Instant and Quantitative Fat Amount Determination in Milk Quality Control Using a BODIPY Fluorescent Sensor-based Detector

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Materials and Methods

All the chemicals were purchased from Sigma Aldrich, Fluka, MERCK, Acros and Alfa Aesar and they were directly used without further purification. The milk samples were purchased from local supermarket and their parameters were listed below. Normal phase column chromatography purification was carried using MERCK silica Gel 60 (Particle size: 230-400 mesh, 0.040-0.063 mm). HPLC-MS was taken on an Agilent-1200 with a DAD detector and a single quadrupole mass spectrometer (6130 series). The analytical method, unless otherwise indicated, is A: H_2O (0.1% HCOOH), B: CH_3CN (0.1% HCOOH), gradient from 10 to 90% B in 10 minutes; C18 (2) Luna column (4.6 * 50 mm^2, 3.5 μm particle size). 1H-NMR, 13C-NMR and 19F-NMR spectra were recorded on Bruker Avance 300 NMR and 500 NMR spectrometers, and chemical shifts are expressed in parts per million (ppm) and coupling constants are reported as a J value in Hertz (Hz). Spectroscopy was performed using a fluorimeter and UV/Vis instrument, SpectraMax M2, Molecular Devices. All the data analysis was performed using GraphPrism 6.0 software.

Experimental Sections

Synthesis of Milk Orange (Scheme S1): BODIPY precursor 1' (25 mg, 60 µmol) and thiophene-2-carbaldehyde 2' (27 mg/21 µL, 240 µmol, 4 equiv.) were dissolved in 2.5 mL acetonitrile, with 4 equiv. of pyrrolidine (29 µL) and 4 equiv. of acetic acid (20 µL). The mixture was shaken at 95 ºC for 5 minutes, followed by immediate cooling down to 0 ºC. The resulting crude mixture was concentrated under vacuum and purified by flash column chromatography on silica gel (dichloromethane-methanol: 9.8:0.2) to afford 3' as a dark purple solid (12.5 mg, 24 µmol, 40% yield, 99.9% purity, Figure S3). 1H NMR (500 MHz, CDCl3) δ 7.47 (s, 1H), 7.46 (s, 1H), 7.43 (s, 1H), 7.38 – 7.32 (m, 2H), 7.06 (s, 1H), 6.88 (d, J = 3.9 Hz, 1H), 6.69 (s, 1H), 6.31 (d, J = 3.9 Hz, 1H), 4.78 (s, 2H), 3.39 (t, J = 7.5 Hz, 2H), 2.96 (t, J = 7.5 Hz, 2H), 2.30 (s, 3H). 13C NMR (126 MHz, CDCl$_3$) δ 171.03, 157.27, 155.73, 143.03, 139.38, 136.80, 133.75, 132.51, 127.24, 126.75, 126.38, 125.52, 122.12, 118.72, 116.66, 116.56, 94.92, 74.10, 31.92, 22.68, 11.38. HRMS (C21H18BCl3F2N2O2S): calc. $[M + Na]^+$: 539.0120, found $[M + Na]^+$: 539.0112; calc. $[M - F]^+$: 497.0236, found $[M-F]^+$: 497.0230.

Milk fat measurement procedures: In high-throughput screening approach, black Greiner 96-well plates were pre-filled with the fluorescence dyes from DOFL at 1 nmol quantity and were dissolved in 1 µL DMSO to make 1 mM solution. In each well, 98 µL DI water was added, mixed well and the plate image was taken one time as dye background. After taking image, 1 µL of milk sample was added into the well, mixed well and the plate image was taken again. Both images were imported into Adobe Photoshop program and compared side-by-side to grant discovery of potential hit compounds. In other non-plate tests such as cuvettes, 1 mM sensor solution was first prepared and diluted with DI water, followed by addition of milk (1% (v/v)). The samples were then inserted into SpectroMax M2 plate reader or measured by constructed fat detector. 365 nm UV excitation was applied in all image-based high-throughput screening whereas 530 nm excitation was used when measuring the spectra of Milk Orange.

Quantum yields measurement: Quantum yield for MO was measured by dividing the integrated emission area of their fluorescent spectrum against the area of Rhodamine B in DMSO excited at 500 nm ($\Phi_{\text{rho-B}} = 0.49$). Quantum yield was then calculated using equation (1), where F represents the integrated emission area of fluorescent spectrum, η represents the refractive index of the solvent, and Abs represents absorbance at excitation wavelength selected for standards and samples. Emission was integrated from 560 nm to 680 nm.

$$\Phi_{flu}^{sample} = \Phi_{flu}^{reference}\left(\frac{F^{sample}}{F^{reference}}\right)\left(\frac{\eta^{sample}}{\eta^{reference}}\right)\left(\frac{Abs^{reference}}{Abs^{sample}}\right)$$

19F NMR test: 18 mg MO compound was dissolved in 30 µL DMSO-d6 solvent and then divided into 5 equal aliquots, each aliquot diluted with D$_2$O to 500 µL. Its concentration was determined to be 14 mM. Milk fat component was extracted from fresh milk sample by dichloromethane and dried under vacuum. It was weighted and 2.5 mg, 5 mg, 10 mg and 20 mg fat was added into each

MO sample, respectively, thus making MO solutions containing 0.0%, 0.5%, 1.0%, 2.0% and 4.0% fat by mass. 19F NMR was taken on a Bruker DRX 300 NMR spectrometer to afford spectra of each sample, followed by analysis on MestReNova software.

Dynamic light scattering measurement: The dynamic radius of MO-milk samples was measured at 25℃ in deionized water using quartz cell. 1 mM DMSO solution of MO was slowly added to water (1% (v/v)) and gives 10 μM concentration. Milk samples were also added to the solution (1% (v/v)) and mixed well. All measurements were performed in triplicate in Zetasizer Nano ZS.

Construction of milk fat detector: each single piece of detector components were purchased separately. The Ultra High Power LED Light was bought from Prizmatix and emits bright white light covering 400-700 nm spectrum. The green light filter (520 +/- 5 nm) was purchased from ASAHI. Both optical fiber and the grating fluorescence detector were purchased from Ocean Optics. During measurement, just dipping the probe inside the sample, its spectra from 400-1100 nm could be instantly shown on the computer for analysis.

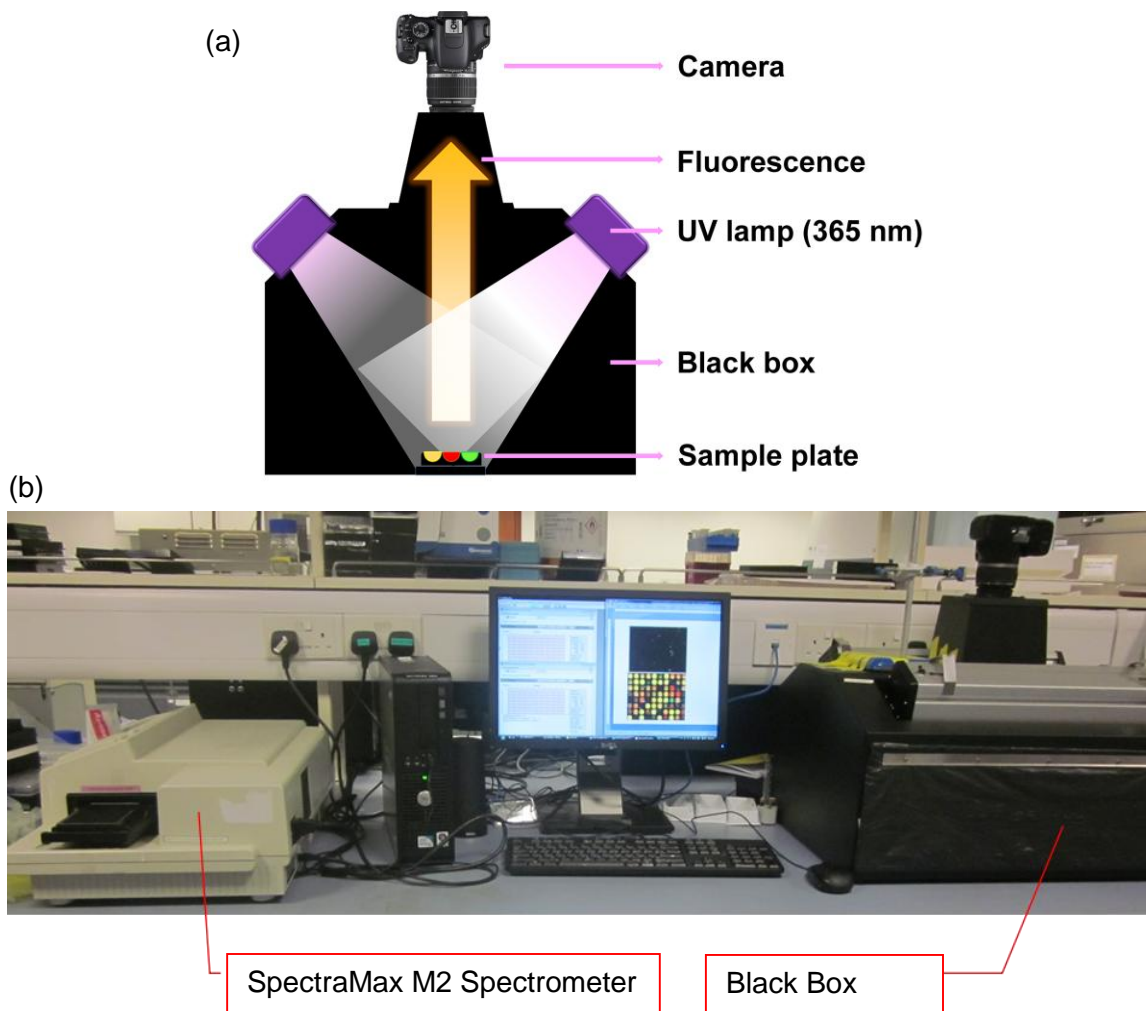**Diagram of Imaging Black Box and Hyper-Throughput Screening**

(a)

(b)

Figure S1. Diagram of imaging black box. (a) Diagram of each components in the black box. The black box is Spectroline Model CL-150 UV-fluorescence analysis cabinet. It is equipped with two UV lamps - 15W long wave tube of 365 nm (model: xx-15NF) – peak intensity of 550 µW/cm². The images are taken with Canon EOS 400D (Digital Rebel XTi). (b) The whole high-throughput screening and validation set. It is composed of image black box and the spectrometer machine.
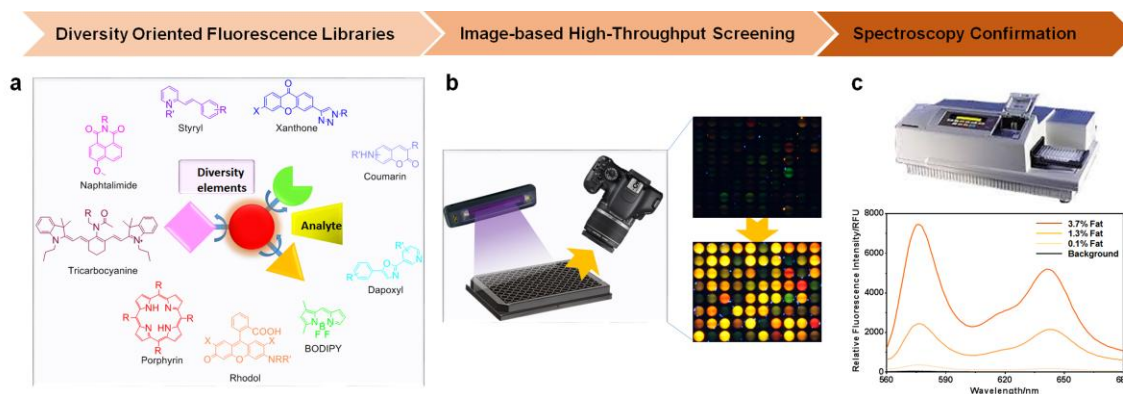
Figure S2. Scheme of hyper-throughput screening approach. (a) Construction of DOFL dyes based on fluorescence scaffolds of various emission colors and diversity elements. (b) Scheme of image-based screening. The plate is pre-filled with dyes, irradiated with a 365 nm UV lamp and taken image both before and after addition of milk samples. (c) Selected hit compounds are subjected to validation using a professional spectrometer. The milk fat sensor that shows best linearity and highest intensity change has been selected.
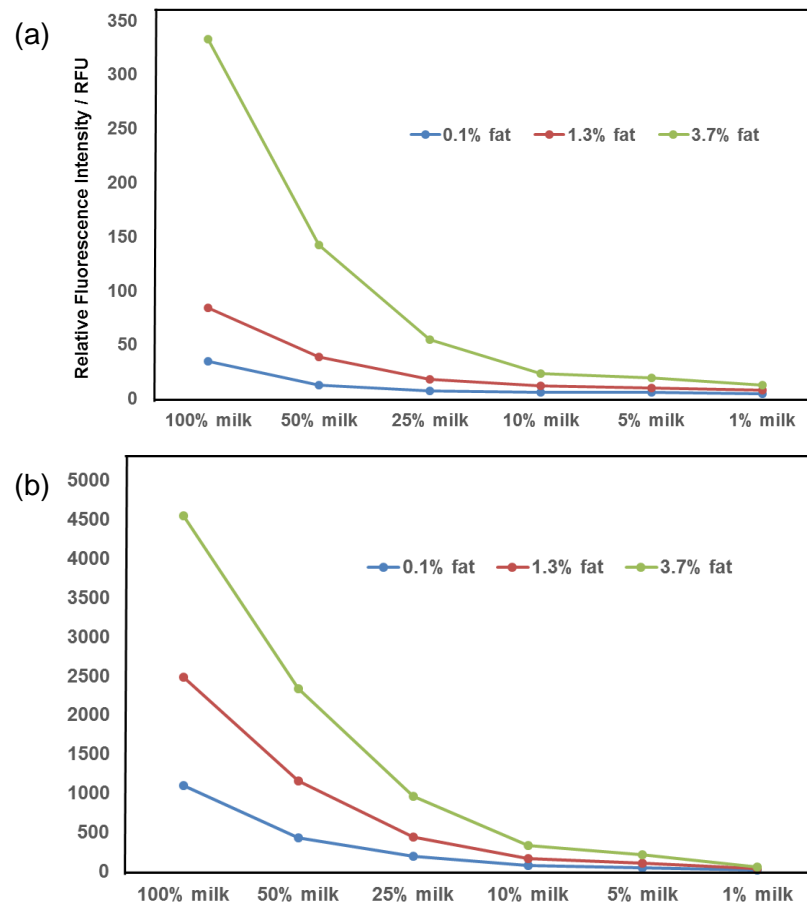
Milk Auto-fluorescence Background

(a)

(b)

Figure S3. Milk auto-fluorescence background excited at (a) 530 nm; (b) 365 nm.

MO Characterization
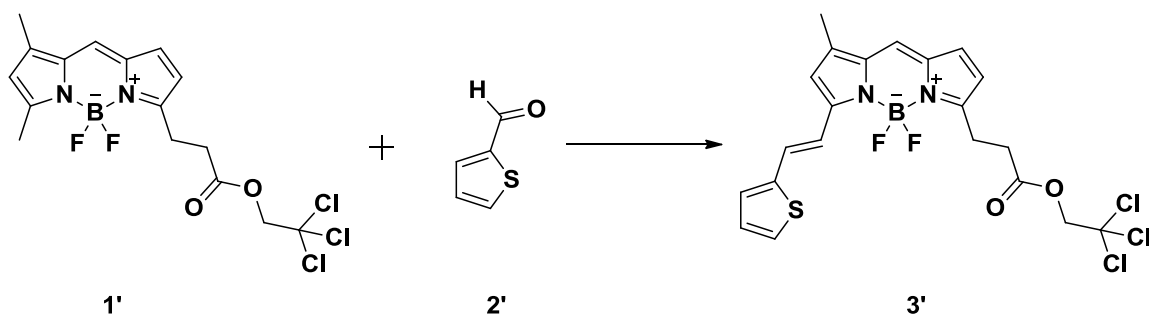
Scheme S1. Synthetic route to form **3'** (MO).

Figure S4. HPLC and HRMS characterization of MO. (a) chromatogram of MO. It indicates that MO purity is more than 99%. HPLC conditions: A: H_2O-HCOOH: 99.9:0.1. B: CH_3CN-HCOOH: 99.9:0.1; gradient 5% B to 95% B (10 min), isocratic 95% B (2.5 min). Reverse phase Phenomenex C18 Luna column (4.6 x 50 mm^2) 3.5μm, flow rate: 1.0 mL/min. (b) HRMS of MO.

MO Applicability Chart

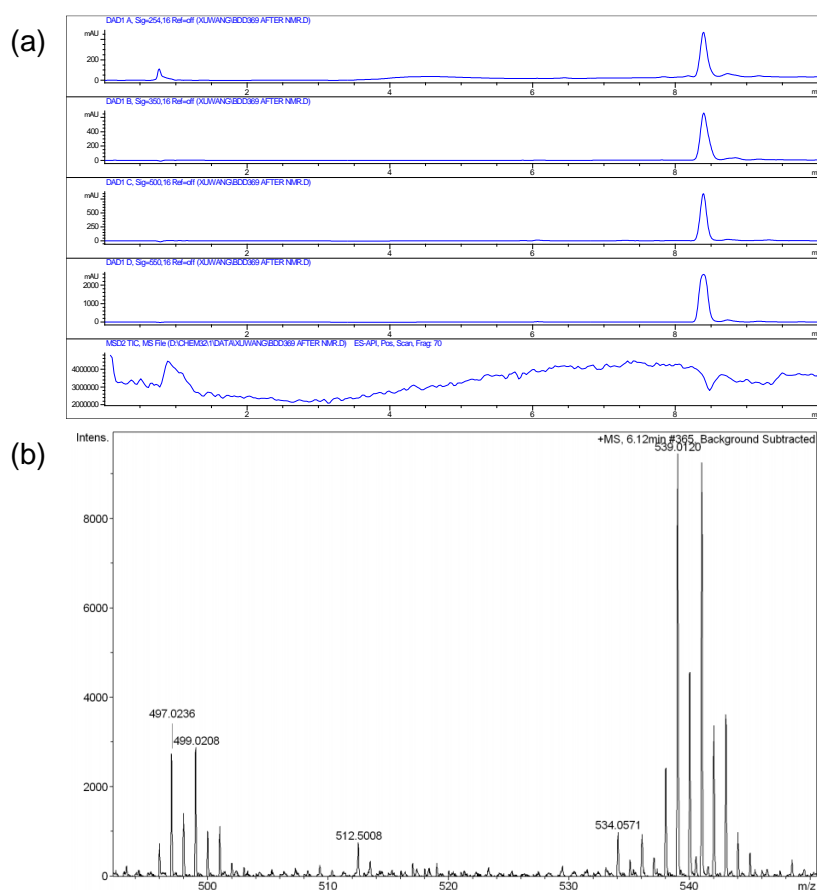Table S1. Comparison of fat concentrations acquired from fluorescence measurement and ingredient tables listed on milk package.

Brands of milk		Official report (g/100ml)			Fluorescence test (g/100ml)
		Protein	Carbohydrate	Fat	Fat
Greenfields®	Skim	3.9	4.2	0.1	0.10±0.01
	Low fat	4.0	3.9	1.3	1.31±0.01
	Fresh	4.0	3.9	3.7	3.69±0.02
Pura®	Skim	4.0	5.4	0.1	0.11±0.01
	Low fat	3.6	5.0	1.4	1.42±0.01
	Fresh	3.2	4.9	3.4	3.42±0.02
Meiji®	Skim	3.2	6.9	0.1	0.10±0.01
	Low fat	3.2	7.0	1.2	1.18±0.01
	Fresh	3.3	5.0	4.1	4.09±0.02
Farmhouse®	Low fat	4.0	5.5	1.5	1.52±0.01
	Fresh	3.2	4.8	3.8	3.80±0.02
Marigold®	Low fat	5.0	5.3	1.0	1.00±0.01
	Fresh	3.5	5.0	3.6	3.59±0.01
Magnolia®	Low fat	3.7	5.0	1.5	1.52±0.00
	Fresh	4.0	5.0	4.0	3.98±0.01
Daisy®	Low fat	3.5	5.2	1.0	1.00±0.01

MO Colour Change towards Milk Fat

(a)

(b)

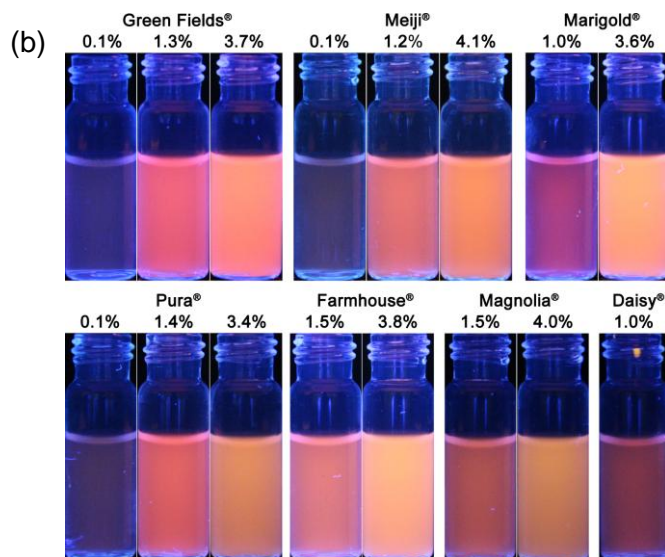Figure S5. (a) Dose-dependent image of MO-milk fat composites. Upper row is the milk fat concentration by mass. The image is taken in a Greiner U-shape 96 well black plate and the exposure time is 30s. MO concentration is 10 μM. (b) Image of MO towards various brands of milk under irradiation of UV lamp. Volume is 3 mL for each bottle and MO concentration is 10 μM. Exposure time is 0.2s in all cases.

Detailed Ingredients of All Milk Brands

Table S2. Greenfields ingredients.

Greenfields	Fresh milk		High Calcium Low fat		High Calcium Skimmed milk	
	Per serving	Per 100mL	Per serving	Per 100mL	Per serving	Per 100mL
Energy	163 kcal	65 kcal	110 kcal	44 kcal	82 kcal	33 kcal
Protein	10.1 g	4.0 g	10.1 g	4.0 g	9.8 g	3.9 g
Total fat	9.3 g	3.7 g	3.4 g	1.3 g	0.3 g	0.1g
-Trans Fat	0.0 g	0.0 g	0.0 g	0.0 g	0.0 g	0.0 g
-Saturated Fat	6.5 g	2.6 g	2.3 g	0.9 g	0.1 g	0.04 g
Cholesterol	~	~	11.4 mg	4.6 mg	0mg	0 mg
Carbohydrate	9.8 g	3.9 g	9.8 g	3.9 g	10.5 g	4.2 g
Sugars	8.5 g	3.4 g	7.8 g	3.1 g	7.8 g	3.1 g
Dietary Fibre	0.0 g	0.0 g	0.0 g	0.0 g	0.0 g	0.0 g
Sodium	89 mg	36 mg	86 mg	34 mg	95 mg	38 mg
Vitamin B1	0.15 mg	0.06 mg	0.16 mg	0.06 mg	0.16 mg	0.06 mg
Vitamin B2	0.34 mg	0.13 mg	0.31 mg	0.12 mg	0.31 mg	0.12 mg
Calcium	375 mg	150 mg	378 mg	151 mg	370 mg	148 mg
Iron	1.4 mg	0.6 mg	1.2 mg	0.5 mg	0.7 mg	0.3 mg
Phosphorus	191 mg	76 mg	197 mg	79 mg	188 mg	75 mg
Magnesium	23.5 mg	9.4 mg	24.3 mg	9.7 mg	23 mg	9 mg
Zinc	0.9 mg	0.4 mg	0.9 mg	0.4 mg	0.8 mg	0.3 mg
Potassium	313 mg	125 mg	331 mg	133 mg	334 mg	134 mg

Table S3. PURA ingredients.

PURA	Fresh milk		High Calcium Low fat		High Calcium Skimmed milk	
	Per 250ml serving	Per 100mL	Per serving	Per 100mL	Per serving	Per 100mL
Energy	156 kcal	63 kcal	118 kcal	47 kcal	97 kcal	39 kcal
Protein	8.0 g	3.2 g	9.0 g	3.6 g	10.0 g	4.0 g
Total fat	8.5 g	3.4 g	3.5 g	1.4 g	0.25 g	0.10 g
-Trans Fat	0.5 g	0.2 g	0.3 g	0.1 g	0.03 g	0.01 g
-Saturated Fat	5.5 g	2.2 g	2.3 g	0.9 g	0.15 g	0.06 g
Cholesterol	~	~	~	~	7.5 mgL	3.0 mg
Carbohydrate	12.2 g	4.9 g	12.5 g	5.0 g	13.5 g	5.4 g
Sugars	12.2 g	4.9 g	12.5 g	5.0 g	13.5 g	5.4 g
Sodium	110 mg	44 mg	115 mg	46 mg	125 mg	50 mg
Vitamin D	~	~	2.0 ug	0.8 ug	~	~
Vitamin B2	500 ug	200 ug	0.31 mg	0.12 mg	~	~
Calcium	295 mg	118 mg	500 mg	200 mg	350 mg	140 mg
Phosphorus	232 mg	93mg	197 mg	79 mg	~	~

Table S4. Meiji ingredients.

Meiji	Fresh milk		High Calcium Low fat		High Calcium Skimmed milk	
	Per serving	Per 100mL	Per serving	Per 100mL	Per serving	Per 100mL
Energy	140 kcal	70 kcal	103 kcal	52 kcal	83 kcal	41 kcal
Protein	6.6 g	3.3 g	6.4 g	3.2 g	6.4 g	3.2 g
Total fat	8.2 g	4.1 g	2.4 g	1.2 g	0.2 g	0.1 g
-Trans Fat	~	~	0.0 g	0.0 g	0.0 g	0.0 g
-Saturated Fat	4.8 g	2.4 g	1.8 g	0.9 g	0.2 g	0.1 g
Cholesterol	23.0 mg	11.5 mg	9 mg	5 mg	4 mg	2 mg
Carbohydrate	10.0 g	5.0 g	14.0 g	7.0 g	13.8 g	6.9 g
-Sugars	~	~	14.0 g	7.0 g	13.8 g	6.9 g
Dietary Fibre	0.0 g	0.0 g	0.0 g	0.0 g	0.0 g	0.0 g
Sodium	89.2 mg	44.6 mg	67 mg	33 mg	66 mg	33 mg
Calcium	218 mg	109 mg	302 mg	151 mg	374 mg	187 mg

Table S5. Farmhouse ingredients.

Farmhouse	Fresh milk		High Calcium Low fat	
	Per serving	Per 100mL	Per serving	Per 100mL
Energy	165 kcal	66 kcal	130 kcal	52 kcal
Protein	8.0 g	3.2 g	10.0 g	4.0 g
Total fat	9.5 g	3.8 g	3.8 g	1.5 g
-Trans Fat	0.5 g	0.2 g	0.5 g	0.2 g
-Saturated Fat	~	~	2.3 g	0.9 g
Cholesterol	~	~	13 mg	5 mg
Carbohydrate	12.0 g	4.8 g	13.8 g	5.5 g
Dietary Fibre	~	~	0.0 g	0.0 g
Sodium	89.2 mg	44.6 mg	123 mg	49 mg
Calcium	300 mg	120 mg	500 mg	200 mg

Table S6. Magnolia ingredients.

F&N Magnolia	Fresh milk		High Calcium Low fat	
	Per serving	Per 100mL	Per serving	Per 100mL
Energy	180 kcal	72 kcal	120 kcal	48 kcal
Protein	10.0 g	4.0 g	9.3 g	3.7 g
Total fat	10.0 g	4.0 g	3.8 g	1.5 g
-Trans Fat	0.5 g	0.2 g	0.5 g	0.2 g
-Saturated Fat	~	~	2.3 g	0.9 g
Cholesterol	~	~	13 mg	5 mg
Carbohydrate	12.5 g	5.0 g	12.5 g	5.0 g
Dietary Fibre	~	~	0.0 g	0.0 g
Sodium	89.2 mg	44.6 mg	123 mg	49 mg
Calcium	300 mg	120 mg	500 mg	200 mg
Vitamin D3	~	~	1.7 mcg	0.7 mcg

Table S8. Marigold fresh ingredients.

MARIGOLD HL	High Calcium Low fat Low in Lactose with 9 Vitamins	
	Per serving	Per 100mL
Energy	100 kcal	50 kcal
Protein	10 g	5 g
Total fat	2.0 g	1.0 g
-Trans Fat	0.06 g	0.03 g
-Saturated Fat	1.4 g	0.7 g
Cholesterol	8 mg	4mg
Carbohydrate	10.6 g	5.3 g
-Lactose	2.4 g	1.2 g
Dietary Fibre	0.0 g	0.0 g
Sodium	152 mg	76 mg
Calcium	400 mg	200 mg
Vitamin A	375 mcg	188 mcg
Thiamine (Vit B1)	1 mg	0.5 mg
Pyridoxine (Vit B6)	2 mg	1 mg
Vitamin C	30 mg	15 mg
Vitamin D3	2.5 mcg	1.3 mcg
Vitamin E	6 mg	3 mg
Vitamin K1	27 mcg	13.5 mcg
Niacin	11 mg	5.5 mg
Pantothenic Acid	7 mg	3.5 mg

Table S7. Marigold fresh ingredients.

MARIGOLD	Fresh milk 100% fresh	
	Per serving	Per 100mL
Energy	134 kcal	67 kcal
Protein	7.0 g	3.5 g
Total fat	7.2 g	3.6 g
-Trans Fat	0.2 g	0.1 g
-Saturated Fat	4.8 g	2.4 g
Carbohydrate	10.0 g	5.0 g
Sodium	70 mg	35 mg
Calcium	240 mg	120 mg

Table S9. Daisy ingredients.

F&N Daisy	99% Fat Free	
	Per serving	Per 100mL
Energy	110 kcal	44 kcal
Protein	8.8 g	3.5 g
Total fat	2.5 g	1.0 g
-Trans Fat	0.13 g	0.05 g
-Saturated Fat	1.3 g	0.5 g
Cholesterol	8 mg	3 mg
Carbohydrate	13.0 g	5.2 g
-Sugars	9.3 g	3.7 g
-Lactose	6.0 g	2.4 g
Dietary Fibre	0.0 g	0.0 g
Sodium	113 mg	45 mg
Calcium	500 mg	200 mg
Vitamin A	470 ug	188 ug
Thiamine (Vit B1)	1.3 mg	0.5 mg
Nicotamide (Vit B3)	10.0 mg	4.0 mg
Pantothenic Acid (Vit B5)	8.8 mg	3.5 mg
Pyridoxine (Vit B6)	2.5 mg	1 mg
Vitamin C	38.0 mg	15.0 mg
Vitamin D3	3.3 ug	1.3 ug
Folate	250 ug	100 ug
Vitamin K	35 ug	14 ug

Milk Fat Detector Parts
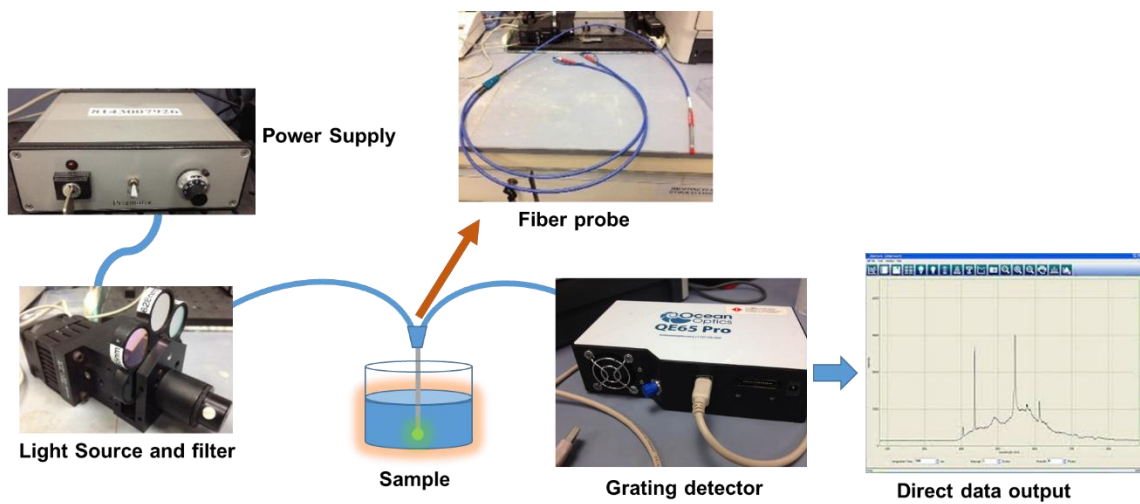
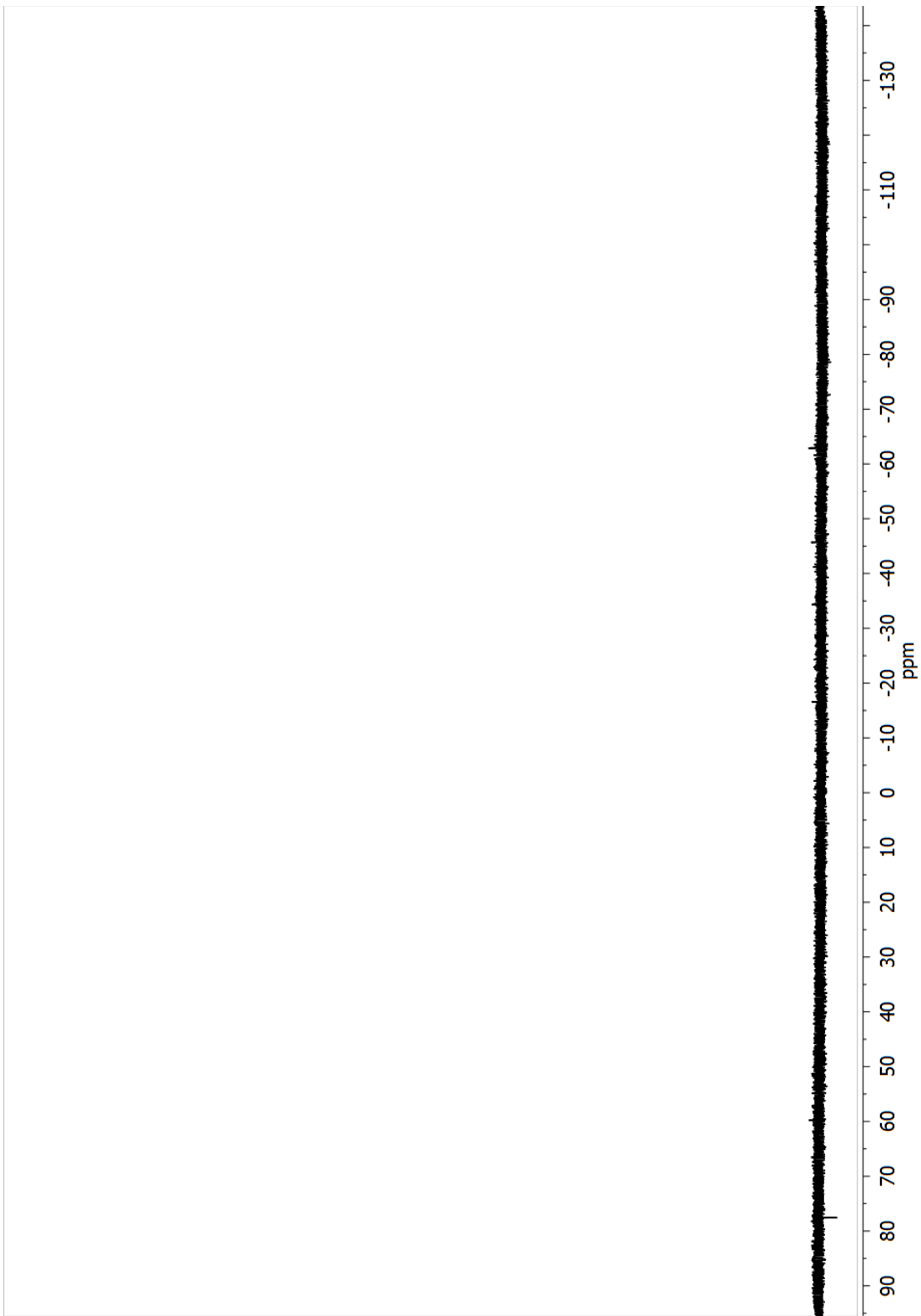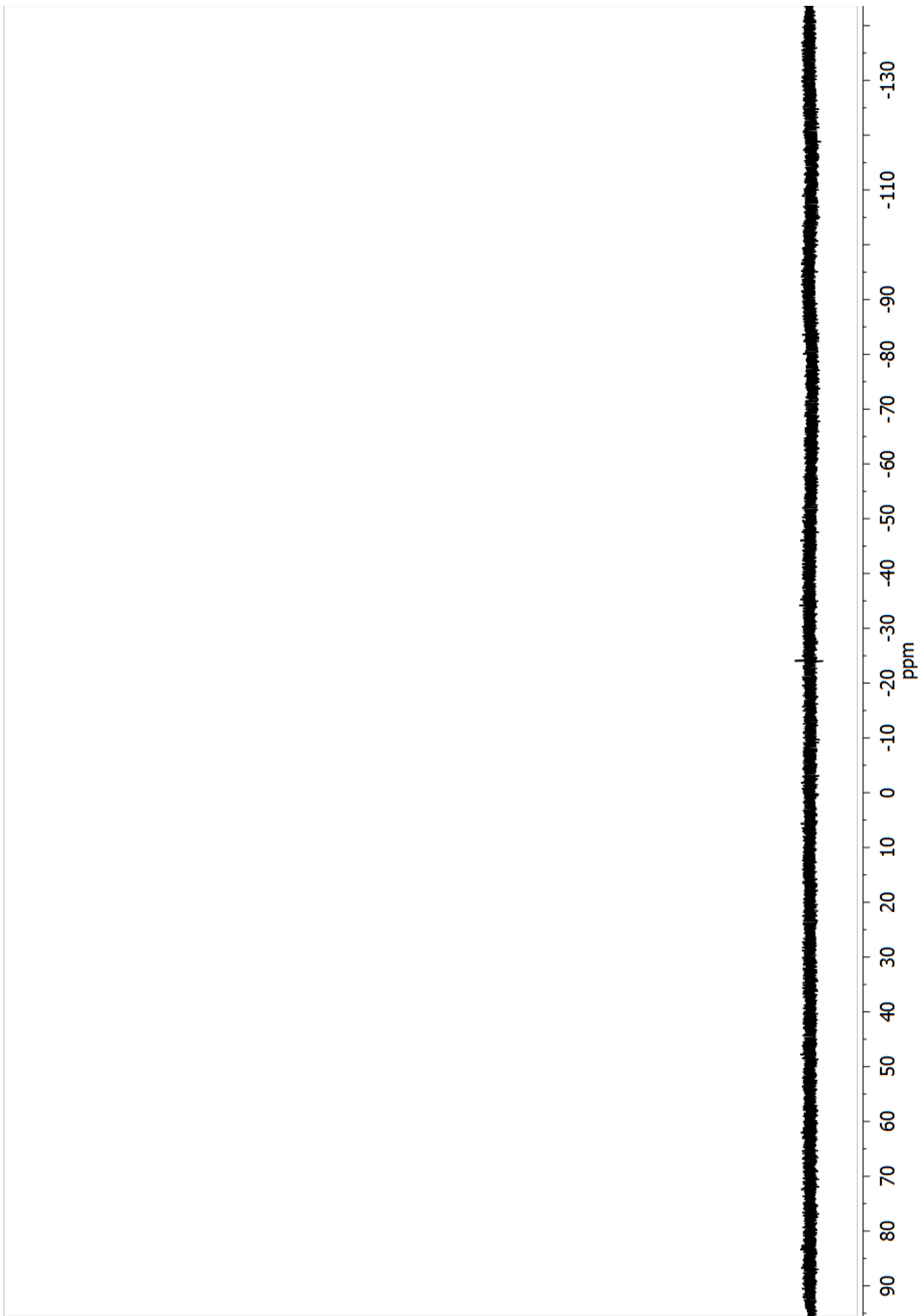Figure S6. Scheme of each component of the milk fat detector.

NMR spectra

1H NMR spectra of MO in CDCl₃.
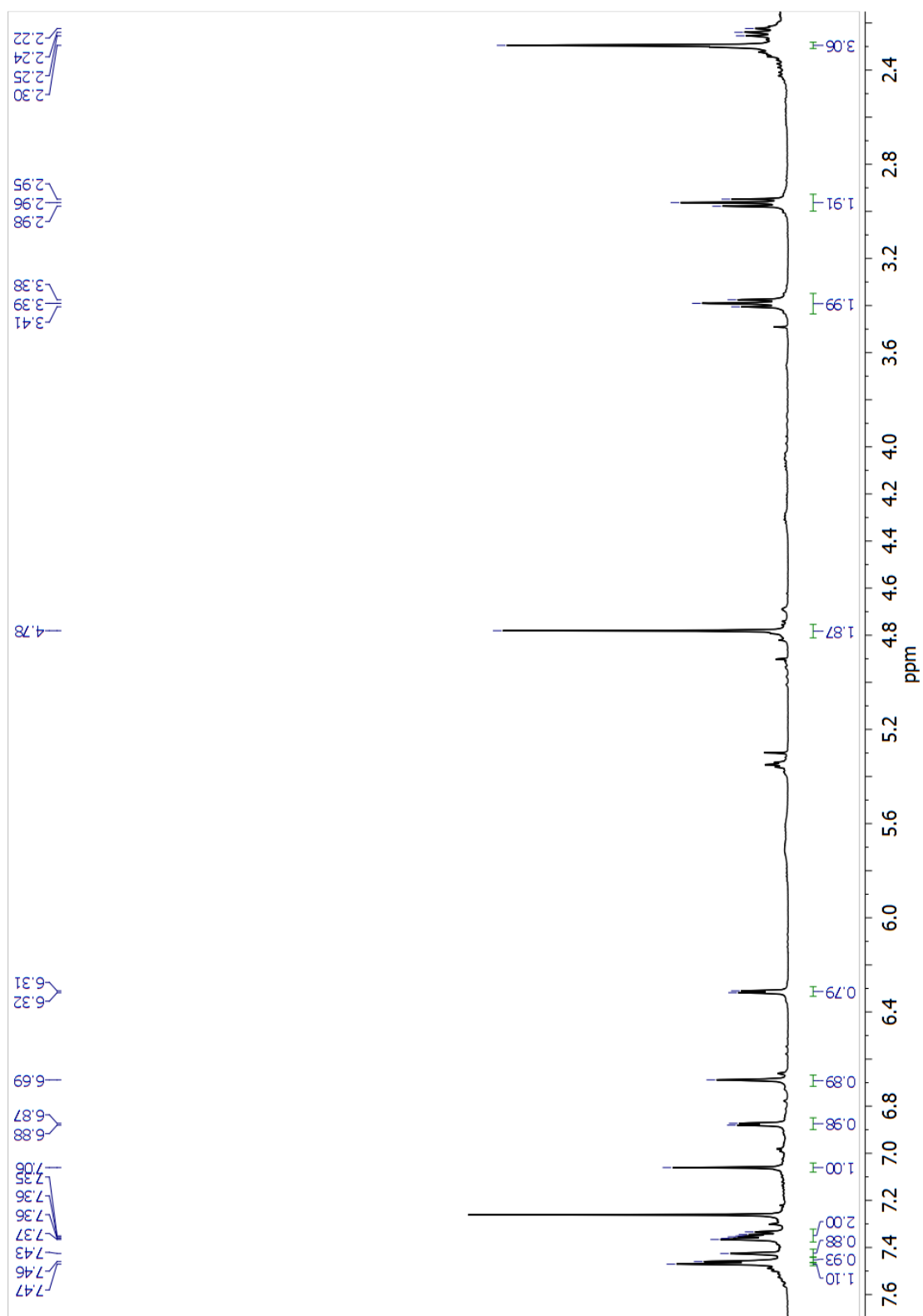
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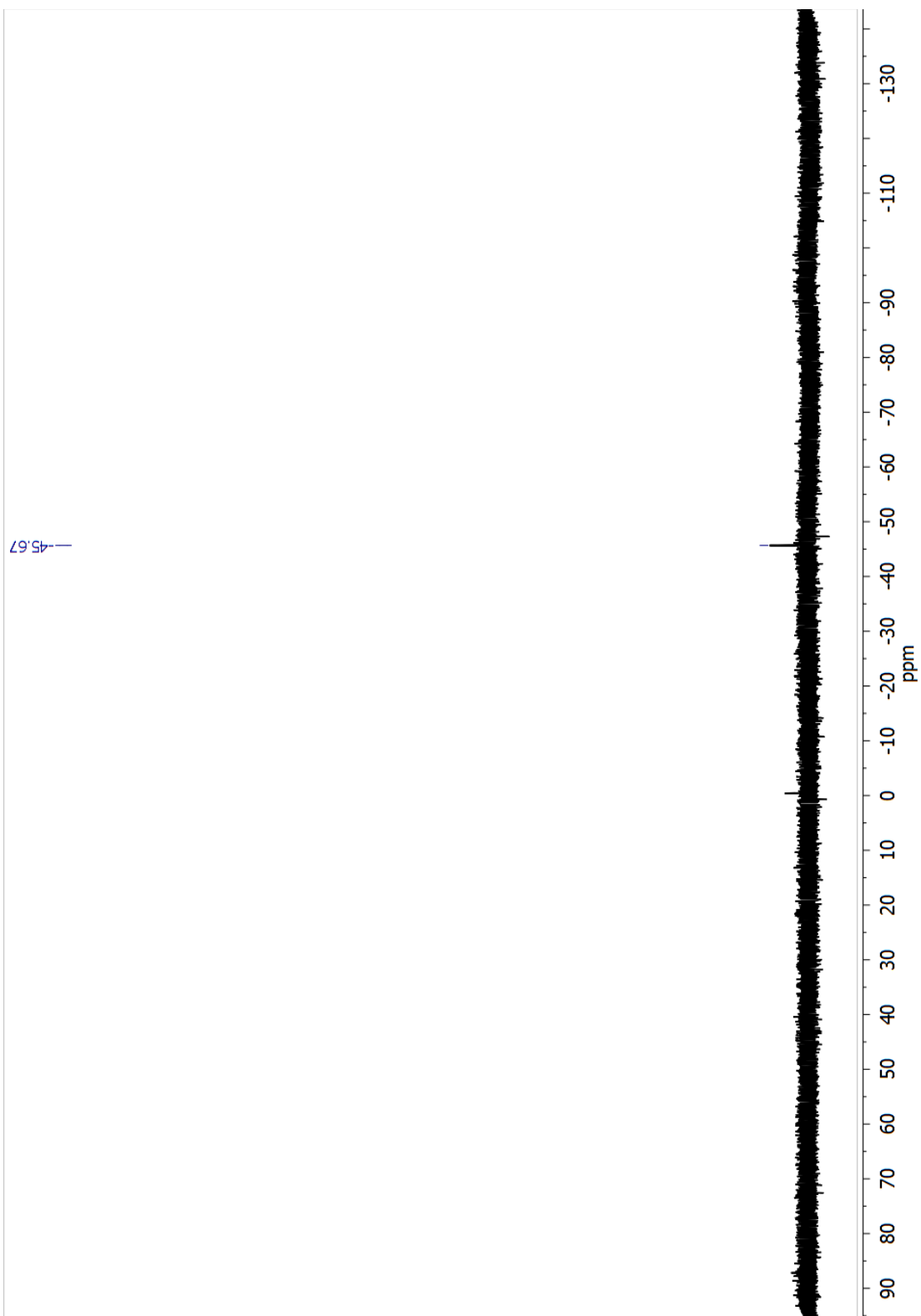126.75
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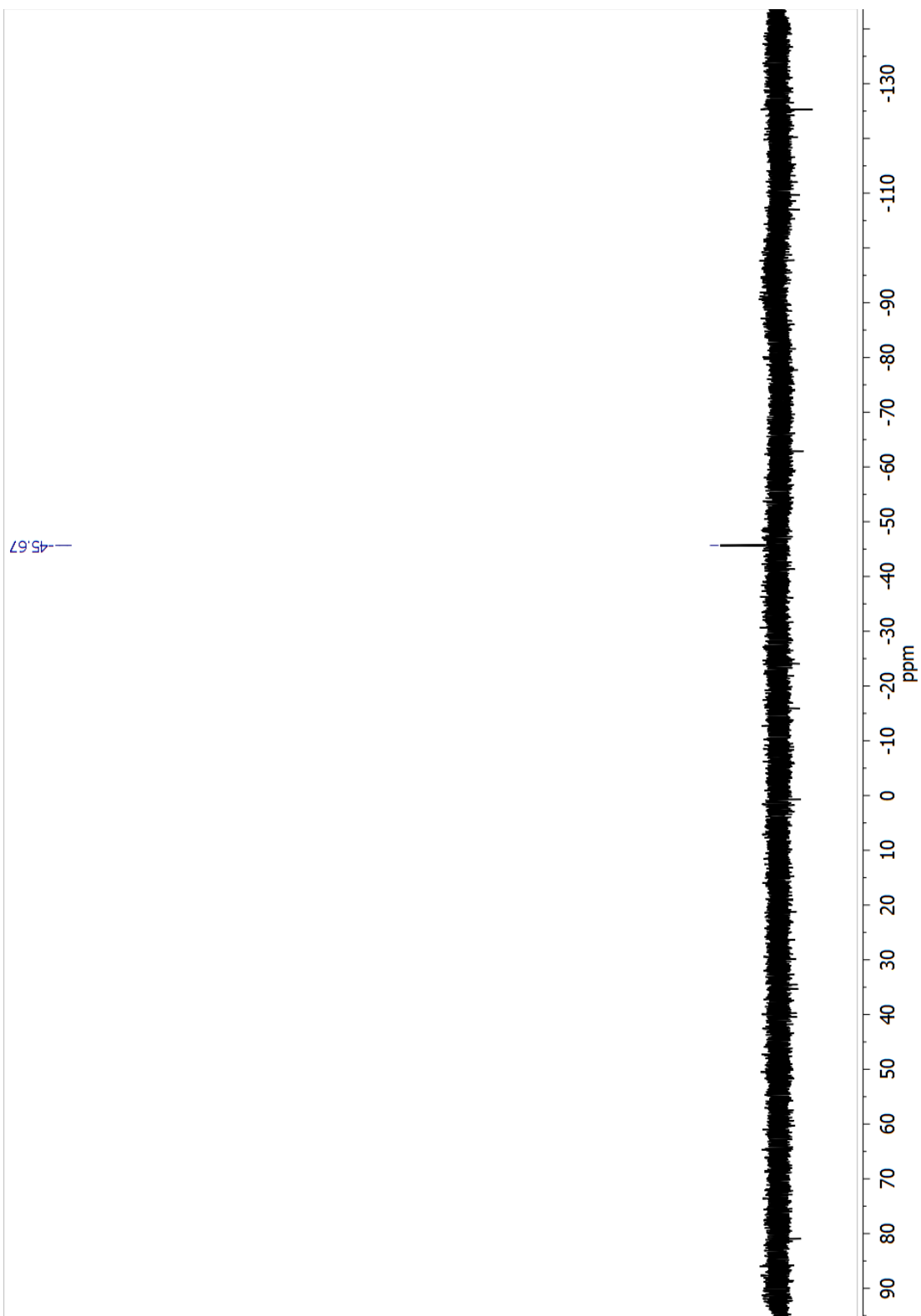143.03
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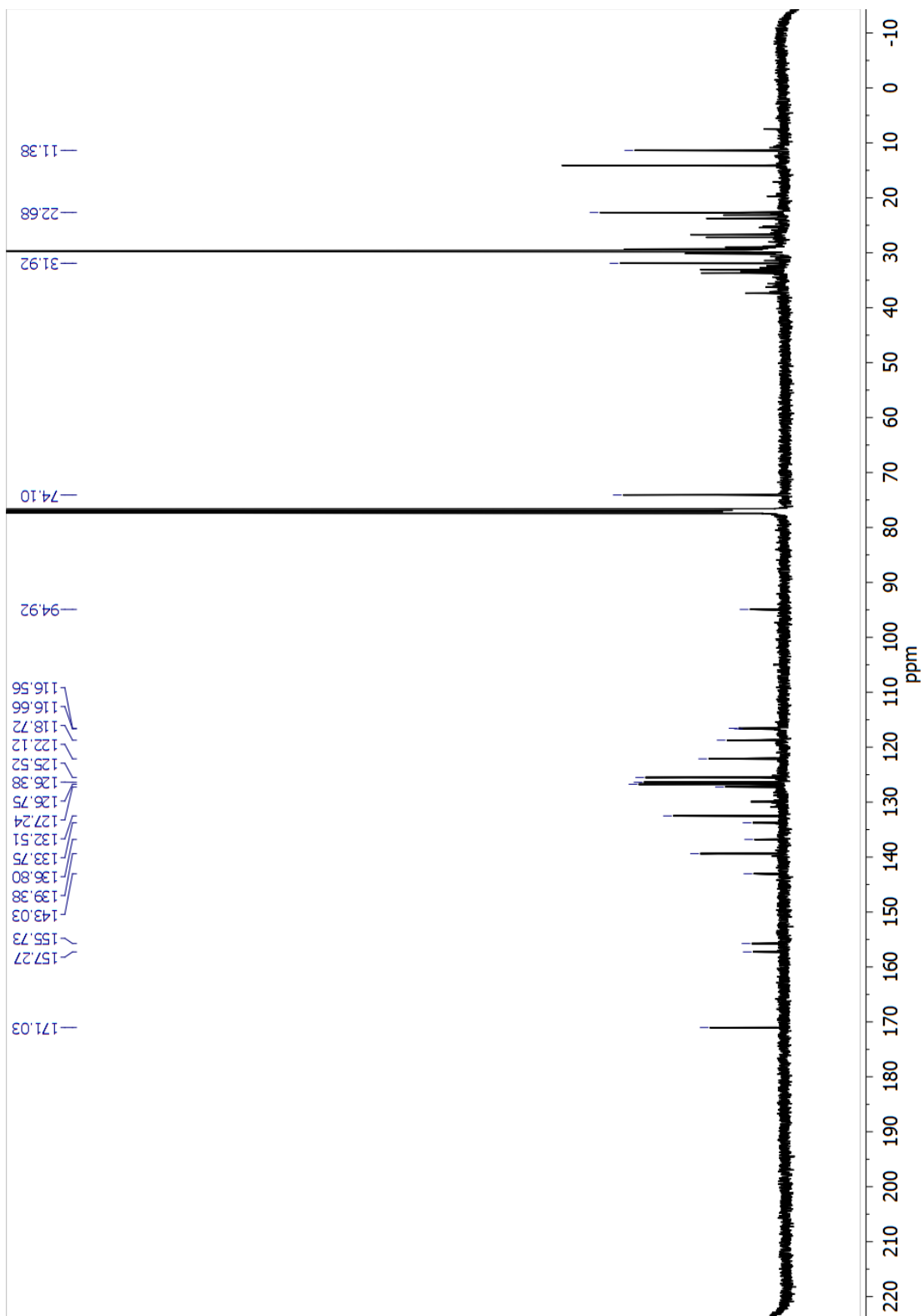
13C NMR spectra of MO in CDCl₃.

19F NMR of MO in D$_2$O (1% DMSO-d6) with no fat.
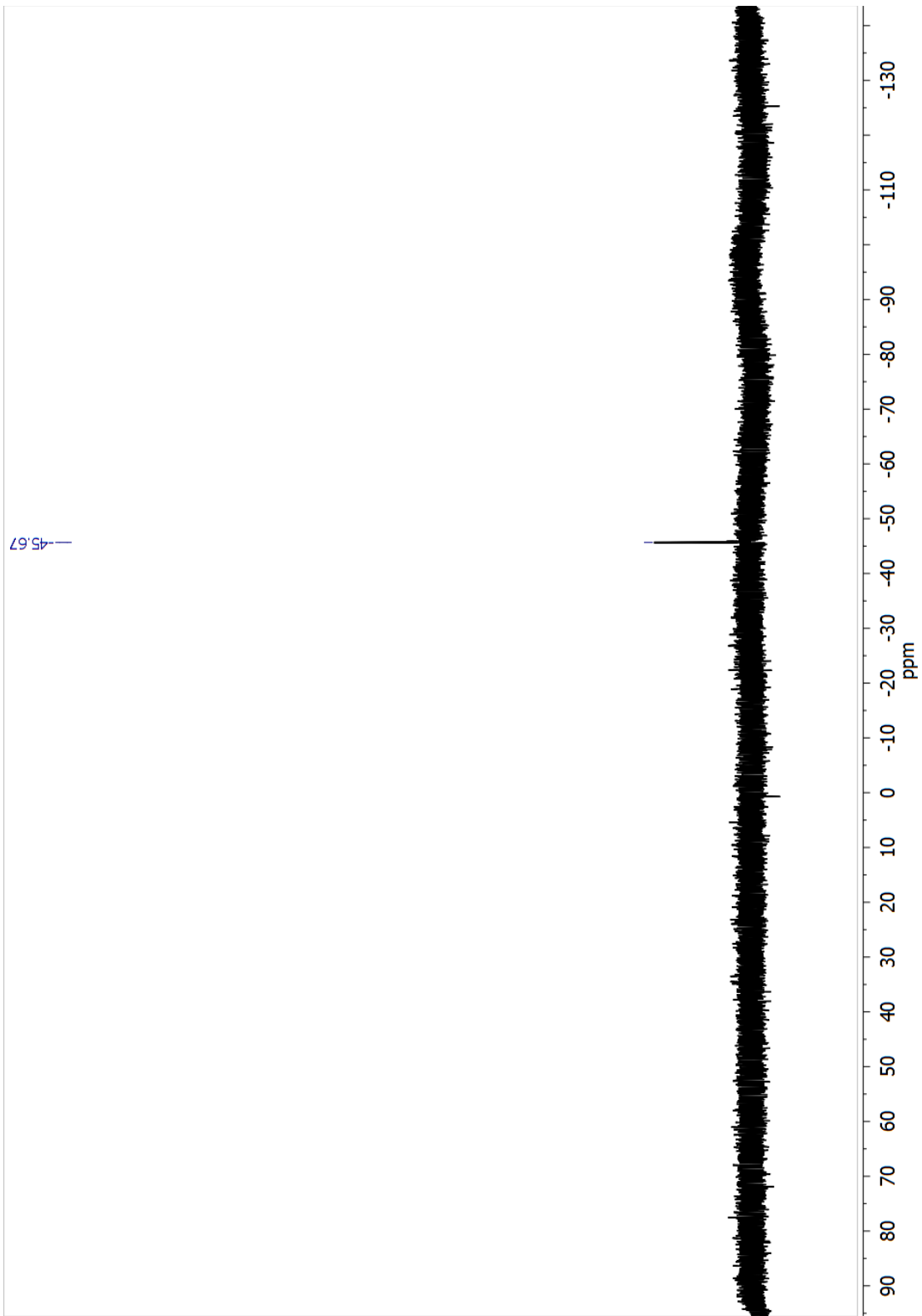
19F NMR of MO in D$_2$O (1% DMSO-d6) with 0.5% fat.

19F NMR of MO in D$_2$O (1% DMSO-d6) with 1.0% fat.

19F NMR of MO in D$_2$O (1% DMSO-d6) with 2.0% fat.

19F NMR of MO in D$_2$O (1% DMSO-d6) with 4.0% fat.