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Prediction of relative retention times of triacylglycerols in non-aqueous reversed-phase high-performance liquid chromatography

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Abstract

Accurate and simple methods for prediction of relative retention times of triacylglycerols in non-aqueous reversed-phase HPLC are presented here. The prediction is based on a simple calculation using the experimental relative retention time (min) of the triacylglycerol with the closest corresponding structure and taking into account the contributions (min) of functional groups present in the triacylglycerol for which the standard is not available to predict the relative retention time. The contribution of functional groups on the fatty acyl in the *sn*-2 position were greater than those at the *sn*-1(3) position, especially for polar functional groups such as the hydroxy group of ricinoleate. Another method of prediction which is based on the experimental relative retention time of tristearin and disregards the difference of *sn*-1(3) and *sn*-2 is also presented here.

Keywords: Relative retention times; Retention prediction; Triacylglycerols

1. Introduction

Triacylglycerols in living systems are complex mixtures and their analyses are tedious and usually require high-performance liquid chromatographic separation. We have recently reported the non-aqueous reversed-phase high-performance liquid chromatography (HPLC) of 45 synthetic triacyglycerols and diacylglycerols and their relative retention times (RRTs) [1]. This HPLC system allowed the simultaneous detection by UV at 205 nm and flow-through liquid scintillation counting, and we have used it to follow fatty acid metabolism and incorporation into triacylglycerols [2]. The eluent used in this HPLC system, methanol–isopropanol, is the least toxic and least hazardous environmentally among the eluents that have been used for the HPLC separation of triacylglycerols. The elution order of triacylglycerols corresponded closely with chain length, degree of unsaturation and presence of polar groups on fatty acid chains, and was similar to the order of elution for fatty acids on methanol–water elution of C_{18} HPLC which we reported [3]. Since it appeared from the elution characteristics of triacylglycerols reported [1] that there was the possibility to predict the RRTs of triacylglycerols based on fatty acid composition, we have extended that study to test the possibility.

The prediction of RRTs of triacylglycerols provides valuable information for the identification of radioactive metabolites when it is known what the

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likely structures are. This predictive value includes useful information for the tentative identification of triacylglycerols in complex mixtures of lipids in living systems, such as the prediction of possible components of a HPLC peak. Methods used to predict the RRTs of triacylglycerols on reversed-phase HPLC have been reviewed [4–6]. They included partition number (PN) [7], equivalent carbon number (ECN) [8], theoretical carbon number (TCN) [9], and matrix model [10]. Wada et al. [7] showed that the elution of triacylglycerols in reversed-phase HPLC was according to ascending order of PN and PN=CN (carbon number)−2ND (number of double bonds). Herslof et al. [8] estimated ECN on the basis of the linear relationship between RRT and the total number of carbons (CN) in saturated triacylglycerols. ECN=CN−a'ND, where a' is determined experimentally, close but not equal to 2. El Hamdy and Perkins [9] introduced the correction of ECN as TCN=ECN−(ΣU_i). Here ΣU_i is the sum of experimentally determined increments for each of the three fatty acid residues. Goiffon et al. [11] estimated the RRTs of a mixed acid triacylglycerol assuming it was the sum of one third of the RRTs of the respective monoacid triacylglycerols. Stolyhwo et al. [12] estimated the RRT as: log RRT=$A+B(CN_a+CN_b+CN_c)$. Here, A is the contribution of the functional group(s), B is the contribution to the retention due to the increase of a chain length by one methylene unit, ($CN_a+CN_b+CN_c$) is the total carbon number of the three fatty acid chains. Takahashi et al. [10] used a matrix model which accounted separately for the chain length and for the number of double bonds of each fatty acid chain. None of the previous models have taken into account the minor factors affecting the RRTs such as *sn* positions, positions of double bonds and/or configurations of double bonds. In addition, the RRTs of triacylglycerols with fatty acid chain containing hydroxy-, keto-, epoxy group, triple bond, cyclopropenyl- or cyclopropanyl group has not been estimated previously. Since the accuracy of prediction is important for identification purposes, we introduce here new empirical methods to estimate RRTs of triacylglycerols for which the structures are known but the standards are not available. The new method can differentiate *sn*-1(3) and *sn*-2 positions, as well as position and configuration of double bonds present in the fatty acids of triacylglycerols. One of the two new methods can be used to estimate the RRTs of triacylglycerols containing ricinoleate (fatty acid with a hydroxy group). We also compared the predicted RRTs with the experimental values of triacylglycerol standards to be sure that the new methods are reliable.

2. Experimental

The HPLC RRTs of synthetic triacylglycerols used here were from our recent report [1]. They are in the column of experimental RRTs of Table 1. The HPLC system was as we recently reported [1]. A C_{18} column (25 cm×0.46 cm, 5 μm, Ultrasphere C_{18}, Beckman Instruments, Fullerton, CA, USA) was used. The eluent was a linear gradient starting at 100% methanol to 100% isopropanol in 40 min at a flow-rate of 1 ml/min. A photodiode array detector (Waters Associates, Milford, MA, USA) detecting at 205 nm was used. When necessary an evaporative light scattering detector (MKIII, Varex, Rockville, MD, USA) was used to detect saturated triacylglycerols.

Since retention times are not reproducible under the same HPLC conditions, RRTs were used to correct for different HPLC runs, different columns, different instruments, different days, minor column temperature variation and long-term column usage. The RRTs used here were described in the experimental section of our recent report [1] where the present data come from. Briefly, RRTs of the five reference triacylglycerols were the retention times from a single HPLC run of the mixture, and the RRTs of all other compounds were based on normalization to the one of these five triacylglycerols that eluted most closely but resolved. Since the retention times from another HPLC run under the same HPLC conditions might differ, use of the RRTs [13] provides a means for normalizing retention times for the sake of comparison. There was minor column temperature variation for these HPLC experimental data which were run at room temperature (22±2°C) in a central air-conditioned laboratory. We have shown recently that the elution sequence of methyl esters of fatty acids were the same at different column temperatures [14]. However, the

Table 1
Prediction of the relative retention times of triacylglycerols in a non-aqueous reversed-phase HPLC

No.	Fatty acids[a]			Relative retention times (min)		
	sn-1	*sn*-2	*sn*-3	Experimental	Method (a)	Method (b)
1	18:0	18:0	18:0	38.86*	38.86	38.86
2	18:0	18:0	16:0	37.07*	37.07	37.09
3	16:0	16:0	16:0	33.27*	33.27	33.27
4	18:0	16:0	18:0		36.85	37.09
5	16:0	16:0	18:0		35.06	35.23
6	16:0	18:0	16:0		35.28	35.23
7	16:0	16:0	14:0	31.34*	31.34	31.21
8	14:0	14:0	14:0	26.80*	26.80	26.81
9	14:0	14:0	16:0	29.06	28.73	29.06
10	18:0	18:0	14:0	35.20	35.14	35.23
11	18:0	14:0	18:0		34.24	33.27
12	14:0	14:0	18:0		30.52	31.21
13	14:0	18:0	14:0		31.42	31.21
14	18:0	18:0	$18:1^{\Delta9}$	36.72	36.82	36.79
15	18:0	$18:1^{\Delta9}$	18:0	36.64	36.77	36.79
16	$18:1^{\Delta9}$	18:0	$18:1^{\Delta9}$	34.76	34.78	34.72
17	$18:1^{\Delta9}$	$18:1^{\Delta9}$	18:0	34.73	34.73	34.72
18	$18:1^{\Delta9}$	$18:1^{\Delta9}$	$18:1^{\Delta9}$	32.83	32.69	32.65
19	$18:1^{\Delta9}$	$18:1^{\Delta9}$	$18:2^{\Delta9,12}$	31.27*	31.27	31.01
20	$18:2^{\Delta9,12}$	$18:2^{\Delta9,12}$	$18:1^{\Delta9}$	29.41*	29.41	29.37
21	$18:2^{\Delta9,12}$	$18:2^{\Delta9,12}$	$18:2^{\Delta9,12}$	27.92	27.85	27.73
22	$18:2^{\Delta9,12}$	$18:1^{\Delta9}$	$18:2^{\Delta9,12}$		29.71	29.37
23	$18:2^{\Delta9,12}$	$18:2^{\Delta9,12}$	18:0		31.45	31.44
24	$18:2^{\Delta9,12}$	18:0	$18:2^{\Delta9,12}$		31.80	31.44
25	$18:3^{\Delta9,12,15}$	$18:3^{\Delta9,12,15}$	$18:3^{\Delta9,12,15}$	23.28*	23.28	23.08
26	$18:3^{\Delta9,12,15}$	$18:3^{\Delta9,12,15}$	$18:2^{\Delta9,12}$		24.73	24.63
27	$18:3^{\Delta9,12,15}$	$18:3^{\Delta9,12,15}$	$18:1^{\Delta9}$		26.29	26.27
28	$18:2^{\Delta9,12}$	$18:2^{\Delta9,12}$	$18:3^{\Delta9,12,15}$		26.47	26.18
29	$18:3^{\Delta9,12,15}$	$18:3^{\Delta9,12,15}$	18:0		28.33	28.27
30	$16:1^{\Delta9}$	$16:1^{\Delta9}$	$16:1^{\Delta9}$	26.69*	26.69	26.70
31	$16:1^{\Delta9}$	$16:1^{\Delta9}$	$18:1^{\Delta9}$		28.62	28.78
32	$18:1^{\Delta9}$	$18:1^{\Delta9}$	$16:1^{\Delta9}$		30.75	30.76
33	$16:1^{\Delta9}$	$18:1^{\Delta9}$	18:0		32.67	32.83
34	$16:1^{\Delta9(t)}$	$16:1^{\Delta9(t)}$	$16:1^{\Delta9(t)}$	27.43*	27.43	27.42
35	$18:1^{\Delta9}$	$18:1^{\Delta9}$	$16:1^{\Delta9(t)}$		31.04	31.00
36	$16:1^{\Delta9(t)}$	$16:1^{\Delta9(t)}$	$18:1^{\Delta9}$		29.07	29.26
37	$16:1^{\Delta9(t)}$	$18:1^{\Delta9}$	18:0		33.09	33.07
38	$18:1^{\Delta6}$	$18:1^{\Delta6}$	$18:1^{\Delta6}$	33.66*	33.66	33.67
39	$18:1^{\Delta9}$	$18:1^{\Delta9}$	$18:1^{\Delta6}$		33.01	32.97
40	$18:1^{\Delta9}$	$18:1^{\Delta9}$	16:0	32.93	32.94	32.95
41	$18:1^{\Delta9}$	16:0	$18:1^{\Delta9}$	32.96	32.75	32.95
42	$18:1^{\Delta9}$	14:0	$18:1^{\Delta9}$		30.14	31.09
43	$18:1^{\Delta6(t)}$	$18:1^{\Delta6(t)}$	$18:1^{\Delta6(t)}$	34.08*	34.08	34.09
44	$18:1^{\Delta6(t)}$	$18:1^{\Delta6(t)}$	18:0		35.63	35.68
45	$18:1^{\Delta6(t)}$	$18:1^{\Delta6(t)}$	$18:1^{\Delta9}$		33.59	33.61
46	$18:1^{\Delta9}$	$18:1^{\Delta9}$	$18:1^{\Delta6(t)}$		33.18	33.13
47	$18:1^{\Delta9(t)}$	$18:1^{\Delta9(t)}$	$18:1^{\Delta9(t)}$	33.43*	33.43	33.43
48	16:0	$18:1^{\Delta9(t)}$	$18:2^{\Delta9,12}$	31.56*	31.56	31.57
49	$18:1^{\Delta9}$	$18:1^{\Delta9}$	$18:1^{\Delta9(t)}$		32.97	32.91

(Cont.)

Table 1. Continued

No.	Fatty acids[a]			Relative retention times (min)		
	sn-1	*sn*-2	*sn*-3	Experimental	Method (a)	Method (b)
50	$18:1^{\Delta 9(t)}$	$18:1^{\Delta 9(t)}$	$18:1^{\Delta 9}$		33.15	33.17
51	$18:2^{\Delta 9(t),12(t)}$	$18:2^{\Delta 9(t),12(t)}$	$18:2^{\Delta 9(t),12(t)}$	28.41*	28.41	28.42
52	$18:2^{\Delta 9(t),12(t)}$	$18:2^{\Delta 9(t),12(t)}$	18:0		31.80	31.90
53	$18:2^{\Delta 9(t),12(t)}$	$18:2^{\Delta 9(t),12(t)}$	$18:1^{\Delta 9}$		29.76	29.83
54	$18:1^{\Delta 9}$	$18:1^{\Delta 9}$	$18:2^{\Delta 9(t),12(t)}$		31.48	31.24
55	12-OH-$18:1^{\Delta 9}$	12-OH-$18:1^{\Delta 9}$	12-OH-$18:1^{\Delta 9}$	7.63*	7.63	7.45
56	12-OH-$18:1^{\Delta 9}$	12-OH-$18:1^{\Delta 9}$	$18:1^{\Delta 9}$	14.52*	14.52	15.85
57	$18:1^{\Delta 9}$	$18:1^{\Delta 9}$	12-OH-$18:1^{\Delta 9}$		25.94	24.25
58	12-OH-$18:1^{\Delta 9}$	12-OH-$18:1^{\Delta 9}$	$18:2^{\Delta 9,12}$	12.93	12.96	14.21
59	12-OH-$18:1^{\Delta 9}$	12-OH-$18:1^{\Delta 9}$	$18:3^{\Delta 9,12,15}$	11.74	11.48	12.66

[a] *cis* Double bonds are not indicated, while *trans* double bonds are indicated as (t) after the double bond positions.
Experimental relative retention times marked with an (*) were used to estimate the contribution of functional groups of fatty acids in Table 2 (method a). Relative retention times are reproducible to ±0.03 min.

effect of column temperature on the RRTs of triacylglycerols has not been studied yet. Usually the accuracy of the experimental RRTs as shown in Table 1 and our recent report [1] could be better than ±0.03 min. The exact experimental RRTs with second place of decimals [1] (Table 1) may not be reproducible, although, the elution sequence of 45 triacylglycerols and diacylglycerols should be the same. For the purpose of reproducing our experimental RRTs [1], or for predicting the RRTs, a C_{18} monomeric column (spherical, 5 μm) or preferably an Ultrasphere C_{18} column should be used. We have used different columns of Ultrasphere C_{18} (monomeric) from different batches of preparation (Beckman) in the studies of the biosynthesis of triacylglycerols containing ricinoleate over a period of one year [2]. The HPLC of castor oil (containing triricinolein, 1,2-diricinoleoyl-3-linolenoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-linoleoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol and other triacylglycerols) and radioactive triacylglycerol mixture (containing the castor triacylglycerols and 1-palmitoyl-2-oleoyl-*sn*-glycerol) were routinely performed as shown in our recent reports [1,2]. The retention times of these triacylglycerols and diacylglycerol were always about the same. Their elution sequence and the RRTs were always the same. However, we have not used C_{18} column from other manufacturers in these studies.

3. Results and discussion

We have used the experimental RRTs to predict the RRTs of other triacylglycerols as shown in Table 1. We have developed two methods to predict the RRTs when the standard is not available for actual run: (a) estimation from the experimental RRT of triacylglycerol standard with the closest corresponding structure, (b) estimation from the experimental RRT of tristearin (the general trend).

After careful observation of the RRTs of triacylglycerols we obtained recently [1], we found that the contribution to the RRT of functional group (or altered chain length) at a specific location of a specific fatty acid in the same *sn* position of triacylglycerol was approximately constant. Our method (a) was based on this observation. Table 2 shows the negative contributions (in minutes) of functional groups (or chain length shortenings) on the fatty acids to the RRTs of triacylglycerols from those of the closest structures. These contributions (Table 2) were obtained from simple calculations using the experimental RRTs with an asterisk (*) in Table 1. For example, the contribution −1.79 min in Table 2 [$-C_2$, chain shortening from 18:0, for *sn*-1(3)] was obtained from experimental RRTs 37.07−38.86 in Table 1 (compounds 1, 2). The contribution −2.01 min at *sn*-2 in Table 2 ($-C_2$, chain shortening from 18:0) was estimated as 33.27−37.07+1.79=−2.01

Table 2
Contributions of functional groups (or chain shortenings) of fatty acids to the relative retention times of triacylglycerols from those of the closest structures, method (a)

No.	Functional groups[a]	Fatty acids[a]	Contributions (min)	
			sn-1(3)	*sn*-2
1	$-C_2$ (chain shortening)	18:0	−1.79	−2.01
2	$-C_2$ (chain shortening)	16:0	−1.93	−2.61
3	Δ9	18:0	−2.04	−2.09
4	Δ12	$18:1^{\Delta 9}$	−1.56	−1.86
5	Δ15	$18:2^{\Delta 9,12}$	−1.45	−1.73
6	Δ9	16:0	−2.18	−2.22
7	Δ9(t)	16:0	−1.89	−2.06
8	Δ6	18:0	−1.72	−1.76
9	Δ6(t)	18:0	−1.55	−1.68
10	Δ9(t)	18:0	−1.76	−1.91
11	Δ12(t)	$18:1^{\Delta 9(t)}$	−1.63	−1.76
12	12-OH	$18:1^{\Delta 9}$	−6.89	−11.42

[a] *cis* Double bonds are indicated by Δ, while *trans* double bonds are indicated as (t) after the double bond positions.

[compounds 2, 3, adding 1.79 as the contribution at *sn*-1(3)]. The experimental RRTs with an asterisk (*) are exactly the same as the estimated values of method (a) in Table 1 since these are the basis for determining the contributions. The contributions of Δ9 on 18:0 at *sn*-1(3) and *sn*-2, −2.04 and −2.09 in Table 2, respectively, were obtained to best fit the experimental values of compounds 14–18 in Table 1, which were all of the possible triacylglycerol isomers of 18:0 and $18:1^{\Delta 9}$. Some values of functional groups in Table 2 were estimated from only two experimental RRTs in Table 1 and the differences between *sn*-1(3) and *sn*-2 were assumed to be proportionally the same as similar fatty acids. For example, the contributions of Table 2, No. 5 the Δ15 double bond were obtained from RRTs (method a) of Table 1, Nos. 21 (trilinolein) and 25 (trilinolenin), and the difference of *sn*-1(3) and *sn*-2 was assumed to be of the same proportion as Table 2, No. 4 (Δ12).

As shown in Table 2, the negative contribution of a functional group of fatty acid at *sn*-2 position toward the RRTs was larger than that at *sn*-1(3) positions. The contribution difference between *sn*-1(3) and *sn*-2 was large when the functional group was polar such as hydroxy group of ricinoleate (Table 2, No. 12), and the contribution differences of a single *cis* double bond (Table 2, Nos. 3,6,8) were low. As expected, this HPLC system does not separate triacylglycerols that are enantiomeric at *sn*-1 and *sn*-3 positions (e.g., 1-linoleoyl-2-oleoyl-3-stearoyl-*sn*-glycerol and 1-stearoyl-2-oleoyl-3-linoleoyl-*sn*-glycerol). The values of contributions of functional groups in Table 2 can be modified when additional experimental RRTs are known. The values of other functional groups (e.g., epoxy group) can be added to Table 2 when additional experimental RRTs are known.

The predicted RRTs (method a) in Table 1 were derived using the values in Table 2 and the experimental RRTs of the closest structures in Table 1. For example, the RRTs of 1,2-dimyristoyl-3-palmitoyl-*rac*-glycerol (28.73 min, Table 1, No. 9, method a) is calculated from the experimental RRT (26.80 min, Table 1, No. 8)+the factor for chain-lengthening 14:0 to 16:0 in the *sn*-1(3) position, 1.93 min (Table 2, No. 2): 28.73 (Table 1, No. 9, method a)=26.80 (Table 1, No. 8, experimental)+1.93 [Table 2, No. 2, *sn*-1(3)]. The estimated values obtained (Table 1, method a) correspond closely to the experimental values. The greatest discrepancy between the predicted vs. the experimental value was No. 9 (Table 1) which was 0.33 min off. The average of 12 comparisons shown in Table 1 (method a) was 0.11 min off from the experimental values.

The estimations of many other triacylglycerols can be made using this method (a). Based on these results, we expect the accuracies of estimations to be in the range, 0.1 to 0.3 min.

Table 1 also shows the predicted RRTs using method (b), estimation from the experimental RRT of tristearin. All calculations are related to the experimental RRT of tristearin, t_R=38.86 min, and disregard the differences of *sn*-1(3) and *sn*-2 to simplify and to show the general trend. The estimated RRT of triacylglycerol, t_R, is 38.86 min adjusted by the changes of carbon number and functional groups as shown in Eq. (1) as follows:

$$t_R \text{ (triacylglycerol)} = 38.86 + \Delta t_R \text{ (carbon number)} + \text{sum of } \Delta t_R \text{ (functional groups)} \quad (1)$$

$$\Delta t_R \text{ (carbon number)} = -0.86m - 0.012m^2 \quad (2)$$

The change in RRTs Δt_R for carbon number is given as Eq. (2) and m is the decrease in total carbon number from tristearin. Eq. (2) was derived by the plot of the change in RRTs Δt_R from tristearin against the decrease in total carbon number m from tristearin (chain shortening). The data of this plot are from the experimental RRTs of Nos. 1–14 of Table 1, the RRTs of triacylglycerols containing saturated fatty acids. The contributions of functional groups (method b) are shown in Table 3 and disregard differences of regiospecific isomers at *sn*-1(3) and *sn*-2. The values in Table 3 are the averages of the contributions of functional groups from the experimental RRTs in Table 1. The total RRT increment Δt_R (functional groups) is equal to the sum of functional group contributions. For example, RRT of 1,2–dioleoyl-3-palmitoyl-*sn*-glycerol is calculated as 38.86 (Table 1, No. 1, experimental for tristearin) − $0.86\cdot 2 - 0.012\cdot 2^2$ (for loss of two carbons) − $2\cdot 2.07$ (Table 3, No. 1, for addition of two double bonds), equal to 32.95 (Table 2, No. 40, method b). The experimental RRT is 32.93 min which is very close to the estimation 32.95 min. The estimation by method (a), 32.94 min, is also very close as shown in Table 1, No. 40.

Table 3
Contributions of functional groups of fatty acids to the relative retention times of triacylglycerols from tristearin, method (b)

No.	Functional groups[a]	Fatty acids[a]	Contributions (min)
1	Δ9	18:0	−2.07
2	Δ12	$18:1^{\Delta 9}$	−1.64
3	Δ15	$18:2^{\Delta 9,12}$	−1.55
4	Δ9	16:0	−2.19
5	Δ9(t)	16:0	−1.95
6	Δ6	18:0	−1.73
7	Δ6(t)	18:0	−1.59
8	Δ9(t)	18:0	−1.81
9	Δ12(t)	$18:1^{\Delta 9(t)}$	−1.67
10	12-OH	$18:1^{\Delta 9}$	−8.40

[a] *cis* Double bonds are indicated by Δ, while *trans* double bonds are indicated as (t) after the double bond positions.

The predictions of RRTs from method (b) shown in Table 1 are very close to the experimental values except those of triacylglycerols containing the functional group, 12-OH, shown in Table 1, Nos. 55–59. They are about 1 min off from the experimental values. Method (b) is accurate when limited to the predictions of RRT for triacylglycerols containing the functional groups of double bonds and having chain lengths between 18:0 and 14:0. These predictions (method b) of triacylglycerols with or without double bonds (Table 1, Nos. 1–54,) were very close to the experimental values, and the worst prediction was only 0.26 min off (Table 1, No. 19). Polar groups such as hydroxy-, epoxy- and keto groups appear unsuitable for method (b) as shown by 12-OH in Table 1 (Nos. 56–59).

We have studied the relation of group contributions of triacylglycerols in Table 3 and those of fatty acids in Table 1 of our previous report on the HPLC of fatty acids [3]. Even though we observed some proportional relations, the predictions of RRTs were not accurate from the HPLC of fatty acids as we are reporting here. The predictions of RRTs of triacylglycerols containing other functional groups not listed in Table 1, can be done with triacylglycerols containing new functional groups.

Our methods to predict the RRTs of triacylglycerols are simple and accurate. The accuracy of the prediction was checked by comparison of the experimental and predicted RRTs as shown in Table 1. The comparison of the experimental and predicted (calculated) retention times has rarely been given. Fabien et al. [15] made the comparison of logarithms of experimental and calculated selectivities of four mixed-acid triacylglycerols using the method of Goiffon et al. [11], however our predictions are more

accurate. The methods will aid the identification of radioactive metabolites and complex mixture of triacylglycerols in living systems.

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