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Free Fatty Acids / LCMS-2050 New Generation Single Quadrupole MS

Application News

A Novel LC-MS Method for Direct Analysis of Mid-Long Chain Free Fatty Acids

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User Benefits

- A direct LC-MS method was established for the separation and quantitation of free fatty acids on LCMS-2050.
- A phenyl column was adopted for effective separation of eleven representative mid-long chain free fatty acids, i.e., saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and poly-unsaturated fatty acid (PUFA).

■ Introduction

The determination of composition of free fatty acids (FFAs) in food plays an important role in quality control. FFAs are naturally present in many raw ingredients, such as cocoa beans and milk, but high levels of them are responsible for rancidity and unacceptable for sale or consumption [1,2]. While extensive researches have been carried out to analyse FFAs on GC-FID and GC-MS, LC-MS offers an advantage over GC method in that FFAs can be analysed directly without an additional derivatisation step. Most LC-MS analyses published are performed on LC-MS/MS with C18 column. However, many FFAs are not fragmented steadily to give high MRM sensitivity due to the nature of the stable structures. In this application news, we present a SIM mode method to analyse eleven representative mid-long chain free fatty acids, i.e., saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and poly-unsaturated fatty acid (PUFA) (Fig. 1) using a phenyl column on LCMS-2050.

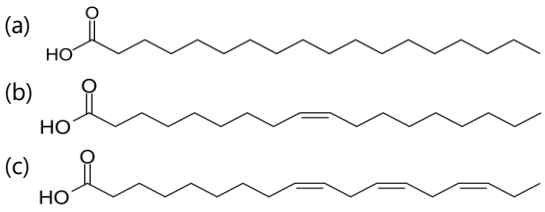

Figure 1. Representative structures of fatty acids, (a) saturated FA (SFA), (b) monounsaturated fatty acid (MUFA) and (c) polyunsaturated fatty acid (PUFA).

■ Experimental

Reagents and chemicals

Acetonitrile (LCMS grade) was obtained from commercial suppliers. Ammonium formate (>99%) of LCMS grade was used as an additive in the mobile phase prepared from Milli-Q water.

Standards preparation

Eleven FFA standards, Lauric acid (C12:0), Myristic acid (C14:0), Palmitic acid (C16:0), Stearic acid (C18:0), Arachidic acid (C20:0), Palmitoleic acid (C16:1), Oleic acid (C18:1), Eicosenoic acid (C20:1), Erucic acid (C22:1), Linoleic acid (C18:2) and Linolenic acid (C18:3) were purchased from commercial suppliers. Stock solutions of 1.0 mg/mL (1000 ppm) were prepared by weighing 15 mg of each standard and dissolving in methanol (MS grade). The stock solutions were used to prepare an intermediate solution containing a mixture of the eleven FFAs by diluting with mixture solvent of Milli-Q water and MeOH (50:50) to get a concentration of 1 ppm. The 11-mix solution was then diluted with a 50:50 (v/v) mixture of mobile phase A and B to obtain the calibration series of 10, 20, 50, 100, 150 and 200 ppb.

Table 1. Analytical conditions on LCMS-2050

LC Conditions	
Column	Shim-pack™ GIST Phenyl (2.1 X 100 mm, 2 µm), P/N: 227-30207-04
Flow Rate	0.5 mL/min
Mobile Phase	A: 2 mM Ammonium Formate in water B: Acetonitrile
Elution mode	B%: 40% (0~0.5 min) → 60% (7~7.5 min) → 40% (7.6 min) → Stop (11.5 min)
Oven Temp.	40°C
Injection Vol.	10 µL
Interface Conditions (LCMS-2050)	
Interface	DUIS (Corona needle off), negative mode
Interface Temp.	150°C
DL Temp.	250°C
Heat Block Temp.	400°C
Nebulizing Gas	2.0 L/min
Heating Gas Flow	7.0 L/min
Drying Gas Flow	5.0 L/min
Data acquisition (SQ)	
MS Mode	SIM
Dwell time	0.050 sec / event
Loop time	0.55 sec / data point

■ Results and Discussion

Optimization for LC separation of fatty acids

Mid-long chain fatty acids are retained relatively strong on C18 columns due to their high hydrophobicity nature. As a result, high concentrations of up to 95% of the organic mobile phase (acetonitrile) was needed to elute the 11 fatty acids. In addition, a high SIM baseline was observed for *m/z* 331.3, which corresponds to FA C20:0. This was suspected to be due to column bleeding under the very high organic phase condition (>90%).

Instead of a C8 column adopted in fatty acids analysis by LC-MS/MS [1], a phenyl column was found to be more appropriate in retaining and separation of the 11 fatty acids studied. An optimized gradient program was adopted with the organic mobile increasing from 40% to 60% and all the compounds eluted out within 7 min (Fig. 2a). The retention times (RT) of the fatty acids are listed in Table 2.

The additive (ammonium formate) in mobile phase A (aqueous) was found to affect the peak intensity significantly. In the absence of the additive, the fatty acids were not detected, indicating that ionization of fatty acids requires the presence of ammonium formate. The optimal concentration of ammonium formate in mobile phase A under this condition was found to be 1~2 mM. Further increase of the concentration would lead to a decrease in peak intensity.

Table 2. Separation and detection of 11 fatty acids on a Phenyl column by SIM (negative) mode with LCMS-2050

Fatty Acid	Formula	Exact Mass	$[M-H]^-$	RT (min)
C12:0	$C_{12}H_{24}O_2$	200.2	199.2	1.63
C14:0	$C_{14}H_{28}O_2$	228.2	227.2	2.53
C16:0	$C_{16}H_{32}O_2$	256.2	255.2	3.26
C16:1	$C_{16}H_{30}O_2$	254.2	253.2	3.40
C18:0	$C_{18}H_{36}O_2$	284.3	283.3	4.84
C18:1	$C_{18}H_{34}O_2$	282.3	281.3	4.28
C18:2	$C_{18}H_{32}O_2$	280.2	279.2	3.73
C18:3	$C_{18}H_{30}O_2$	278.2	277.2	3.65
C20:0	$C_{20}H_{40}O_2$	312.3	311.3	6.04
C20:1	$C_{20}H_{38}O_2$	310.3	309.3	5.42
C22:1	$C_{22}H_{42}O_2$	306.3	337.3	6.57

LCMS chromatograms of fatty acids

The LCMS chromatograms of the 11 fatty acids shown in Fig. 2a can be displayed separately according to the type of fatty acids, i.e., SFA (Fig 2b), MUFA (Fig. 2c) and C18 fatty acids (Fig 2d). The relationship between RT and carbon number are plotted in Fig. 3. For SFAs and MUFAs, the retention time increases with the number of carbons almost linearly on the phenyl column under the LC conditions. The adverse effect of the double bond in the hydrocarbon chain of the fatty acids on retention time is obvious, as seen from the results of the C18 fatty acid series.

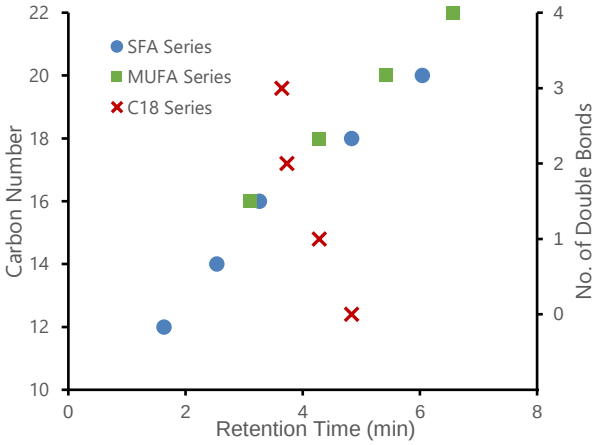

Figure 3. SFA and MUFA series show the relationship between retention time of FAs and their carbon number. C18 series shows relationship between retention time and number of double bonds.

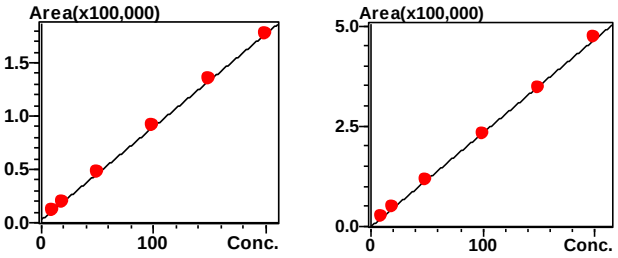

Figure 4. Calibration curves of C18:1 (left) and C22:1 (right)

Quantitation of fatty acids in SIM mode

A 6-point calibration curve was generated for each FFA using mixed standards of 10, 20, 50, 100, 150 and 200 ppb. The calibration curves of the eleven FFAs were linear with coefficient of determination (r^2) values of 0.99

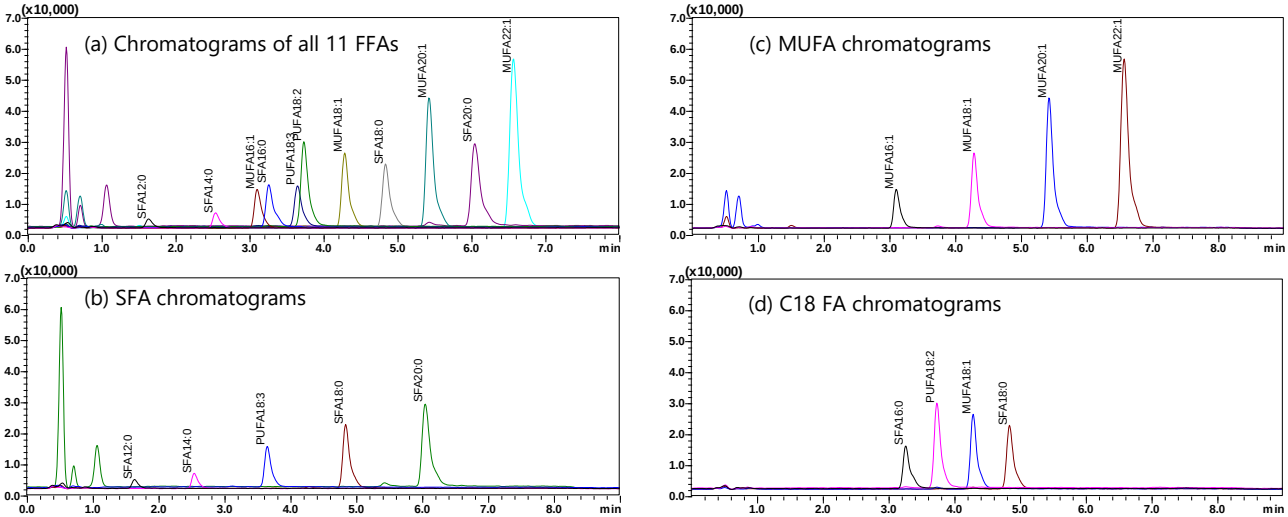

Figure 2. Chromatograms of FFA separation on LCMS (200 ppb each in a mixture). (a) 11 FFAs; (b) SFAs (DB=0); (c) MUFA (DB=1) and (d) C18 FFAs (BD=, 0, 1, 2 and 3).

Table 3. Calibration curve linearity, range, LOD and LOQ of eleven free fatty acids

Fatty Acid	Conc. Range (ppb)	Linearity (r^2)	LOQ	LOD (ppb)
C12:0	20 - 200	0.9999	33.9	11.2
C14:0	10 - 200	0.9996	17.1	5.7
C16:0	10 - 200	0.9997	8.7	2.9
C16:1	10 - 200	0.9997	11.4	3.8
C18:0	10 - 200	0.9968	1.8	0.6
C18:1	10 - 200	0.9999	6.5	2.1
C18:2	10 - 200	0.9999	6.8	2.3
C18:3	10 - 200	0.9890	3.4	1.1
C20:0	10 - 200	0.9998	8.8	2.9
C20:1	10 - 200	0.9993	3.8	1.3
C22:1	10 - 200	0.9997	2.3	0.8

or higher. Figure 4 shows the calibration curves of FFA C18:1 and FFA C22:1. Table 3 shows the concentration ranges of the calibration curves and the r^2 values. The estimated LOD and LOQ of the FFA are obtained from S/N ratios from the lowest calibration levels. Repeatability was evaluated with six repeated analyses at 20 ppb and 100 ppb. Table 4 shows the %RSD values obtained for retention time and peak area.

■ Conclusion

This novel LCMS method adopting a phenyl column is fast and sensitive in the separation and quantitation of mid-long chain free fatty acids (FFAs). Clear patterns of relationship between retention time and the length of hydrocarbon chain as well as the degree of unsaturation were established. Quantitative results also show that the determination of trace amounts of FFAs is possible on LCMS-2050, a new generation of single quadrupole mass spectrometer.

Table 4. Summary of %RSD values of repeatability testing with mixed standards (n = 6)

Fatty Acid	RSD (%) Retention time	RSD (%) Peak Area	
		20 ppb	100 ppb
C12:0	0.58	7.47	3.94
C14:0	0.30	9.29	4.83
C16:0	0.21	7.37	3.73
C16:1	0.35	3.72	3.16
C18:0	0.23	3.86	2.77
C18:1	0.19	1.77	2.09
C18:2	0.20	3.92	2.58
C18:3	0.09	3.69	3.07
C20:0	0.20	6.82	1.66
C20:1	0.14	2.25	2.14
C22:1	0.16	1.54	0.82

■ References

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2. Kokotou, M. G.; Mantzourani, C.; Kokotos, G. Development of a Liquid Chromatography–High Resolution Mass Spectrometry Method for the Determination of Free Fatty Acids in Milk. *Molecules* **2020**, *25* (7), 1548.

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