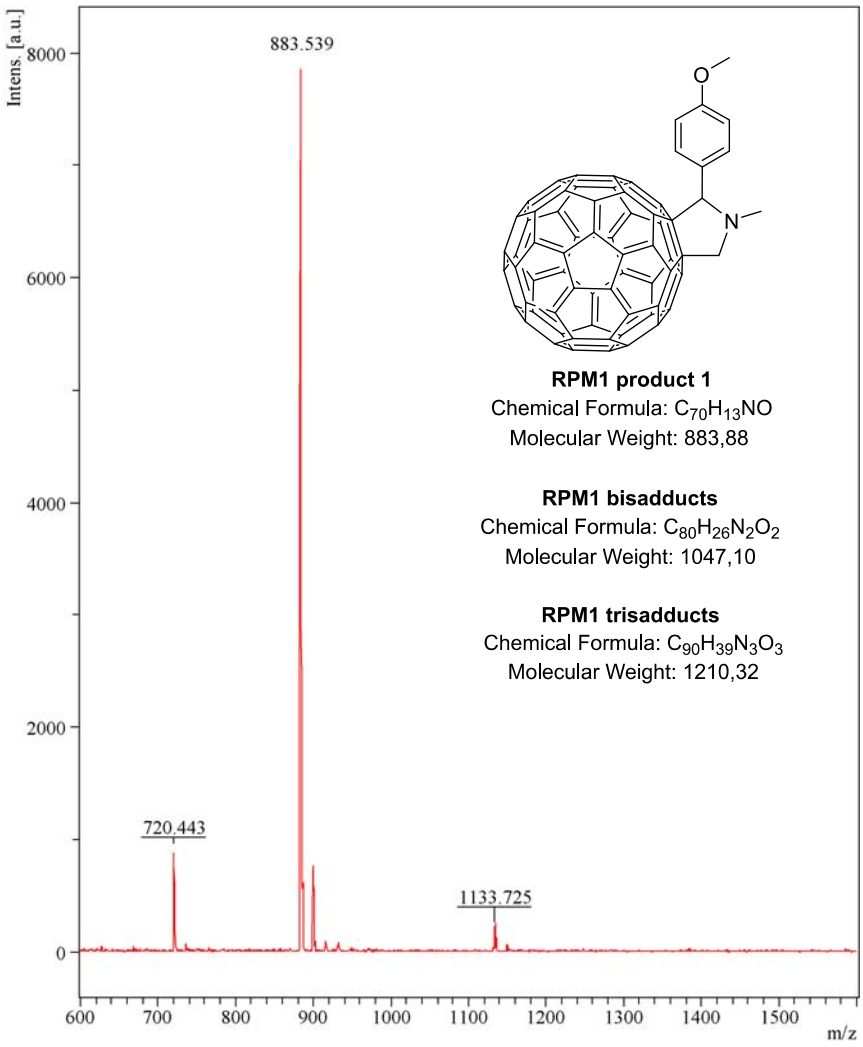


Fig. S 15 MALDI-MS Spectrum of RPM1 product **1** isolated from preparative HPLC

4.3. HPLC Analyses of RPM2

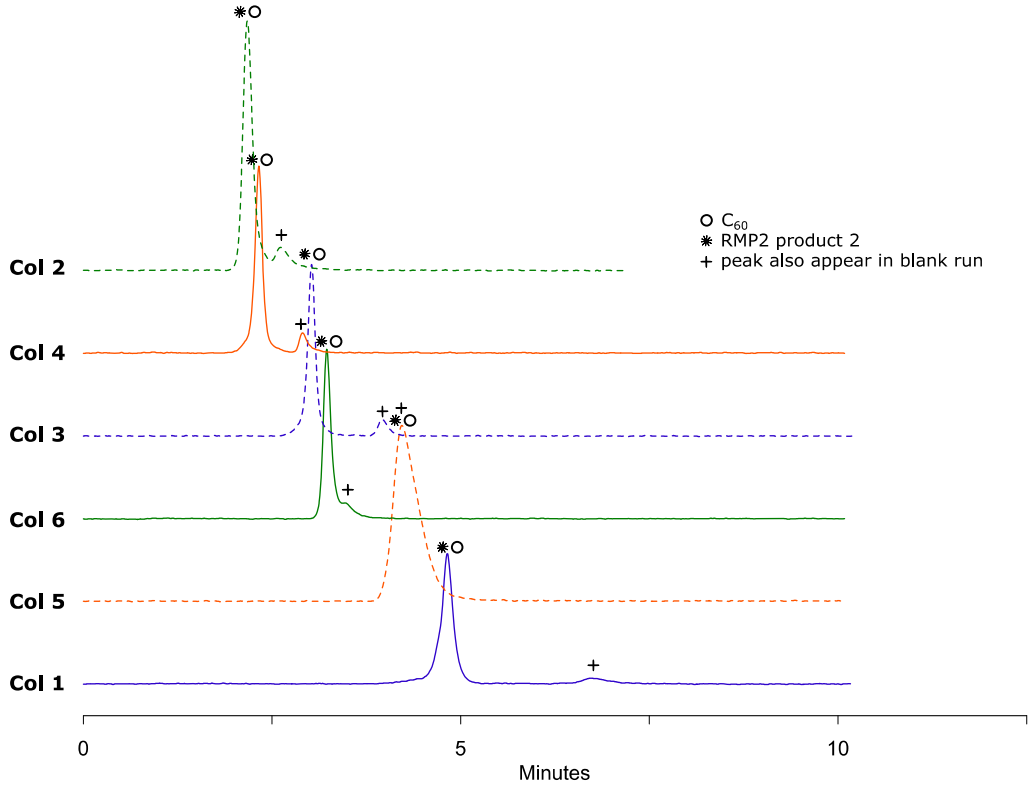


Fig. S 16 RPM2 chromatograms obtained in toluene at 285 nm UV detection showing that there is no separation.

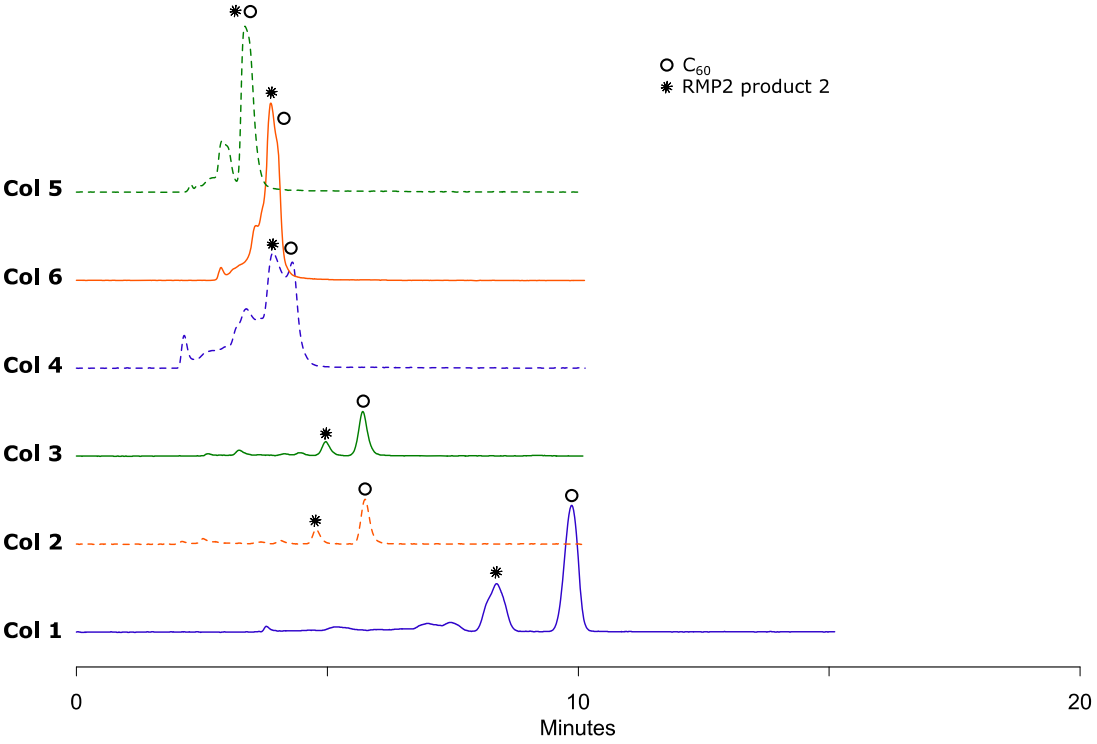


Fig. S 17 RPM2 chromatograms obtained by eluting with 45% (v/v) acetonitrile in toluene at 285 nm UV detection where Col 1, Col 2 and Col 3 show very good separation, Col 4, Col 5 and Col 6 does not separate RPM2 product 2 from bis, tris and polyadducts.

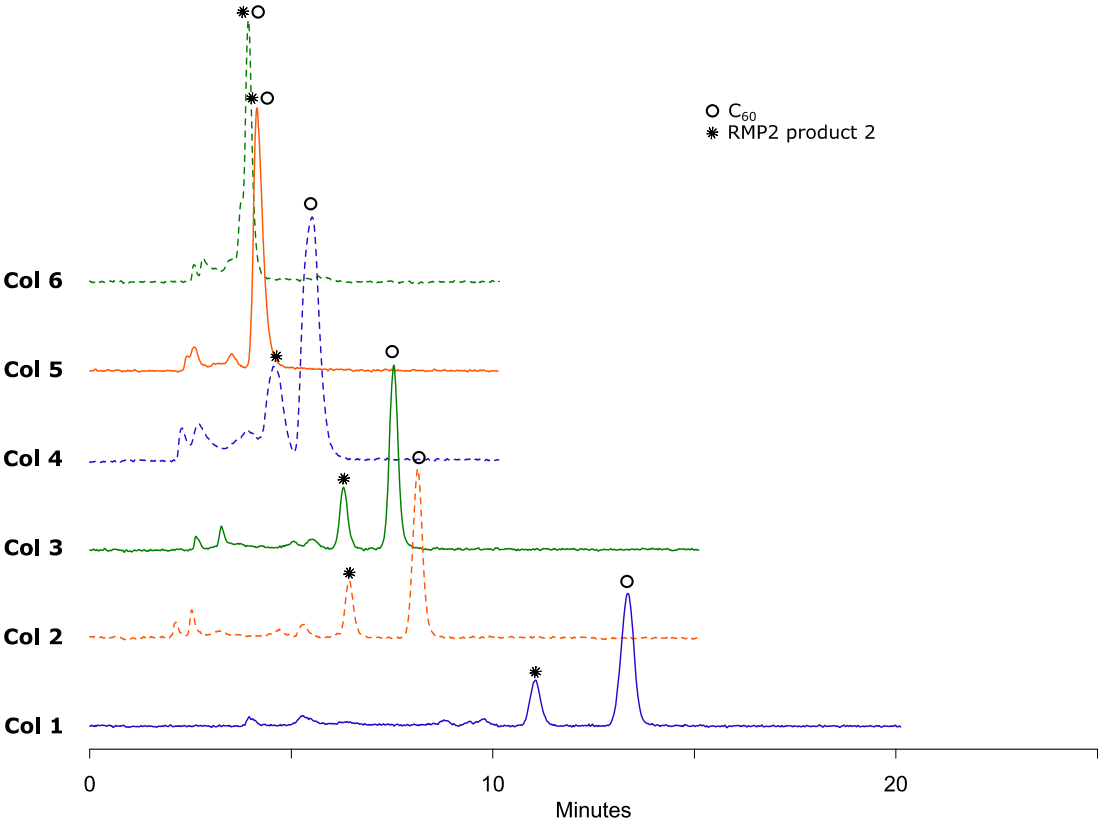


Fig. S 18 RPM2 chromatograms obtained by eluting with 50% (v/v) acetonitrile in toluene at 285 nm UV detection where Col 1, Col 2 and Col 3 show very good separation, Col 4 shows moderate separation and Col 5 and Col 6 does not separate RPM2 product 2 from bis, tris and polyadducts.

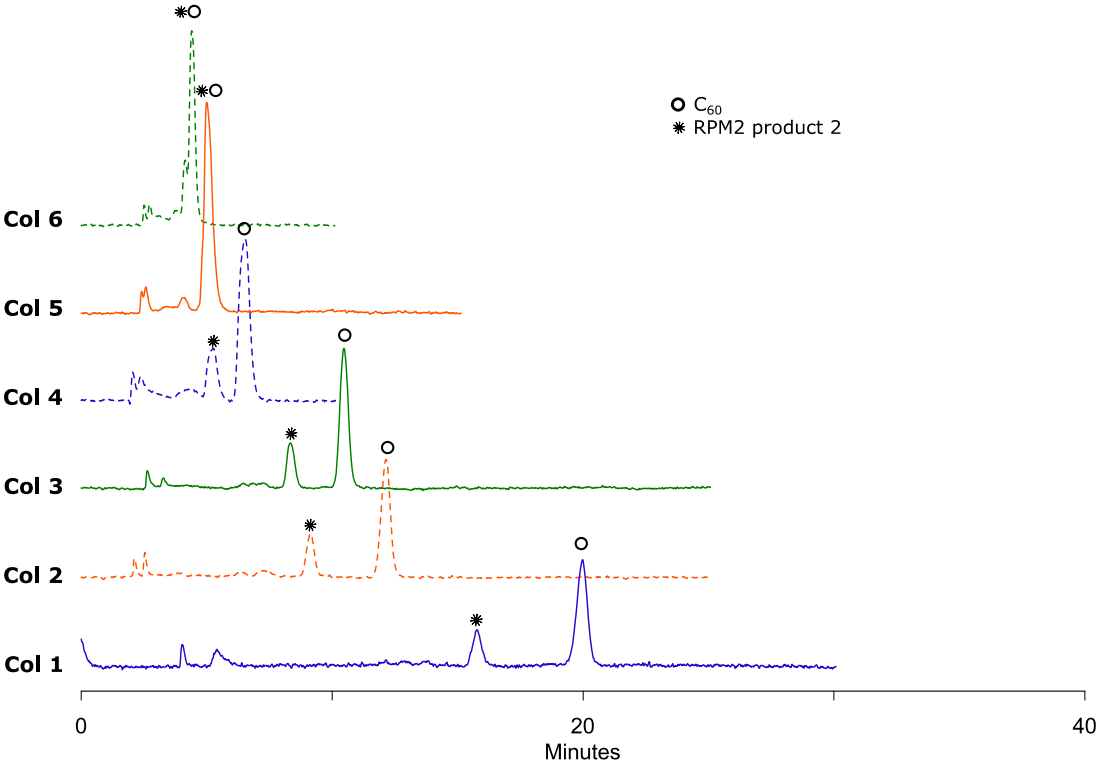


Fig. S 19 RPM2 chromatograms obtained by eluting with 55% (v/v) acetonitrile in toluene at 285 nm UV detection Col 1, Col 2 and Col 3 show very good separation, Col 4 shows moderate separation and Col 5 and Col 6 does not separate RPM2 product 2 from bis, tris and polyadducts.

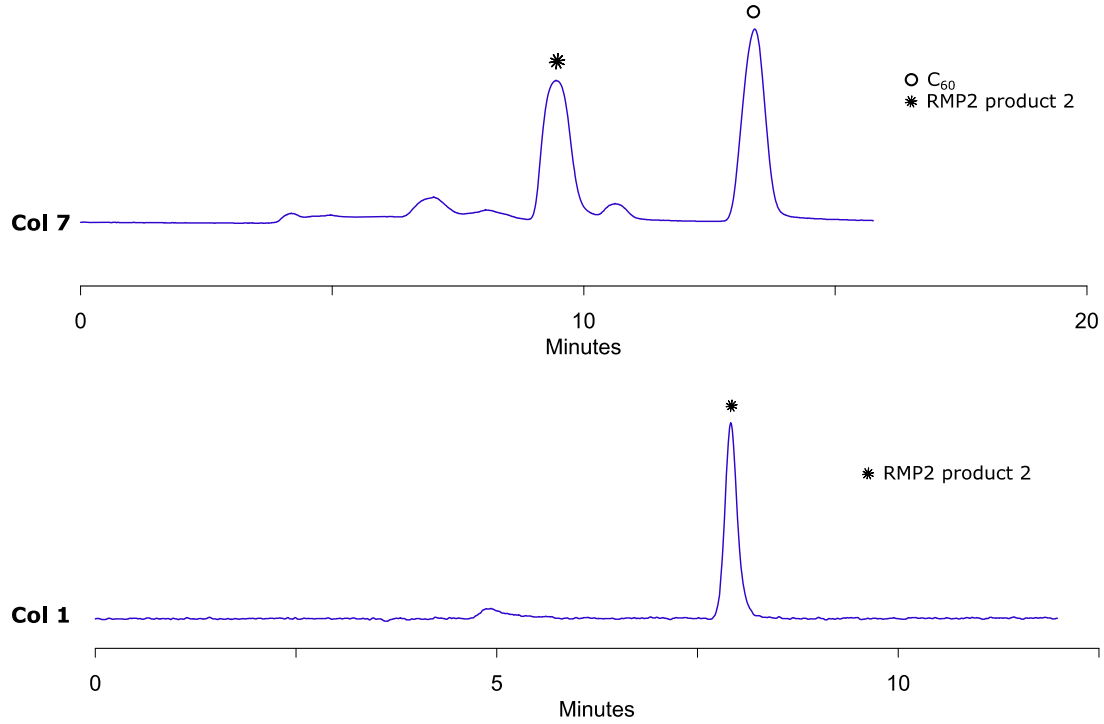


Fig. S 20 Upper trace: Preparative RPM2 chromatogram obtained by Col 7 eluting with 45% (v/v) acetonitrile in toluene at 285 nm UV detection that shows very effective separation of RPM2 product **2** from higher adducts and unreacted C_{60}. Lower trace: Analytical chromatogram obtained by injecting collected RPM2 product **2** fraction from preparative RPM2 injection by Col 1 eluting with 45% (v/v) acetonitrile in toluene at 285 nm UV detection that shows RPM2 product **2** is well separated from other peaks and isolated pure

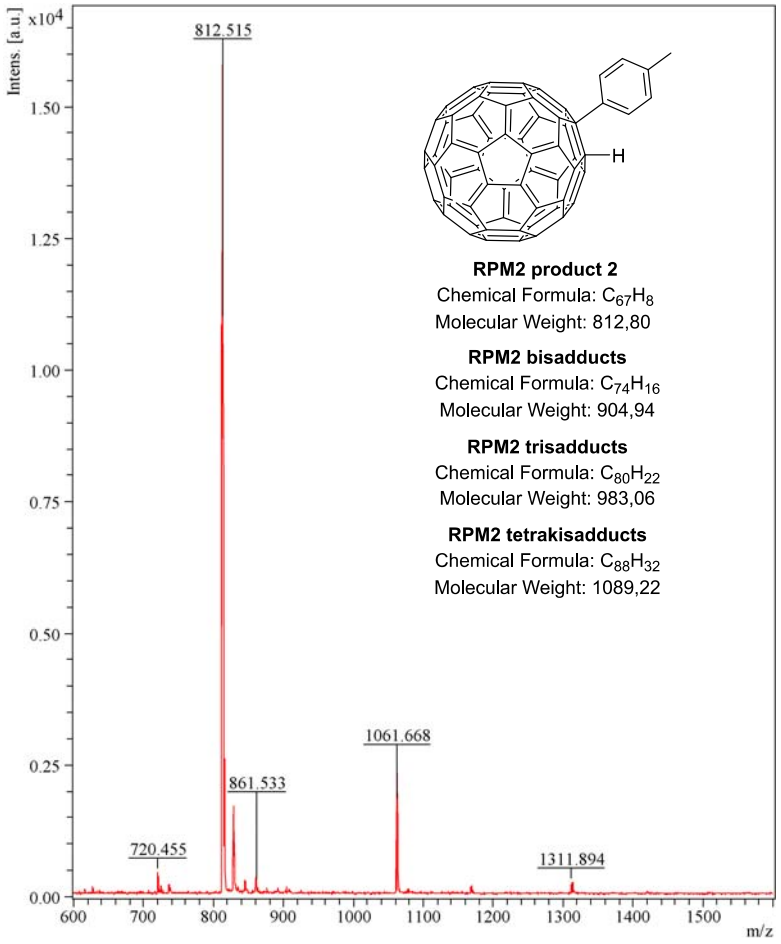


Fig. S 21 MALDI-MS Spectrum of RPM2 product **2** isolated from preparative HPLC

4.4. Summary of HPLC analyses

Table S 2 C_{60}, C_{70}, RPM1 product **1** and RPM2 product **2** peak properties where Rt is retention time, k' is capacity factor[a], N is plate number[b] and Rs is resolution[c] on different columns using 45% (MPA), 50% (MPB) and 55% (v/v) (MPC) acetonitrile in toluene as mobile phase

		Col 1			Col 2			Col 3			Col 4			Col 5			Col 6		
Name of the column		Inertsil ODS 4			Synergi Hydro-RP			Synergi Max-RP			Gemini NX			Kinetex Biphenyl			Kinetex Phenyl-Hexyl		
Column Dimensions		250 x 4.6 mm, 5.0 µm			100 x 4.6 mm, 4.0 µm			150 x 4.6 mm, 4.0 µm			100 x 3.0 mm, 3.0 µm			100 x 4.6 mm, 2.6 µm			150 x 4.6 mm, 5.0 µm		
Mobile phase		MP A	MP B	MP C	MP A	MP B	MP C	MP A	MP B	MP C	MP A	MP B	MP C	MP A	MP B	MP C	MP A	MP B	MP C
t_0		3.9	3.9	3.9	2.1	2.1	2.1	2.7	2.6	2.6	2.1	2.2	2.3	2.4	2.5	2.5	2.9	2.6	2.7
C_{60}	Rt	9.9	13.4	20.0	5.7	8.1	12.1	5.7	7.5	10.5	4.2	5.4	6.5	3.5	4.1	5.0	4.0	3.9	4.4
	k'	1.5	2.4	4.1	1.7	2.9	4.8	1.1	1.9	3.0	1.0	1.5	1.8	*0.5*	*0.6*	1.0	*0.4*	*0.5*	*0.6*
	N	13195	8808	9966	6026	4843	7163	6139	4908	8582	3839	*1042*	*791*	6647	1858	1157	9497	2168	1497
C_{70}	Rt	12.5	18.3	30.6	7.8	12.2	20.2	6.9	9.7	14.9	5.2	7.4	9.8	4.0	5.0	6.4	4.4	4.4	5.2
	k'	2.2	3.7	6.8	2.7	4.8	8.6	1.6	2.7	4.7	1.5	2.4	3.3	0.7	1.0	1.6	*0.5*	*0.7*	0.9
	N	11006	6402	10086	6587	6099	7913	6670	5131	9507	4900	*1430*	*1055*	6790	2030	*936*	9897	*1973*	*1382*
	Rs	6.4	6.6	10.6	6.0	7.4	10.8	3.5	2.4	8.2	3.4	2.6	3.0	3.0	2.1	2.0	2.0	*1.3*	*1.6*
RPM1 Product 1	Rt	6.9	8.8	12.1	3.7	4.7	6.3	4.1	5.1	6.6	3.1	3.7	4.0	3.0	3.3	3.8	3.6	3.3	3.6
	k'	0.8	1.3	2.1	0.8	1.2	2.0	*0.5*	1.0	1.5	*0.5*	*0.7*	*0.7*	*0.3*	*0.3*	*0.5*	*0.2*	*0.3*	*0.3*
	N	13751	8181	6436	4280	4451	4330	4990	5989	5780	3337	*1343*	*1381*	*NS*	*NS*	*NS*	*NS*	*NS*	3669
	Rs	2.2	2.3	4.8	3.2	4.6	2.2	2.2	2.6	3.2	*1.8*	*1.5*	*1.9*	*NS*	*NS*	*NS*	*NS*	*NS*	*1.5*
RPM2 Product 2	Rt	8.3	11.1	15.8	4.8	6.1	8.9	5.0	6.3	8.4	3.6	4.5	5.2	2.9	3.5	4.1	3.7	3.6	4.0
	k'	1.1	1.8	3.1	1.3	1.9	3.2	0.9	1.4	2.2	*0.7*	1.0	1.3	*0.2*	*0.4*	*0.6*	*0.3*	*0.4*	*0.5*
	N	2196	7766	9674	4557	4438	3753	4323	4549	3062	*NS*	*524*	*727*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	Rs	2.2	2.3	6.0	2.4	2.8	2.2	*1.7*	*1.8*	*1.7*	*NS*	*1.2*	*1.6*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

[a] Capacity factor is calculated from; $k' = (Rt - t_0)/t_0$, ideal $k' > 0.8$. [b] Plate number is calculated by the Gilson software, ideal $N > 2000$. [c] Resolution is calculated from; $Rs = 1.18 \times (t_2 - t_1) / (½W_1 + ½W_2)$ using the ½ height widths calculated by Gilson software, ideal $Rs > 2.0$. NS: Not separated. The values marked *red/italic* are outside the ideal range.

5. References

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