Title: A normal phase high performance liquid chromatography method for the separation of hydroxy and non-hydroxy neutral lipid classes compatible with ultraviolet and in-line liquid scintillation detection of radioisotopes

Hari Kiran Kotapati[a,b], Philip D. Bates[a,b]*

[a]Department of Chemistry & Biochemistry, The University of Southern Mississippi
118 College Drive, Box # 5043, Hattiesburg, Mississippi-39406, USA

[b]Current address: Institute of Biological Chemistry, Clark Hall, Washington State University, Pullman, WA 99164

*Correspondence: phil_bates@wsu.edu

Abstract: In this paper, we report a method for the separation of hydroxy fatty acid and non-hydroxy fatty acid containing neutral lipid classes via normal phase HPLC with UV detection on a PVA-Sil column. The hexane/isopropanol/methanol/water based method separates all the neutral lipids in 21 min, and subsequently flushes through the polar lipids by 27 min such that prefractionation of neutral and polar lipids are not required, and the column is re-equilibrated for the next run in 15 min, for a total run time of 45 min per sample. The separation was demonstrated at both 1.0 ml/min and 1.5 ml/min for added applicability for fraction collection or inline analysis. Separation of various hydroxy fatty acid containing lipids was demonstrated from three different plant species *Ricinus communis*, *Physaria fendleri*, and engineered *Arabidopsis thaliana*. Additionally, we have combined this method with an inline liquid scintillation counter for the separation and quantification of ^{14}C labeled lipids obtained from *in vivo* metabolic flux experiments conducted in the developing seeds of *Arabidopsis thaliana*.

Keywords: Hydroxy neutral lipids; PVA-Sil; Normal phase HPLC; Lipid class separation; Metabolic flux analysis; Radio-HPLC; triacylglycerol; diacylglycerol; monoacylglycerol.

Abbreviations: Triacylglycerol (TAG), Diacylglycerol (DAG), Monoacylglycerol (MAG), Hydroxy Fatty Acid Triacylglycerol (HFA TAG), Hydroxy Fatty Acid Diacylglycerol (HFA DAG), Hydroxy Fatty Acid Monoacylglycerol (HFA MAG) Free Fatty Acid (FFA), Hydroxy Free Fatty Acid (HFFA), Phosphatidylcholine (PC), disintegrations per minute (DPM), counts per minute (CPM).

1. Introduction: Lipids are some of the most energy dense compounds produced in nature. Triacylglycerols (TAGs) that are the major component of plant seeds oils are used as food, bio-fuels, and industrial chemicals. The various uses of plant oils are related to the fatty acid composition of TAG. Most plants produce five common fatty acid structures (16-18 carbons long and 0-3 double bonds), leading to many potential molecular species of TAG in the oil. For example, in the model plant *Arabidopsis thaliana* 93 molecular species of TAGs have been identified [1]. Greater than 450 different fatty acid structures have been characterized in nature, indicating 10s of millions of possible molecular species of TAG could be produced [2, 3]. However, most plants that naturally produce these unusual fatty acids only produce a few unique structures, and typically produce very small amounts or have poor agronomic features that limits their use as crops. Therefore, it has been a goal of plant scientists to produce novel crops with valuable TAG compositions through genetic engineering. Much progress toward that goal has been made over the last 20 years, and has been extensively reviewed [4-12].

As plants are engineered to produce different fatty acid compositions, improved analytical methods are needed to identify, quantify, and decipher the metabolism of the novel lipids. In particular, we are interested in developing radio-isotopic labeling approaches that trace the precursor-product

relationships of fatty acid flux through lipid metabolism [13, 14]. This type of analysis requires complete separation of each lipid class for quantification radiolabel incorporation into each step of the pathway. Thin Layer Chromatography (TLC) and HPLC are commonly used separation techniques in lipid analysis. Reverse phase HPLC of lipids leads to the separation molecular species within a lipid class which can overlap with other lipid classes [15]. To achieve lipid class separation with the molecular species of each class grouped together, normal phase HPLC or TLC is preferred. However, normal phase separation of neutral lipid classes is sensitive to the polarity of fatty acids within each molecular species.

The common fatty acids found in found in all plants (16-18 carbon, 0-3 double bonds) do not affect the normal phase migration of molecular species within lipid classes. However, many plants produce valuable unusual fatty acids that accumulate in seed oils that have additional polar functional groups such as hydroxyls or epoxides that can effect normal phase molecular species migration [2]. For example, ricinoleic acid ((9Z,12R)-12-Hydroxyoctadec-9-enoic acid), a hydroxylated fatty acid (HFA) accumulates in seed oil of various species such as castor (*Ricinus communis*) [16]. Transgenic production of HFA in different plants been used as a model to understand the engineering of various unusual fatty acids into crop species [11, 17]. In plants producing HFA, neutral lipid classes will separate into sub-groups of molecular species based on the number of HFA in each molecule. For example, four groupings of TAG, three diacylglycerol groups, and two groups of monoacylglycerols and free fatty acids. Currently, separation of HFA-containing neutral lipid classes by common reverse phase methods used in lipidomics [18, 19], or normal phase HPLC or TLC systems leads to overlapping sub-groups from different lipid classes [20, 21].

Our goal was to develop a normal phase HPLC method that would separate all the HFA-containing neutral lipid classes and sub-groups in a single run for analysis by a continuous flow LSC detector, and to wash through polar lipid classes, such that prior fractionation of neutral and polar lipids is not needed. To limit the use of expensive radioactive standards during method development, we utilized a UV detector with unlabeled standards to determine analyte retention times for identification. The choice of a UV compatible solvent system was to develop a general method that could be used in a wide range of laboratories as UV systems are inexpensive and almost ubiquitous with most laboratory HPLC systems. There are several existing reports of the use of continuous flow detection in combination with HPLC. However, the HPLC methods involve separation of molecular species via non-aqueous reverse phase HPLC, and lead to some overlapping lipid classes [21, 25-28]. To the best of our knowledge there are no published reports on the complete separation of hydroxy and non-hydroxy neutral lipid classes by normal phase HPLC with UV detection on a PVA-Sil column, and its application in the continuous flow LSC. Because most lipids do not contain a chromophore UV detection of lipids is solely dependent on the presence of isolated double bonds in the lipid molecule at 200-210 nm [29]. As a result, HPLC analysis with UV detection requires the use of a small subset of solvents which have low absorbance at 210 nm. Organic solvents such as acetone, chloroform, toluene and ethyl acetate that are widely used in the separation of lipids with detection by mass spectrometry (MS) or evaporative light scatter detection (ELSD) have high absorbance at this wavelength [24, 29], and cannot be used with UV detection. Recent reports of normal phase HPLC methods to separate some lipid classes have used various stationary phases such as PVA-Sil [30-32], or monolithic silica [33]. These methods involve either MS and or ELSD as detectors and the solvents are not suitable for UV detection. There are a few reports for the normal phase lipid HPLC analysis using silica columns with UV detection at lower wavelengths [34-37]. Although the normal phase methods reported previously provide decent

separations of several lipid classes, they do not provide any information on the lipid classes of our interest involving hydroxylated fatty acid containing TAGs, DAGs, and MAGs.

Here we disclose a normal phase HPLC-UV method for class separation of ten lipid classes/sub-groups including hydroxy and non-hydroxylated neutral lipids and the polar lipid phosphatidylcholine, and extended its application to radio-isotopic metabolic labeling studies in transgenic plants. Additionally, we apply the method to lipids from three different plant species, and demonstrate two method variations that are beneficial for increasing separation between select lipids for assorted detection or fractionation applications.

2. Materials and Methods:

2.1 Lipid standards and solvents: 1-2-dioleoyl-*sn*-glycerol (18:1 DAG) was purchased from Sigma (USA) and 95% Soy PC standards were purchased from Avanti Polar Lipids (Alabaster, Al). The neutral lipid mix standard purchased from Sigma (USA) includes the Triacylglycerol (TAG), Diacylglycerol (DAG) and Monoacylglycerol (MAG) all composed of oleic acid ((9Z)-Octadec-9-enoic acid). Hydroxy neutral lipid standards, 3-HFA TAG, 2-HFA TAG and 1-HFA TAG, were prepared from castor oil by TLC [20]. HFA standards, 2-HFA DAG, 1-HFA DAG and 1-HFA MAG, were prepared from the lipase digestion of the 3-HFA TAG and 2-HFA TAG standards isolated from castor oil [20]. All solvents were submicron filtered HPLC or Optima grade purchased from Fisher Chemical. Glacial acetic acid for HPLC was obtained from Fisher chemical (USA)). For radiolabeling assays, [1-^{14}C]acetic acid (Sodium Salt), Specific Activity: 50.5mCi (1.868GBq)/mmol, 1mCi (37MBq) was purchased from PerkinElmer (USA). Liquid scintillation fluid used for on-line radio detection in HPLC, FlowLogic U, was purchased from LabLogic (USA).

2.2 Preparation of ^{14}C labelled lipids: Continuous [^{14}C]acetate metabolic labelling for 24 hrs within developing seeds tissue of *Arabidopsis thaliana* as in [20]. Metabolism was stopped by quenching in 2-Propanol at 85 °C for 10 min. Total lipids were extracted as in [20] The final lipid extract was stored in toluene with 0.05% 3,5-*Di-tert*-4-butylhydroxytoluene (BHT) prior to HPLC analysis.

3. Instrumentation:

HPLC instrumentation setup comprises of Thermo Fisher Ultimate 3000 instrument equipped with: 1. Quaternary pump system (Dionex Ultimate Pump - LPG3400SD), 2. Temperature controlled auto sampler - Dionex WPS-3000TSL (Analytical), 3. Temperature controlled column compartment TCC-3000SD, 4. Variable Wavelength Detector- Dionex VWD-3100, 5. Fraction Collector -Dionex AFC-3000. Columns used for HPLC analysis are YMC-Pack PVA-Sil (250 X 4.6 mm, 5 µm particle size) and Thermo Betasil Diol-100 (250 X 4.6 mm, 5 µm particle size). Each column is equipped with a guard column (10 X 4.0 mm) containing the same stationary phase and similar particle size as that of the column. The operating data system for the HPLC instrument is Chromeleon 7.2 SR4. Radio HPLC detector used for the analysis of ^{14}C samples is LabLogic β-RAM model 5, with an electronically controlled front end splitter which diverts flow to the fraction collector. The β-RAM counting flow cell equipped to the setup is 500 µL Liquid Cell, and the acquisition software for the Radio-HPLC analysis is Laura 4.2.11.129.

4. Results and Discussion:

HPLC method optimization was performed using UV detection at 210 nm where isolated double bonds in the lipids are detected. The analysis was performed on various mixtures of the lipid classes and the individual lipid standards that were either purchased or prepared chemically/enzymatically from the

respective standards. The hydroxy fatty acid (HFA) containing lipid standards were isolated by TLC from castor oil of which is composed of 3-HFA TAG, 2-HFA TAG and 1–HFA TAG where the hydroxy fatty acid is ricinoleic acid ((9Z,12R)-12-Hydroxyoctadec-9-enoic acid). Each component of the castor oil was identified and the assignments were made based on the retention times of individual standards that were prepared by TLC of castor oil. For the normal phase HPLC method development, we initially experimented with two columns Betasil Diol-100 and YMC-Pack PVA-Sil, and started with general UV compatible solvent systems found in the literature [39]. After examining both stationary phases, we have determined YMC-Pack PVA-Sil provides excellent separation of neutral lipid classes containing hydroxy fatty acids (Fig. 1).

4.1. Stationary phase and solvent screening: Our initial method development was performed using Betasil Diol-100 column utilizing a hexane-isopropanol system. Although Betasil diol-100 provided decent separations for lipid classes with non-hydroxy fatty acids no significant separation between DAG and 2-HFA TAG was observed. We tried various modifiers within the running solvent with the Betasil Diol-100 to improve separation between DAG and 2-HFA TAG. For example, we included methanol up to 5% of the mobile phase, or methanol containing acetone (up to 5%), or acetonitrile (up to 5%) but they did not improve separation to our liking. Therefore, we turned our focus towards the YMC-Pack PVA-Sil column. PVA-Sil is a silica based stationary phase with poly vinyl alcohol groups chemically bonded to it. This column has been used for the separation of various polar and neutral lipid classes using ELSD and MS detectors [32, 40-42], but to the best of our knowledge has not been demonstrated with a UV compatible lipid separation method. Although PVA-Sil phase is said to have similar separation properties as that of silica and diol [41], we demonstrate much better separations with PVA-Sil over diol stationary phases for the hydroxy neutral lipid classes under the same separation parameters (Table 1). Figure 1 illustrates the separation properties of Betasil Diol-100 (Fig. 1A) and PVA-Sil column (Fig. 1B) in the analysis of a mixture of castor oil and 18:1 DAG. PVA-Sil column seems to retain the analytes for longer periods compared to that of Betasil Diol-100. The separation between 1,2-DAG (Peak 2) and 2-HFA-TAG (Peak 3) is about 2 minutes on PVA-Sil column (Fig.1B) while the two peaks overlapped on Betasil Diol-100 (Fig. 1A).

4.2. Optimized HPLC-UV method on PVA-Sil column:

We have developed a twelve step linear gradient method that includes four isocratic hold points to ensure complete class separation of the hydroxy and non-hydroxy lipids, and also proper mobile phase switching between the gradients (Table 1). The solvents used in this method gave minimal background at 210 nm across gradient time course. 0.065% Acetic acid was added to solvent D, mixture of (60/40) 2-propanol/water, however higher levels will lead to increased background. The mobile phase flow rate was 1.5 mL/min, the column compartment was maintained at 35 °C, and the auto sampler at 20 °C. The total run time for the optimized HPLC method is 45 minutes. This includes 15 minutes of column equilibration prior to analysis, and 30 minutes of analysis time. To ensure proper equilibration of the column, a blank run was performed in the beginning of each sequence prior to the sample analysis.

4.3. HPLC-UV analysis of hydroxy and non-hydroxy neutral lipid classes:

Using the developed method that involves a twelve step linear gradient with isocratic hold points, operated by a quaternary gradient pump, we were able to completely separate nine neutral lipid classes based on the number of hydroxy fatty acids in each TAG, DAG or MAG lipid class. The neutral lipid classes that were separated on the PVA-Sil column include TAG, DAG (1,3 and 1,2 isomers), MAG, 1-

HFA TAG, 2-HFA TAG, 3-HFA TAG, 1-HFA DAG, 2-HFA DAG, 1-HFA MAG. Besides the neutral lipid classes we were also able to elute phosphatidylcholine (PC, tR = 25.5 min) as a separate peak from other polar lipids at a later time during the run (Supplementary information Figure S1). The individual standards for the lipid classes were analyzed by HPLC to confirm the identity of the lipid classes in the mixture based on the retention times (See Supplementary information Figures S2.a and S2.b for individual standard retentions). The gradients for the method, solvents used and gradient slope are as indicated in Table 1. Standards for each lipid class, and mixture of these standards were analyzed by the current method. The injection solvent for the analysis of standards and their mixture is hexane/2-propanol (60/40). For the standards and their mixtures 150-300 µg of sample was loaded on to the column in 25-40 µL of injection solvent. We have also used toluene (up to 10 µL) as injection solvent, and the retention times of most of the lipid classes remained the same. TAG appeared to elute slightly earlier with Toluene, and interestingly 1-HFA TAG appeared to elute later when toluene was used as the injection solvent. Even after a slight change in the retention times of these standards with different injection solvent, we did not observe them coeluting with other components in the mixture. The separations between the lipid classes of interest were baseline and of at least 0.5 min. However non-hydroxy free fatty acids appeared to co-elute with 1,3-DAG. 1,2-DAG is the biologically relevant form, and 1,3-DAG is formed through acyl migration *in vitro* [43]. 2-HFA DAG and HFFA which are difficult to separate by TLC [20] were completely separated (Figure 2).

Another aspect of the current method on PVA-Sil is that 2-HFA TAG elutes after 1,3 and 1,2-DAG moieties whereas it elutes in between 1,3-DAG and 1,2-DAG on the TLC system that has been reported which complicates analysis when significant 1,3-DAG is formed *in vitro* [20]. 2-HFA TAG elutes as multiple combined peaks on the PVA-Sil column suggesting a possible partial molecular species separation within the lipid class (Figure 2), and may be related to molecular species where the hydroxy fatty acids are on the 1,2 vs 1,3 positions of the TAG backbone. The retention times for each of these lipid classes from UV chromatogram are listed below in Table 2.

The method gradient in the current method is created in such a way that after the elution of all neutral lipid classes, the mobile phase is adjusted to 10% water, 40% methanol and 50% 2-propanol to flush any polar lipids which will remain and clog the column, and eliminates the need for a prefractionation of lipid extracts into neutral and polar lipid fractions prior to HPLC. During the process of polar lipid flush PC eluted as a pure component, and this was confirmed by TLC analysis of a collected fraction (Supplementary information, Figure S3).

4.4. Method application-Radio HPLC analysis: The developed method for the separation of neutral lipid classes has been applied to the analysis of radioactive lipid extracts from [^{14}C]acetate metabolic labeling experiments conducted in developing seeds of *Arabidopsis thaliana*. Two different plant lines of *A. thaliana* were used in the experiments. The first line is the wild-type (WT) Columbia-0 ecotype which does not contain HFA, and the second line (RcPDAT1a) is a previously genetically engineered to accumulate HFA-containing neutral lipids [44]. Continuous metabolic labeling with [^{14}C]acetate was carried out according to [20]. For WT, developing seeds were incubated in [^{14}C]acetate media and collected after 30 minutes. For the RcPDAT1a line, the seeds were continuously labeled for 21 hrs.

4.4.1. Method for radio detection: To analyze both WT and RcPDAT1a extracts containing ^{14}C labelled lipids, a common method was created for the detection with β-ram detector. We have used 1:2 eluant to scintillant flow rate ratio for the continuous flow detection. The residence time in the counting cell for this flow rate is 6.7 seconds. When the flow rate ratio was adjusted to 1:1 for eluant: scintillant, the residence time went up to 10 seconds, and this resulted in radioactive peak overlap during the analysis. The details of the method parameters for each sample in Radio-HPLC analysis are illustrated in Table

3. The ^{14}C activity for each sample was measured on a static liquid scintillation counter prior to analysis, and known amount of total sample radioactivity was loaded for the HPLC analysis.

4.4.2. Analysis of ^{14}C labelled wild-type *Arabidopsis thaliana* seeds:

Figure 3 shows the ^{14}C and UV traces for the 30 min [^{14}C]acetate labeled seeds. The differences in each trace reflect the differences in specific activity (DPM/mass) for each lipid class. TAG has high mass (>90% of total lipid mass in the extract) and can be detected by the UV, but it has a low specific activity as compared to the other lipids where the radioactivity can be detected but the low mass cannot. The differences in specific activity at 30 min of labeling reflect both endogenous lipid pool size and the pathway structure where TAG is an endpoint that is labeled slowly. Identification of each radioactive peak was based on the retention times of the non-labeled standards from the UV chromatogram.

We have also performed time based fraction collection of the individual peaks followed by TLC - phosphor imaging analysis to confirm the identities and purities of the individual peaks. (See Supplementary information; Figure S 3 for TLC and Table S 1 for fraction collection method). The peaks observed on the radio chromatogram correspond to TAG (Peak 1), DAG (Peak 2), and PC (Peak 4). The retentions of the peaks from Radio-HPLC analysis and HPLC-UV analysis are compared in the Table 4.

4.4.3. Analysis of ^{14}C labeled *Arabidopsis thaliana RcPDAT1a* seeds: For injection purpose, sample was dissolved in toluene. When sample was dissolved in hexane/2-propanol (60/40), and injected 1-HFA TAG eluted as a broad peak from 8-10 minutes. Although there was no co-elution of the 1-HFA TAG with other components during the run, we have decided to use the solvent that provided better peak shape. When the analysis was performed on the sample, without a blank run, there was a peak overlap between 1-HFA TAG and free fatty acids. The 21 hrs RcPDAT1a labeled seeds crude extract in 10 µL of injection solvent (Toluene) was analyzed by the developed Radio-HPLC method (Figure 4).

Each one of the components on the radio chromatogram were identified by the comparison of retention times to the standards on HPLC-UV analysis. Also, time based fraction collection was performed followed by TLC-phosphor imaging analysis of the individual components. TLC data confirmed the identities and purities of the individual components (See Supplementary information; Figure S4 for TLC and Table S2 for fraction collection method). The retention times of the components from the Radio-HPLC of ^{14}C crude extract and the standards in HPLC-UV analysis are compared in Table 5.

4.5 Variations of the General Method, Reproducibility, and Various Applications

While we have extensively used the general method to analyze lipid extracts obtained from *A. thaliana* seeds, slight modifications to this method were made as per our research needs. One of these modified methods involve the separation of hydroxylated and non-hydroxylated lipid classes at a lower mobile phase flow rate of 1 mL/min (Supplementary information; Table S3). This method however provided a minimum baseline separation between DAG & 2-HFA TAG (Supplementary information; Figure S5), and is not suitable for application to inline radio detection with a 500 µL counting cell, but it would be valuable for use with other detectors such as MS or ELSD, and could be used for purification of lipid classes with fraction collection. PVA-Sil stationary phase demonstrated excellent reproducibility with a very slight change (<0.15 min) in TAG peak retention after 72 runs in the analysis of crude silique extract of non-hydroxy fatty acid producing *A. thaliana* (Supplementary Information; Figure S6). Due to the less complex nature of non-hydroxy fatty acid producing *A. thaliana*, in these runs, we were able to shorten the analysis time to 18 minutes (Supplementary Information; Tables S4 and S5). We have also applied slightly modified and extended method (39 minutes analysis time) to separate the ^{14}C

labeled HFA containing lipid classes from the natural HFA-accumulating species *Physaria fendleri* (Supplementary Information Figure S7). In this method, the initial separation conditions for HFA containing neutral lipids were similar to that of the original method but the polar lipid flush was extended (Supplementary Information Tables S6 and S7).

Conclusion:

The unusual hydroxylated fatty acid, ricinoleic acid, has been found in lipids from at least 85 plant species [45], and engineering of various valuable unusual fatty acids into high yielding crops has been a key goal of plant lipid biotechnology [4-12]. To enhance analysis of HFA-containing lipids we have developed a PVA-Sil based UV compatible, normal phase HPLC method for the separation of nine different neutral lipid classes that include both hydroxy and non-hydroxy lipids, and a polar lipid class phosphatidylcholine (PC) that is suitable for detection, fraction collection, or in-line liquid scintillation counting as part of *in vivo* metabolic labeling experiments. We have demonstrated the functionality of the general method and slight variations, with lipid separations from three HFA accumulation species, *Ricinus communis*, *Physaria fendleri*, and engineered *Arabidopsis thaliana*. This method will be valuable to characterize plant lipids, and to understand changes in plant metabolism due to unusual fatty acid engineering.

Supplementary Information: Supplementary Information for this manuscript is available at

Funding Disclosures: This work was supported by the National Science Foundation (MCB #1613923), and the United States Department of Agriculture (AFRI #2016-67013-24605).

References:

[1] M. Li, E. Baughman, M.R. Roth, X. Han, R. Welti, X. Wang, Quantitative profiling and pattern analysis of triacylglycerol species in Arabidopsis seeds by electrospray ionization mass spectrometry, The Plant journal : for cell and molecular biology, 77 (2014) 160-172.
[2] F.D. Gunstone, J.L. Harwood, A.J. Dijkstra, Editors, The Lipid Handbook with CD-ROM, Third Edition, CRC Press LLC2007.
[3] R.C. Badami, K.B. Patil, Structure and occurrence of unusual fatty acids in minor seed oils, Prog. Lipid Res., 19 (1980) 119-153.
[4] E.B. Cahoon, J.M. Shockey, C.R. Dietrich, S.K. Gidda, R.T. Mullen, J.M. Dyer, Engineering oilseeds for sustainable production of industrial and nutritional feedstocks: solving bottlenecks in fatty acid flux, Current Opinion in Plant Biology, 10 (2007) 236-244.
[5] D.J. M., S. Sten, G.A. G., C.A. S., High-value oils from plants, The Plant Journal, 54 (2008) 640-655.
[6] J.A. Napier, I.A. Graham, Tailoring plant lipid composition: designer oilseeds come of age, Curr. Opin. Plant Biol., 13 (2010) 329-336.
[7] J.A. Napier, R.P. Haslam, F. Beaudoin, E.B. Cahoon, Understanding and manipulating plant lipid composition: Metabolic engineering leads the way, Current Opinion in Plant Biology, 19 (2014) 68-75.
[8] H.R. P., R.L. Noemi, E. Peter, M. Maurice, S. Olga, N.J. A., The modification of plant oil composition via metabolic engineering—better nutrition by design, Plant biotechnology journal, 11 (2013) 157-168.

[9] C.A. S., Y.J. Lindberg, G.A. G., S. Sten, H. Per, Replacing fossil oil with fresh oil – with what and for what?, European Journal of Lipid Science and Technology, 113 (2011) 812-831.
[10] V. Thomas, W.C. C., S. Sten, S.S. P., G.A. G., Metabolic engineering of plant oils and waxes for use as industrial feedstocks, Plant biotechnology journal, 11 (2013) 197-210.
[11] P.D. Bates, Understanding the control of acyl flux through the lipid metabolic network of plant oil biosynthesis, Biochim. Biophys. Acta, Mol. Cell Biol. Lipids, 1861 (2016) 1214-1225.
[12] J.A. Aznar-Moreno, T.P. Durrett, Review: Metabolic engineering of unusual lipids in the synthetic biology era, Plant Sci. (Shannon, Irel.), 263 (2017) 126-131.
[13] P.D. Bates, S.R. Johnson, X. Cao, J. Li, J.-W. Nam, J.G. Jaworski, J.B. Ohlrogge, J. Browse, Fatty acid synthesis is inhibited by inefficient utilization of unusual fatty acids for glycerolipid assembly, Proc. Natl. Acad. Sci. U. S. A., 111 (2014) 1204-1209.
[14] D.K. Allen, P.D. Bates, H. Tjellström, Tracking the metabolic pulse of plant lipid production with isotopic labeling and flux analyses: Past, present and future, Progress in Lipid Research, 58 (2015) 97-120.
[15] A. Fauland, H. Köfeler, M. Trötzmüller, A. Knopf, J. Hartler, A. Eberl, C. Chitraju, E. Lankmayr, F. Spener, A comprehensive method for lipid profiling by liquid chromatography-ion cyclotron resonance mass spectrometry, Journal of Lipid Research, 52 (2011) 2314-2322.
[16] L.J. Morris, The mechanism of ricinoleic acid biosynthesis in Ricinuscommunis seeds, Biochemical and Biophysical Research Communications, 29 (1967) 311-315.
[17] K.R. Lee, G.Q. Chen, H.U. Kim, Current progress towards the metabolic engineering of plant seed oil for hydroxy fatty acids production, Plant cell reports, 34 (2015) 603-615.
[18] T. Cajka, O. Fiehn, Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry, Trends in analytical chemistry : TRAC, 61 (2014) 192-206.
[19] T. Cajka, O. Fiehn, Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics, Analytical Chemistry, 88 (2016) 524-545.
[20] P.D. Bates, J. Browse, The pathway of triacylglycerol synthesis through phosphatidylcholine in Arabidopsis produces a bottleneck for the accumulation of unusual fatty acids in transgenic seeds, The Plant Journal, 68 (2011) 387-399.
[21] J.-T. Lin, G.Q. Chen, Identification of TAG and DAG and their FA Constituents in Lesquerella (Physaria fendleri) Oil by HPLC and MS, J. Am. Oil Chem. Soc., 90 (2013) 1819-1829.
[22] W.W. Christie, G. Dobson, Thin-layer chromatography re-visited, Lipid Technol., 11 (1999) 64-66.
[23] M. Holčapek, P. Jandera, J. Fischer, B. Prokeš, Analytical monitoring of the production of biodiesel by high-performance liquid chromatography with various detection methods, Journal of Chromatography A, 858 (1999) 13-31.
[24] W.W. Christie, Detectors for high-performance liquid chromatography of lipids with special reference to evaporative light-scattering detection, Oily Press, 1992, pp. 239-271.
[25] M. Kiffe, A. Jehle, R. Ruembeli, Combination of High-Performance Liquid Chromatography and Microplate Scintillation Counting for Crop and Animal Metabolism Studies: A Comparison with Classical On-Line and Thin-Layer Chromatography Radioactivity Detection, Analytical Chemistry, 75 (2003) 723-730.
[26] A.E. Nassar, Y. Parmentier, M. Martinet, D.Y. Lee, Liquid chromatography-accurate radioisotope counting and microplate scintillation counter technologies in drug metabolism studies, Journal of chromatographic science, 42 (2004) 348-353.
[27] J.-T. Lin, C.L. Woodruff, T.A. McKeon, Non-aqueous reversed-phase high-performance liquid chromatography of synthetic triacylglycerols and diacylglycerols, Journal of Chromatography A, 782 (1997) 41-48.
[28] W.C. Byrdwell, W.E. Neff, Analysis of hydroxy-containing seed oils using atmospheric pressure chemical ionization mass spectrometry, J. Liq. Chromatogr. Relat. Technol., 21 (1998) 1485-1501.
[29] F.B. Jungalwala, J.E. Evans, R.H. McCluer, High-performance liquid chromatography of phosphatidylcholine and sphingomyelin with detection in the region of 200 nm, Biochemical Journal, 155 (1976) 55-60.

[30] W.W. Christie, S. Gill, J. Nordback, Y. Itabashi, S. Sanda, A.R. Slabas, New procedures for rapid screening of leaf lipid components from Arabidopsis, Phytochem. Anal., 9 (1998) 53-57.
[31] F.S. Deschamps, P. Chaminade, D. Ferrier, A. Baillet, Assessment of the retention properties of poly(vinyl alcohol) stationary phase for lipid class profiling in liquid chromatography, Journal of Chromatography A, 928 (2001) 127-137.
[32] J. Jones, S. Manning, M. Montoya, K. Keller, M. Poenie, Extraction of Algal Lipids and Their Analysis by HPLC and Mass Spectrometry, J. Am. Oil Chem. Soc., 89 (2012) 1371-1381.
[33] M. Graeve, D. Janssen, Improved separation and quantification of neutral and polar lipid classes by HPLC–ELSD using a monolithic silica phase: Application to exceptional marine lipids, Journal of Chromatography B, 877 (2009) 1815-1819.
[34] J.R. Yandrasitz, G. Berry, S. Segal, High-performance liquid chromatography of phospholipids with UV detection: optimization of separations on silica, Journal of Chromatography B: Biomedical Sciences and Applications, 225 (1981) 319-328.
[35] J.G. Hamilton, K. Comai, Separation of neutral lipids and free fatty acids by high-performance liquid chromatography using low wavelength ultraviolet detection, Journal of Lipid Research, 25 (1984) 1142-1148.
[36] T.L. Kaduce, K.C. Norton, A.A. Spector, A rapid, isocratic method for phospholipid separation by high-performance liquid chromatography, Journal of Lipid Research, 24 (1983) 1398-1403.
[37] E.J. Lesnefsky, M.S.K. Stoll, P.E. Minkler, C.L. Hoppel, Separation and Quantitation of Phospholipids and Lysophospholipids by High-Performance Liquid Chromatography, Analytical Biochemistry, 285 (2000) 246-254.
[38] J.-T. Lin, J.M. Chen, L.P. Liao, T.A. McKeon, Molecular Species of Acylglycerols Incorporating Radiolabeled Fatty Acids from Castor (Ricinus communis L.) Microsomal Incubations, Journal of Agricultural and Food Chemistry, 50 (2002) 5077-5081.
[39] C. Silversand, C. Haux, Improved high-performance liquid chromatographic method for the separation and quantification of lipid classes: application to fish lipids, J Chromatogr B Biomed Sci Appl, 703 (1997) 7-14.
[40] U. Sommer, H. Herscovitz, F.K. Welty, C.E. Costello, LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures, Journal of Lipid Research, 47 (2006) 804-814.
[41] J. Nordback, E. Lundberg, W.W. Christie, Separation of lipid classes from marine particulate material by HPLC on a polyvinyl alcohol-bonded stationary phase using dual-channel evaporative light-scattering detection, Mar. Chem., 60 (1998) 165-175.
[42] C. Beermann, A. Green, M. Moebius, J.J. Schmitt, G. Boehm, Lipid class separation by HPLC combined with GC FA analysis: Comparison of seed lipid compositions from different Brassica napus L. varieties, J. Am. Oil Chem. Soc., 80 (2003) 747-753.
[43] D.R. Kodali, A. Tercyak, D.A. Fahey, D.M. Small, Acyl migration in 1,2-dipalmitoyl-sn-glycerol, Chem. Phys. Lipids, 52 (1990) 163-170.
[44] H. van Erp, P.D. Bates, J. Burgal, J. Shockey, J. Browse, Castor Phospholipid:Diacylglycerol Acyltransferase Facilitates Efficient Metabolism of Hydroxy Fatty Acids in Transgenic Arabidopsis, Plant Physiology, 155 (2011) 683.
[45] J. Ohlrogge, N. Thrower, C. Wilkerson, M. Baxter, W. Yang, K. McGlew, J. Liu, D. Higgin, B. Matthaus, M. Zhang, X. Cheng, C. Li, V. Mhaske, B. Shorrosh, A.W. Tumaney, Plant FA database, https://plantfadb.org/, DOI.

Tables

Table 1: *Gradient for HPLC separation of hydroxy and non-hydroxy neutral lipids*

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Gradient Curve
0.00	1.50	0.5	99.5	0.0	0.0	5
1.00	1.50	1.0	99.0	0.0	0.0	5
4.00	1.50	1.0	99.0	0.0	0.0	5
8.00	1.50	2.5	97.0	0.5	0.0	5
10.00	1.50	2.5	97.0	0.5	0.0	5
12.00	1.50	5.0	94.0	1.0	0.0	5
16.00	1.50	7.0	92.0	1.0	0.0	5
21.00	1.50	99.0	0.0	1.0	0.0	5
22.00	1.50	50.0	0.0	25.0	25.0	5
25.00	1.50	50.0	0.0	25.0	25.0	5
26.00	1.50	100	0.0	0.0	0.0	5
29.00	1.50	100	0.0	0.0	0.0	5
30.00	1.50	0.5	99.5	0.0	0.0	5

λ_{max} = 210 nm;
Equilibration time: 15 min; prior to the start of the run
Equilibration mobile phase flow: 1.5 mL/min**;**
Equilibration solvent: 0.5% A and 99.5%B
Solvent A = 2-Propanol; **Solvent B** = Hexanes; **Solvent C** = Methanol;
Solvent D = 2-Propanol/Water/Acetic acid (60/40/0.065)
Column compartment: 35 °C; **Sampler Temperature:** 20 °C

Table 2*: Retention times of individual neutral lipid classes and PC on PVA-Sil column*

	Lipid Class	tR of standard on UV chromatogram (min)
Peak 1	TAG	3.43
Peak 2	1-HFA TAG	9.90
Peak 3	FFA	11.00
Peak 3	1,3-DAG	11.00
Peak 4	1,2-DAG	12.60
Peak 5	2-HFA TAG	14.64
Peak 6	HFFA	15.57
Peak 7	1-HFA DAG	16.08
Peak 8	3-HFA TAG	16.82
Peak 9	MAG	18.29
Peak 10	2-HFA DAG	19.52
Peak 11	1-HFA MAG	20.29
Peak 9*	PC	25.50
*For PC standard's chromatogram refer to Supplementary Information Figure S1		

***Table 3**: Radio detection method parameters for the analysis of Arabidopsis seed extracts*

	***At* Wild Type**	***At* RcPDAT1a**
Eluant Flow (mL/min)	1.500	1.500
Scintillant Flow (mL/min)	3.000	3.000
Residence time (Seconds)	6.7	6.7
Injection Solvent	2-PrOH/Hexanes (40/60)	Toluene
Injection volume (µL)	25	10
^{14}C Activity loaded (DPM)	6612	10730

***Table 4**: Identities of ^{14}C labeled lipid classes in Arabidopsis thaliana wild type seed extract*

	Identity	**^{14}C Count (CPM)**	**tR on radio chromatogram (min)**	**tR of standard on UV chromatogram (min)**
Peak 1	TAG	2196	3.83	3.43
Peak 2	DAG	1683	12.13	12.60
Peak 3	Unidentified Polar Lipids	378	24.87	n/a
Peak 4	PC	1764	25.83	26.00

***Table 5**: Identities of ^{14}C labeled lipid classes in Arabidopsis thaliana RcPDAT1a seed extract*

	Identity	**^{14}C Count (CPM)**	**tR on radio chromatogram (min)**	**tR of standard on UV chromatogram (min)**
Peak 1	TAG	2502	4.08	3.43
Peak 2	1-HFA TAG	1080	10.10	9.90
Peak 3	FFA	1872	11.25	11.00
Peak 4	1,2-DAG	1116	12.83	12.60
Peak 5	2-HFA TAG	468	14.72	14.64
Peak 6	MAG	333	18.50	18.30
Peak 7	1-HFA MAG	459	19.67	20.30
Peak 9	PC	2007	25.15	26.00

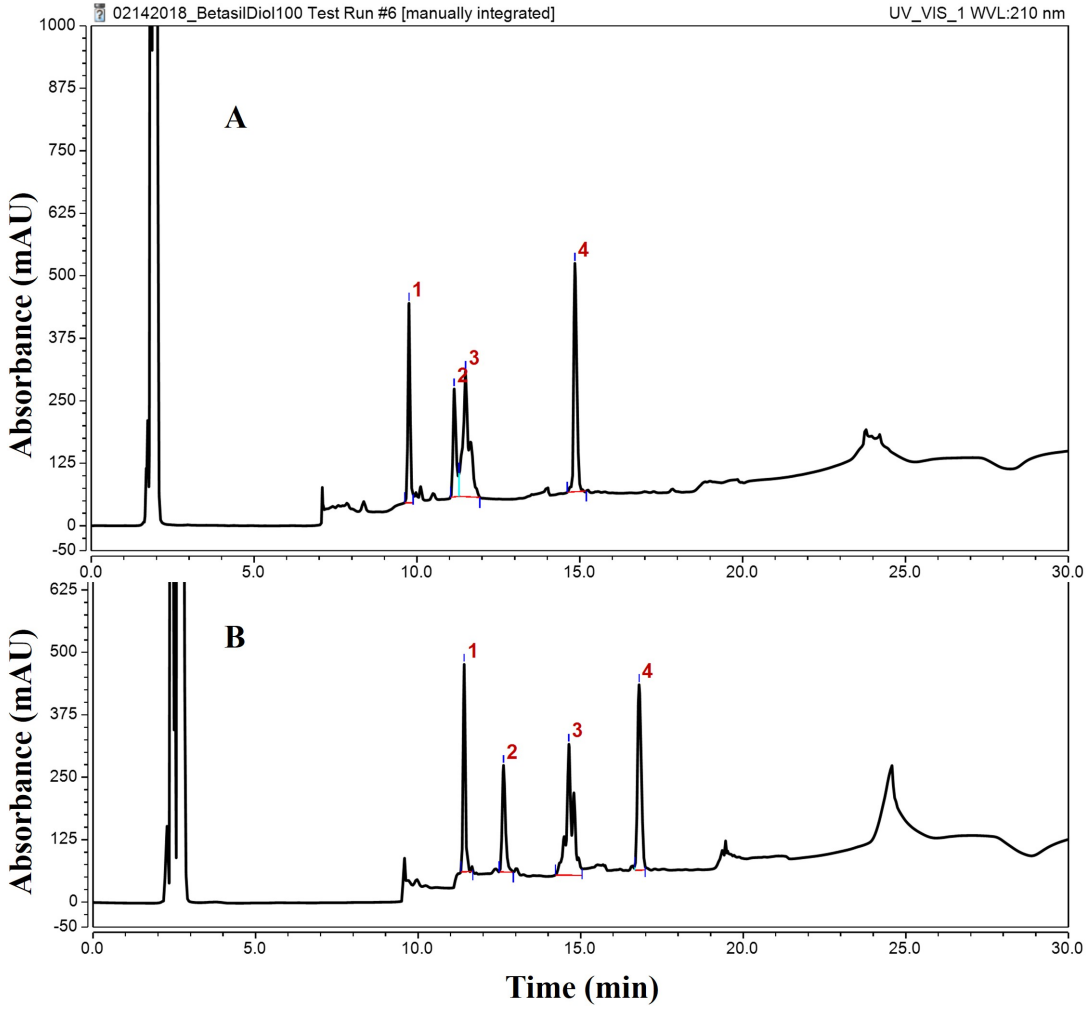
Figure Captions

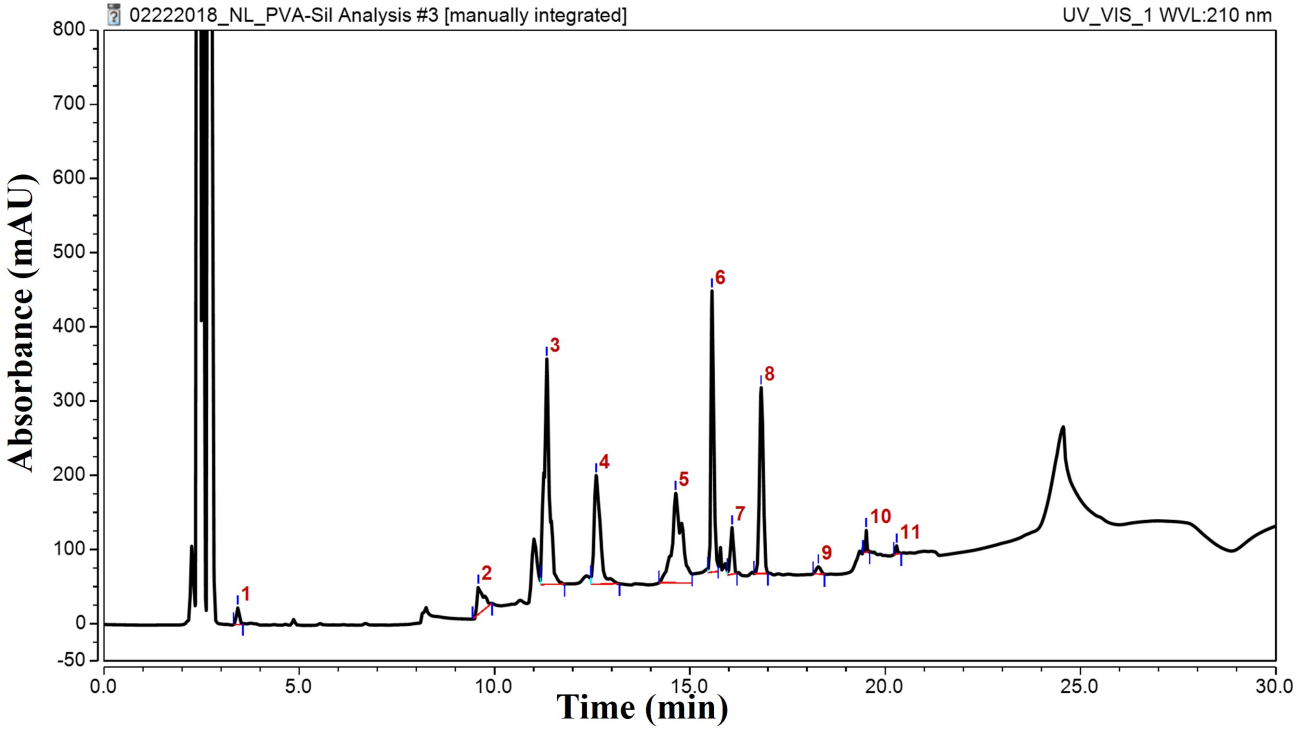
Figure 1: HPLC-UV traces of mixture of castor oil and 18:1 DAG on Betasil Diol-100 (A) and PVA-Sil column (B). Identified peaks in **Figure 1** are: **1** – 1, 3-DAG + FFA; **2** – 1, 2-DAG; **3** – 2-HFA TAG; 4 – 3-HFA TAG.

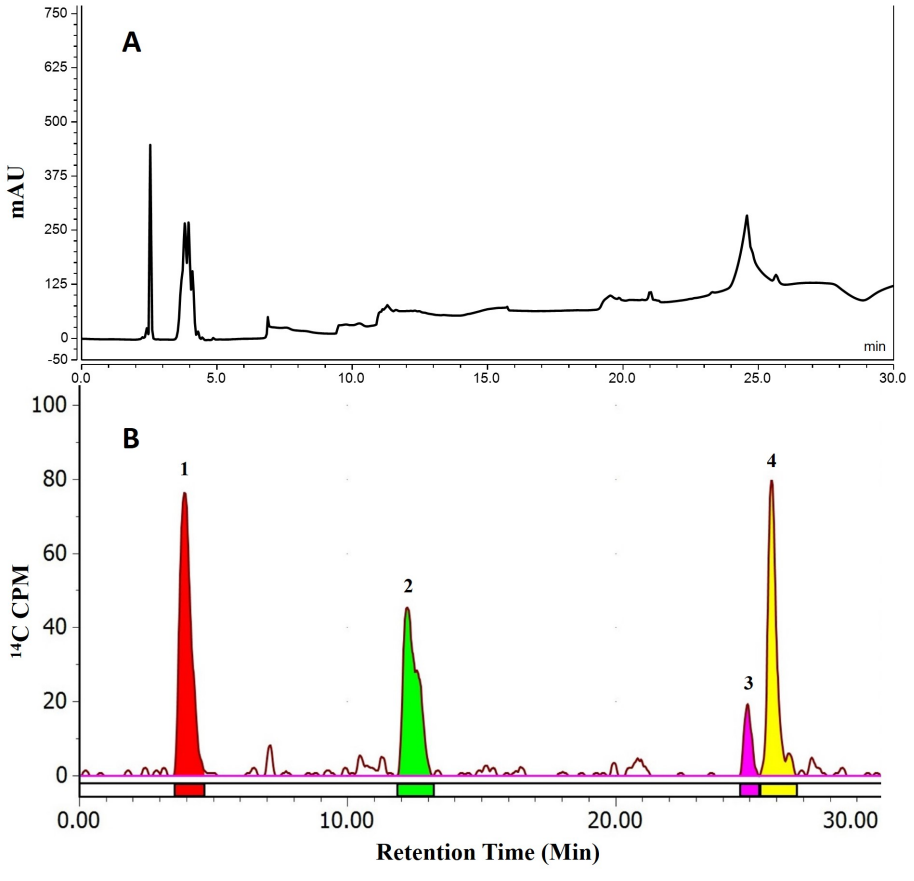
Figure 2: UV trace of the mixture of hydroxy and non-hydroxy neutral lipid class standards. Identified peaks in **Figure 2** are: **1** – TAG; **2** – 1-HFA TAG; **3** – FFA + 1, 3-DAG; **4** – 1, 2-DAG; **5** – 2-HFA TAG; **6** – HFFA; **7** – 1-HFA DAG; **8** – 3-HFA TAG; **9** – MAG; **10** – 2-HFA DAG; **11** – 1-HFA MAG.

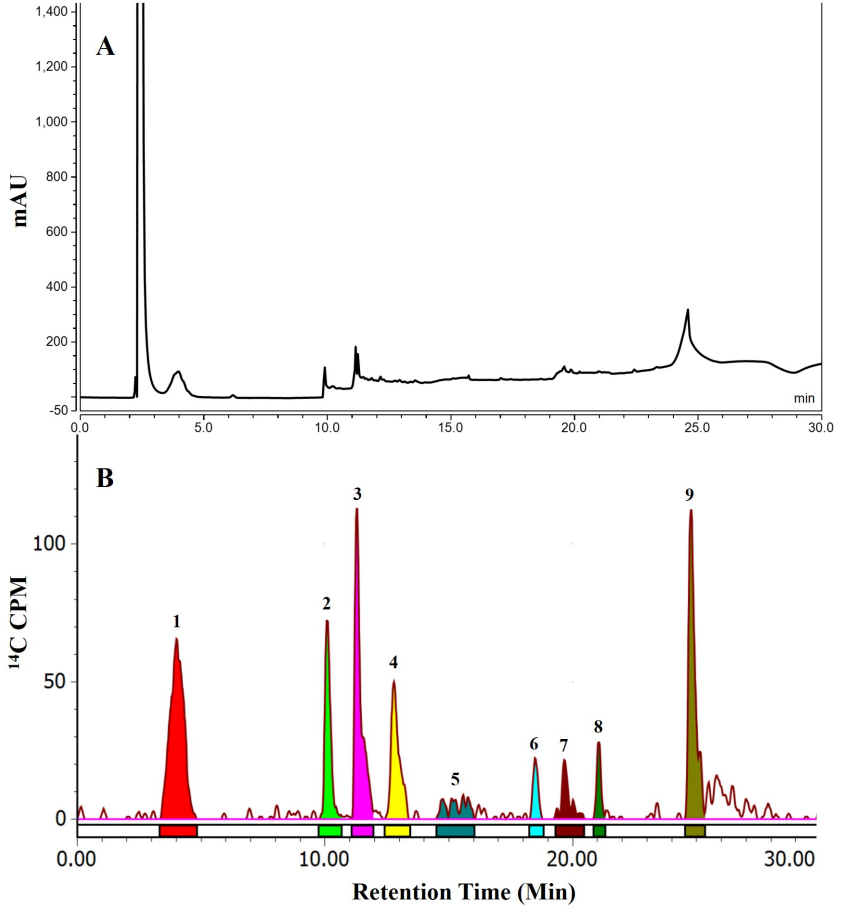
Figure 3: UV (Trace A) and Radio (Trace B) chromatograms of ^{14}C labelled Arabidopsis thaliana wild type seed extract showing the sensitivity of radio detection. Trace B y-axis is in units of counts per minute (CPM). Identified peaks in **Figure 3** are: **1** – TAG; **2** – DAG; **3** – Unidentified; **4** – PC.

Figure 4: UV (Trace A) and Radio (Trace B) chromatograms of ^{14}C labeled HFA producing Arabidopsis thaliana RcPDAT1a seed extract showing the sensitivity of radio detection. Trace B y-axis is in units of counts per minute (CPM). Identified peaks in **Figure 4** are: **1** – TAG; **2** – 1-HFA TAG; **3** – FFA; **4** – 1, 2-DAG; **5** – 2-HFA TAG; **6** – MAG; **7** – 1-HFA MAG; **8** – Unidentified; **9** – PC.


02142018_BetasilDiol100 Test Run #6 [manually integrated]
UV_VIS_1 WVL:210 nm
A
B
Absorbance (mAU)
Absorbance (mAU)
Time (min)
1
2
3
4
1000
875
750
625
500
375
250
125
0
-50
0.0
5.0
10.0
15.0
20.0
25.0
30.0


02222018_NL_PVA-Sil Analysis #3 [manually integrated]
UV_VIS_1 WVL:210 nm
Absorbance (mAU)
Time (min)
800
700
600
500
400
300
200
100
0
-50
0.0
5.0
10.0
15.0
20.0
25.0
30.0
1
2
3
4
5
6
7
8
9
10
11


A
mAU
750
625
500
375
250
125
0
-50
min
0.0
5.0
10.0
15.0
20.0
25.0
30.0
B
^{14}C CPM
100
80
60
40
20
0
1
2
3
4
0.00
10.00
20.00
30.00
Retention Time (Min)


A
mAU
1,400
1,200
1,000
800
600
400
200
-50
0.0
5.0
10.0
15.0
20.0
25.0
30.0
min
B
^{14}C CPM
100
50
0
1
2
3
4
5
6
7
8
9
0.00
10.00
20.00
30.00
Retention Time (Min)

Supplementary Information

A normal phase high performance liquid chromatography method for the separation of hydroxy and non-hydroxy neutral lipid classes compatible with ultraviolet and in-line liquid scintillation detection of radioisotopes

Hari Kiran Kotapati[a,b], Philip D. Bates[a,b]*

[a]Department of Chemistry & Biochemistry, The University of Southern Mississippi
118 College Drive, Box # 5043, Hattiesburg, Mississippi-39406, USA

[b]Institute of Biological Chemistry, Clark Hall, Washington State University, Pullman, WA 99164

*Correspondence: phil_bates@wsu.edu

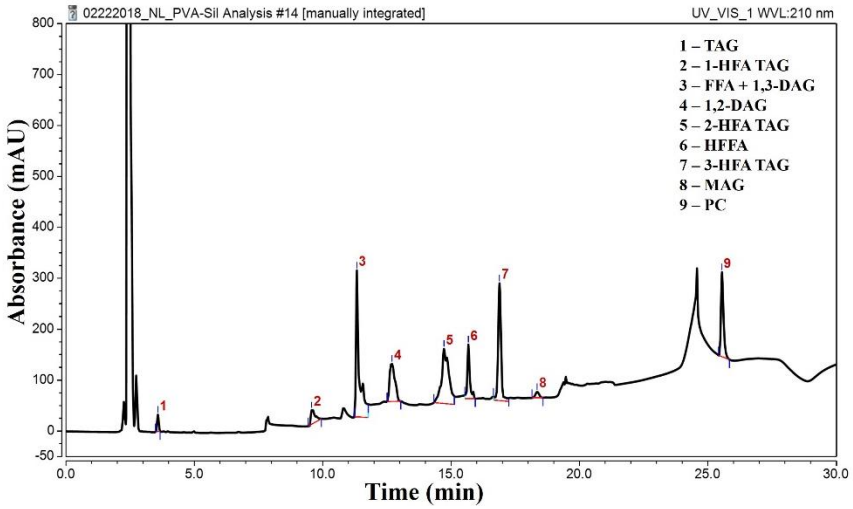


Figure S1*: HPLC-UV method : UV trace of analysis of neutral lipid standards mixture and PC. Identities of individual standards are in the figure insert. For the method parameters see Table 1 in the article.*

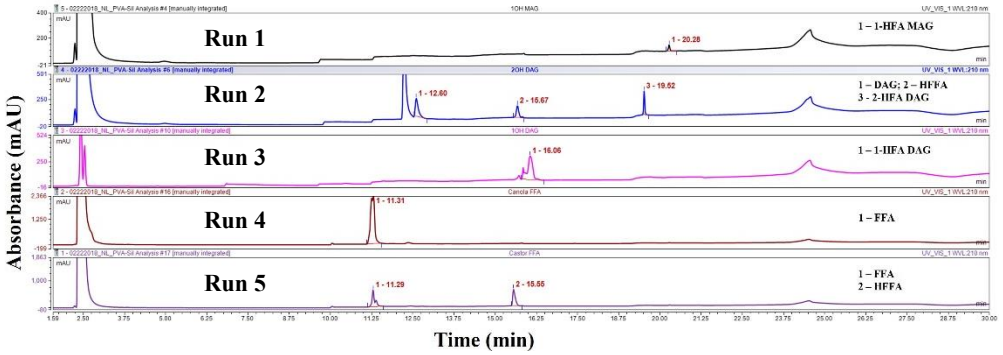


Figure S2.a: *HPLC-UV chromatogram of individual neutral lipid standards; 1-HFA MAG (Run1;tR=20.28min), 2-HFA DAG (Run2;tR=19.52min), 1-HFA DAG (Run 3;tR=16.06min), FFA (Run4;tR=11.31min), HFFA (Run5;tR=15.55min)*

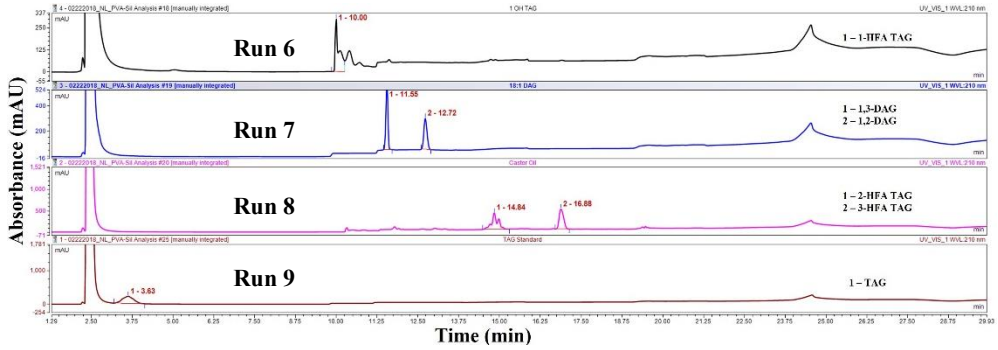


Figure S2.b: *HPLC-UV chromatogram of individual neutral lipid standards; 1-HFA TAG (Run 6; tR=10.00min), 1,3- DAG (Run 7; tR=11.55min), 1,2-DAG (Run 7; tR=12.72min),2-HFA TAG(Run 8; tR=14.84min), 3-HFA TAG(Run 8; tR=16.88min), TAG (Run 9; tR=3.63min)*

Table S1: *Fraction collection parameters for ^{14}C labeled seed extract analysis of wild type A.thaliana*

Fraction #	Start Collection (Min)	End Collection (Min)
1	0.5	2.5
2	2.9	5
3	5.1	8
4	11.7	13.3
5	14	16.5
6	20	23
7	24.4	25.3
8	25.35	25.5
9	25.55	26.7
10	27	28.5

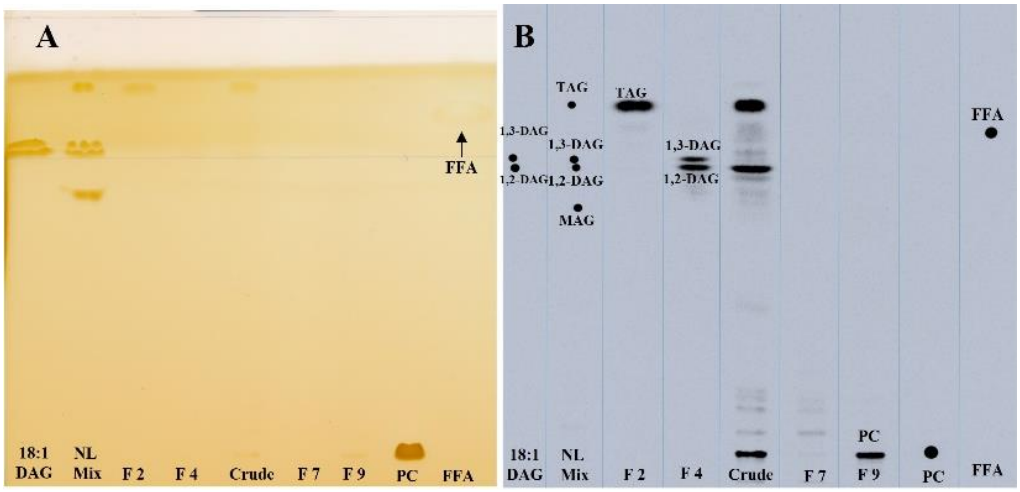


Figure S3: *HPLC-TLC; Iodine stain (A) and Phosphorimaging (B) analysis of 14C lipid extract HPLC fractions of wild type A.thaliana (Col-0B)*

TLC: Dual development method.

Development 1: Chloroform/Methanol/Acetic acid/Water (75/25/4/4, v/v/v/v), 3/4th of plate.

Development 2: Hexanes/Diethyl ether/Acetic acid, (70/30/1, V/V/V) to top of plate.

***Table S2**: Fraction collection parameters for ^{14}C labeled seed extract analysis of HFA producing A.thaliana (RcPDAT1a)*

Fraction #	Start Collection (Min)	End Collection (Min)
1	1.5	2.9
2	3	5
3	5.2	9.2
4	9.6	10.6
5	10.75	10.95
6	11	12.1
7	12.3	12.4
8	12.5	13.6
9	13.8	14.3
10	14.5	16.5
11	16.65	18
12	18.2	19
13	19.15	19.3
14	19.5	20.65
15	20.75	21.75
16	22	24
17	24.3	25.4
18	25.6	27.5

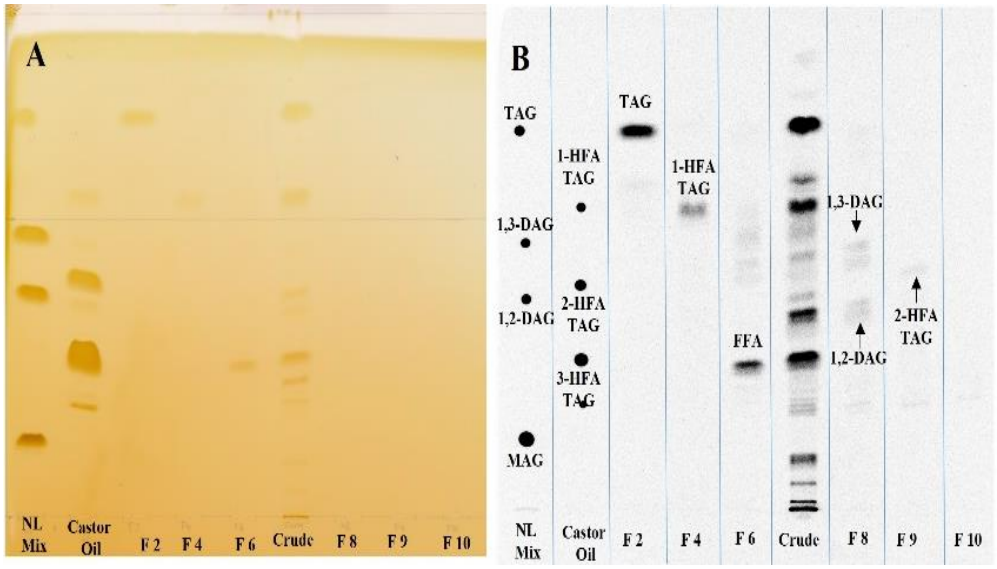


***Figure S4** : HPLC-TLC; Iodine stain (A) and Phosphorimaging (B) analysis of 14C lipid extract HPLC fractions of HFA producing transgenic A.thaliana (RcPDAT1a)*

*TLC protocol adapted from[1]

***Table S3**: HPLC-UV 1mL/min method; analysis of neutral lipid standards mixture on PVA-Sil column*

Time (min)	Flow Rate (mL/min)	%A	%B	Gradient Curve
0.00	1.00	1.5	98.5	5
10.00	1.00	10.0	90.0	5
14.00	1.00	10.0	90.0	5
17.00	1.00	100	0.0	5
20.00	1.00	100	0.0	5
22.00	1.00	1.5	98.5	5
25.00	1.00	1.5	98.5	5

λ_{max} = 210 nm
Solvent A: 2-Propanol; **Solvent B:** Hexanes
Column Oven Temperature: 35 °C
Sampler Temperature: 20 °C
Equilibration Time: 10 min; prior to start of each run
Equilibration Solvent: 1.5%A : 98.5%B

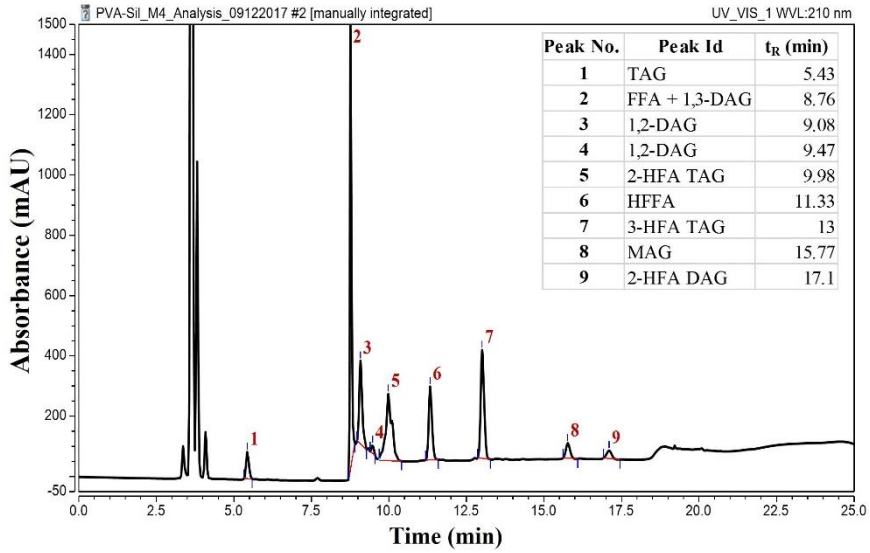


***Figure S5:** UV trace of neutral lipid standards mixture using method at 1mL/min mobile phase flow on PVA-Sil column. Identified Peaks and retention times are listed in the figure insert. HPLC method details are in Table S3 above.*

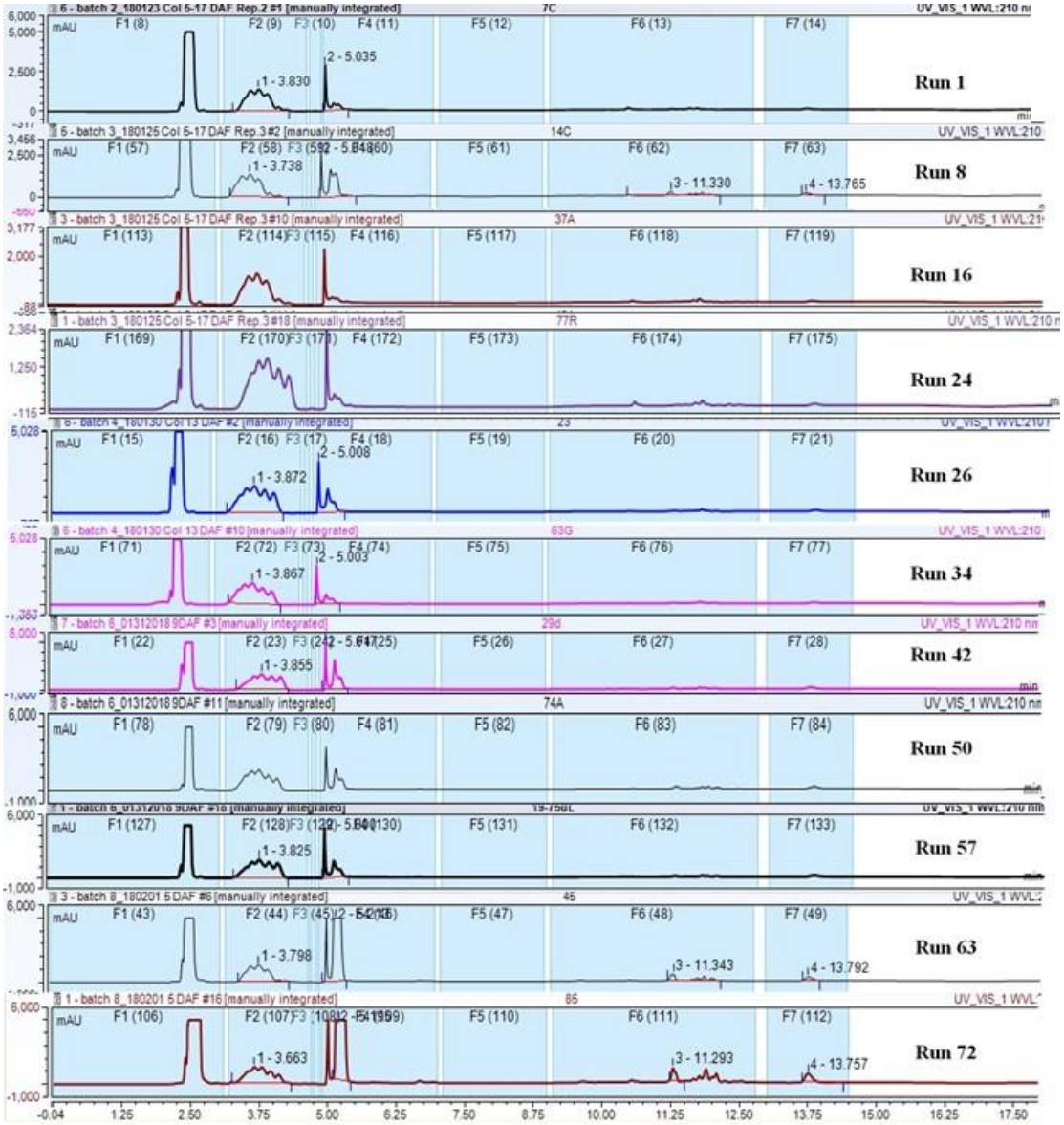


Figure S6*: HPLC-UV traces of crude silique extract of A.thaliana containing non-hydroxy neutral lipids. Lipid classes identified are TAG (Peak 1, Fraction2, tR=3.75min), and PC (Peak 4, Fraction 7, tR=13.76min)*

Table S4: *Method gradient for HPLC analysis of crude silique extract of non-HFA producing A.thaliana*

Time (min)	Flow Rate (mL/min)	%A	%B	%C	%D	Gradient Curve
0.00	1.50	0.5	99.5	0	0	5
6.00	1.50	8.0	92.0	0	0	2
7.00	1.50	20.0	78.0	1.0	1.0	5
9.00	1.50	61.0	15.0	12.0	12.0	7
11.00	1.50	50.0	0.0	25.0	25.0	5
13.00	1.50	50.0	0.0	25.0	25.0	5
14.00	1.50	100	0.0	0	0	5
17.00	1.50	100	0.0	0	0	5
18.00	1.50	0.5	99.5	0	0	2

λ_{max} = 210 nm
Solvent A: 2-Propanol; **Solvent B:** Hexanes; **Solvent C:** Methanol;
Solvent D: 2-Propanol/Water/Acetic Acid (60/40/0.065)
Column Oven Temperature: 35 °C
Sampler Temperature: 20 °C
Equilibration Time: 12 min; prior to start of each run
Equilibration Solvent: 0.5%A : 99.5%B

Table S5: *HPLC-fraction Collection parameters of crude silique extract in non-HFA producing A.thaliana*

Fraction #	Start Collection (Min)	End Collection (Min)	Lipid class in the corresponding fraction
1	0	3.10	-
2	3.20	4.70	TAG
3	4.75	4.85	-
4	4.90	7.00	-
5	7.10	9.00	-
6	9.10	12.80	-
7	13.00	14.50	PC

Table S6: *HPLC method parameters for the analysis of ^{14}C labeled Physaria fendleri seed extract*

Time (min)	Flow Rate (mL/min)	%A	%B	%C	%D	Gradient Curve
0.00	1.50	0.5	99.5	0.0	0.0	5
1.00	1.50	1.0	99.0	0.0	0.0	5
4.00	1.50	1.0	99.0	0.0	0.0	5
8.00	1.50	2.5	97.0	0.5	0.0	5
10.00	1.50	2.5	97.0	0.5	0.0	5
12.00	1.50	5.0	94.0	1.0	0.0	5
16.00	1.50	9.0	90.0	1.0	0.0	5
24.00	1.50	66.0	20.0	7.0	7.0	7
27.00	1.50	66.0	20.0	7.0	7.0	7
31.00	1.50	50.0	0.0	25.0	25.0	2
33.00	1.50	50.0	0.0	25.0	25.0	5
34.00	1.50	100	0.0	0.0	0.0	5
38.00	1.50	100	0.0	0.0	0.0	5
39.00	1.50	0.5	99.5	0.0	0.0	5

λ_{max} = 210 nm
Solvent A: 2-Propanol; **Solvent B:** Hexanes; **Solvent C:** Methanol;
Solvent D: 2-Propanol/Water/Acetic Acid (60/40/0.065)
Column Oven Temperature: 35 °C
Sampler Temperature: 20 °C
Equilibration Time: 15 min; prior to start of each run
Equilibration Solvent: 0.5%A: 99.5%B

Table S7: *Fraction collection parameters for ^{14}C labeled seed extracts of Physaria fendleri*

Fraction #	Start Collection (Min)	End Collection (Min)
1	0.5	3.40
2	3.55	4.28
3	4.40	7.00
4	7.17	8.12
5	8.25	12.10
6	12.22	13.25
7	13.36	13.78
8	13.92	15.05
9	15.20	15.60
10	15.75	16.37
11	16.50	21.50
12	21.70	26.30
13	26.45	27.82
14	27.93	30.00
15	30.02	30.93
16	31.05	31.45
17	31.57	32.48
18	32.60	36.0

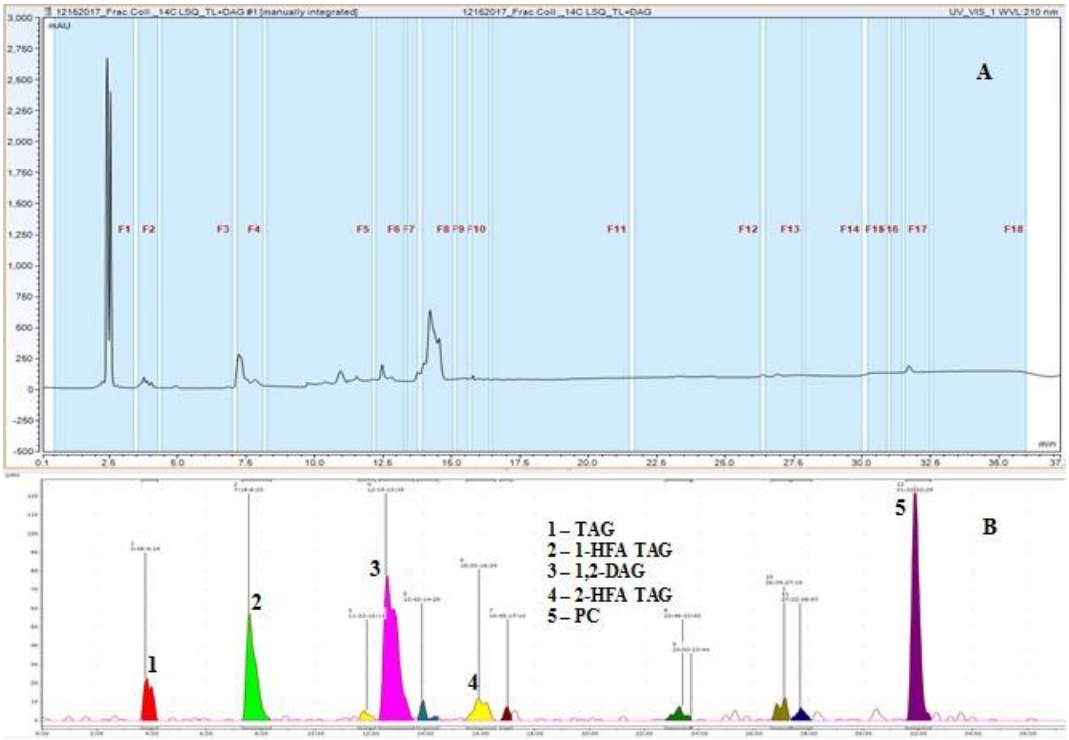


Figure S7: *HPLC-UV (A) and Radio (B) traces of ^{14}C labeled seed extract of Physaria findleri. Lipid classes on radio chromatogram (Trace B) are TAG (Peak1; tR=3.7min), 1-HFA TAG(Peak2; tR=7.75min), 1,2-DAG (Peak3; tR=12.5min), 2-HFA TAG (Peak 4; tR=15.5min), PC (Peak5; tR=31.75min)*

Reference:

[1] P.D. Bates, J. Browse, The pathway of triacylglycerol synthesis through phosphatidylcholine in Arabidopsis produces a bottleneck for the accumulation of unusual fatty acids in transgenic seeds, The Plant Journal, 68 (2011) 387-399.