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In-Silico-Generated Library for Sensitive Detection of 2-Dimethylaminoethylamine Derivatized FAHFA Lipids Using High-Resolution Tandem Mass Spectrometry

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Abstract

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Author Contributions

J.D. and T.K designed the experiment. Q.Z., Y.W., and J.Y. performed experimental measurements. T.K. developed the in silico library. J.D. performed the library validation and application. J.D., O.F., and T.K wrote the paper with contributions from all other authors.

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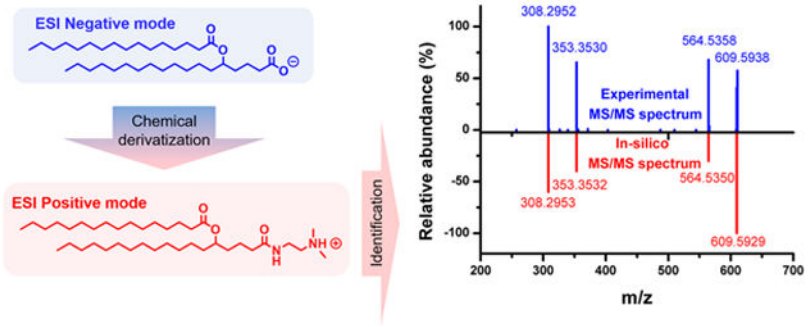
Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c00172.
Experimental MS/MS spectra of 12 DMED-FAHFA standards under direct infusion; validation of in silico DMED-FAHFA library with MS/MS spectra obtained from direct infusion of 35 DMED-FAHFA standards; detailed information on identified DMED-FAHFA standards; annotated DMED-FAHFA information in *Arabidopsis thaliana* by MRM transition and in silico library (PDF)

The authors declare no competing financial interest.

Fatty acid esters of hydroxy fatty acids (FAHFAs) are a family of recently discovered lipids with important physiological functions in mammals and plants. However, low detection sensitivity in negative ionization mode mass spectrometry makes low-abundance FAHFA challenging to analyze. A 2-dimethylaminoethylamine (DMED) based chemical derivatization strategy was recently reported to improve the MS sensitivity of FAHFAs by labeling FAHFAs with a positively ionizable tertiary amine group. To facilitate reliable, high-throughput, and automatic annotation of these compounds, a DMED-FAHFA in silico library containing 4290 high-resolution tandem mass spectra covering 264 different FAHFA classes was developed. The construction of the library was based on the heuristic information from MS/MS fragmentation patterns of DMED-FAHFA authentic standards, and then, the patterns were applied to computer-generated DMED-FAHFAs. The developed DMED-FAHFA in silico library was demonstrated to be compatible with library search software NIST MS Search and the LC–MS/MS data processing tool MS-DIAL to guarantee high-throughput and automatic annotations. Applying the in silico library in *Arabidopsis thaliana* samples for profiling FAHFAs by high-resolution LC–MS/MS enabled the annotation of 19 DMED-FAHFAs from 16 families, including 3 novel compounds. Using the in silico library largely decreased the false-positive annotation rate in comparison to low-resolution LC–MS/MS. The developed library, MS/MS spectra, and development templates are freely available for commercial and noncommercial use at https://zenodo.org/record/3606905.

Graphical Abstract



Fatty acid esters of hydroxy fatty acids (FAHFA) are a family of recently discovered bioactive lipids.[1] FAHFAs are formed through the esterification between the hydroxyl group of a hydroxy fatty acid and the carboxyl group of a fatty acid. FAHFAs were first reported in mice adipose tissues and demonstrated to possess antidiabetes and anti-inflammatory effects. Since the initial discovery, FAHFAs have been extensively investigated by both biologists and chemists and discovered in a number of different species. A most recent study revealed FAHFAs, including 9-PAHSA, 9-OAHSA, and 9-SAHSA, could be protective molecules to prevent colon carcinoma cells from apoptotic cell death,[2] highlighting a new role of FAHFAs in cancer research. Chemists found FAHFAs were distributed not only in mammals but also in various plants.[3,4] This newly discovered function and the wide distribution of FAHFAs in nature suggests there could be more potential functions to be uncovered. Therefore, the ability to metabolically profile a wide variety of FAHFAs in any given biological specimens is of great importance.

Mass spectrometry (MS) is one of the most powerful platforms for compound identification, which enabled the initial discovery of FAHFAs.[5] In previous reports, multiple-reaction monitoring (MRM) mode of triple quadrupole MS[6,7] and data-dependent acquisition in high-resolution MS[8] were utilized to identify new FAHFAs. However, due to the low ionization efficiency and the relative low abundance of FAHFAs in biological samples, only 64 endogenous FAHFAs belonging to 17 families were identified endogenously. Chemical derivatization is a strategy to convert a compound into its product using a chemical derivatization reagent. By incorporating a positively charged group into the molecule, the ionization efficiency of the product could be highly improved in positive electrospray ionization (ESI) mode.[9] Specifically, we previously reported 2-dimethylaminoethylamine (DMED) as an effective derivatization reagent for FAHFAs[10] to detect FAHFAs in positive ESI MS instead of the less sensitive negative ionization mode (Figure 1). The ESI detection sensitivities of DEMD derivatized FAHFAs were improved by up to 70-fold in comparison to the free FAHFAs.[10] By combining chemical derivatization and MRM mode analysis, 292 potential FAHFAs from 49 FAHFA families were detected in rice and *Arabidopsis thaliana*.[11] However, due to the low resolution of triple quadrupole MS and the dependence on a single MRM transition for FAHFA identification, the rate of false-positive discoveries remained unclear.[11]

We previously also explored untargeted MS/MS analysis of underivatized FAHFAs using an in silico FAHFA MS/MS library containing 3267 tandem mass spectra for 1089 FAHFA species.[8] Probably due to the poor detection sensitivity of unlabeled FAHFAs and relatively low abundances of FAHFAs in egg yolk, only six FAHFAs were identified.[8] In order to facilitate high coverage as well as accurate and automatic annotation of more potential FAHFAs, we are here combining our strategies of DMED based chemical derivatization with developing and applying a high-resolution in silico MS/MS library. Such in silico libraries are created by learning MS/MS fragmentation rules using experimental MS/MS fragmentation patterns. These fragmentation rules are then imposed onto in-silico-generated structures based on the LipidBlast templates.[8,12] Our newly created DMED-FAHFA in silico library contains 4290 high-resolution tandem mass spectra, covering 264 different FAHFA classes obtained from 33 of the most important fatty acids. We carefully validated the library and show its feasibility for untargeted LC–high-resolution MS/MS screening on plant extracts of *Arabidopsis thaliana*.

EXPERIMENTAL METHODS

Chemicals and Reagents.

The FAHFA standards, including 5-PAHSA, 9-PAHSA, 10-PAHSA, 12-PAHSA, 13-PAHSA, 5-SAHPA, 10-SAHPA, 5-MAHMA, 9-MAHMA, 10-MAHMA, 5-MAHPA, 9-MAHPA, 10-MAHPA, 5-PAHMA, 9-PAHMA, 10-PAHMA, 5-PDAHPA, 10-PDAHPA, 5-PAHPA, 9-PAHPA, 10-PAHPA, 5-SAHMA, 10-SAHMA, 7-PDAHSA, 9-PDAHSA, 10-PDAHSA, 10-POHSA, 5-POHSA, 12-POHSA, 13-POHSA, 5-OAHSA, 9-OAHSA, 12-OAHSA, 13-OAHSA, 5-SAHSA, 10-SAHSA, 12-SAHSA, 13-SAHSA, and 10-OSHSA were purchased from Cayman Chemical (Ann Arbor, MI, USA), RC ChemTecCo., Ltd. (Wuhan, Hubei, China), and XuKang Medical Science and Technology Co., Ltd. (Xiangtan,

Hunan, China). 2-Chloro-1-methylpyridinium iodide (CMPI), triethylamine (TEA), and 2-dimethylaminoe-thylamine (DMED) were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

HPLC grade acetonitrile (ACN), acetone, chloroform, and methanol were purchased from Merck (Darmstadt, Germany). Analytical grade formic acid, and ammonium hydroxide were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Strong anion-exchange solid-phase extraction cartridges (3 mL, 200 mg) were supplied by Weltech Co. (Wuhan, China).

Sample Preparation.

The sample preparation procedure consisted of three steps, including solvent extraction, solid phase extraction (SPE), and DMED derivatization.[11]

The extraction of endogenous FAHFAs was carried out in the following way. Briefly, 100 mg of *Arabidopsis thaliana* shoots were weighed and ground into powder in liquid nitrogen with a mortar and pestle. The homogenized powder was transferred into a 5 mL Eppendorf tube and extracted in a mixture of methanol/chloroform/water (1:2:1, v/v/v, 4 mL) by sonication for 30 s. After that, the extract was centrifuged for 2 min at 14 000 rpm, and the supernatant was transferred into a new tube and dried under a mild stream of nitrogen gas. The residue was reconstituted in ammonium hydroxide/acetonitrile (0.1%, v/v, 1 mL) right before SPE.

A strong anion-exchange SPE cartridge (200 mg, 3 mL) was used for sample purification. The cartridge was first activated with acetonitrile (3 mL) and then loaded with the as-prepared reconstitution solution. After the sample matrix on the cartridge was washed with acetone/water (1:9, v/v, 3 mL) and acetone (3 mL) in sequence, the enriched FAHFAs were eluted with formic acid/acetone (1:99, v/v, 2 mL). The elution was evaporated to dryness under a gentle stream of nitrogen gas and stored in −20 °C until use.

Before DMED derivatization, the SPE elution residue was reconstituted in acetonitrile (100 μL). Then, CMPI (20 μmol/mL in acetonitrile, 10 μL) and TEA (20 μmol/mL in acetonitrile, 20 μL) was added. The mixture was incubated at 40 °C for 5 min. Subsequently, DMED (20 μmol/mL in acetonitrile, 20 μL) was added and shaked at 40 °C for 30 min. Finally, the solution containing DMED-FAHFAs was evaporated to dryness and stored in −20 °C until use.

Instrumentation and Analytical Conditions.

The acquisition of standard reference spectra was performed using direct infusion of DMED-FAHFAs on an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, USA) with a mobile phase of 100% methanol at a flow rate of 0.3 mL/min.

The LC–MS/MS analysis of samples was performed on an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, USA) coupled with an UltiMate 3000 UHPLC System (Thermo Fisher Scientific, USA). The LC separation was conducted using an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters). The mobile phases A and

B were ACN/water (6:4, v/v) containing 0.1% formic acid and IPA/ACN (9:1, v/v) containing 0.1% formic acid, respectively. The flow rate and oven temperature were set at 0.4 mL/min and 40 °C, respectively. The following gradient was applied: 0–26 min from 20 to 90% B, 26–35 min 90% B, and 35–37 min re-equilibrate at 20% B. The MS analysis was performed under positive ion mode with full scan detection (MS^1, m/z 300–700) at the resolution of 60 000. ESI conditions employed were as follows: heater temperature, 300 °C; sheath gas flow, 35 arbitrary; auxiliary gas flow, 15 arbitrary; spray voltage, 3.5 kV; capillary temperature, 350 °C. MS/MS analysis was performed with data-dependent acquisition (DDA) mode in an ion trap with 2 m/z of isolation width, 10 ms of activation time, and 0.25 of activation Q. MS^2 fragmentation of DMED-FAHFAs was acquired under collision induced dissociation (CID) at 30% normalized collision energy (NCE).

Computational Methods.

The original FAHFA in silico library template[8] was downloaded from [https://fiehnlab.ucdavis.edu/projects/fahfa-lipids-library]. The InChI code (IUPAC International Chemical Identifier)[13] from the library was converted with ChemAxon Molconvert to SDF and SMILES format. The ChemAxon SMILES file was utilized by replacing the carboxyl group “(O)=O” to form a [2-(dimethylamino)ethyl]amide group “(=O)NCCN(C)C”. A similar process is also possible by using ChemAxon Reactor and a combinatorial chemistry approach. From the transformed SMILES, the related InChiKeys and all other physicochemical properties were obtained using the cxcalc software (cxcalc calculator, version 18.23.0, ChemAxon Ltd., www.chemaxon.com). Subsequently, the octanol–water partition coefficients (logP) and pH-dependent logD values, accurate masses, and molecular formulas were calculated with ChemAxon cxcalc. Additional SMILES and standard InChiKeys were calculated with OpenBabel.[14]

The library itself was created with 33 fatty acids ranging from C14:0 to C24:6, with the numbers describing the carbon number count followed by the degree of unsaturation. The abbreviations of the involved fatty acids are summarized in Table 1. A total of 4290 unique structures were obtained, covering 264 individual FAHFA classes. Depending on the carbon count, between 12 to 22 regioisomers are contained in each class. These isobars have the same precursor and also the same MS/MS fragmentation pattern, but different structures and InChiKeys. In many cases, the logP and logD values are very similar within each class. The nomenclature for FAHFAs was derived from abbreviations of the individual fatty acids; however, different abbreviations[15] can be added to the library if needed.

The original FAHFA EXCEL template was then modified to add specific precursor information for protonated molecules $[M + H]^+$ and their associated product ions and peak abundances. The product ion information was based on fragmentation investigation using the Mass Frontier 8.0 Spectral Interpretation Software (HighChem LLC and Thermo Fisher Scientific Inc.). The information for the fragments was based on 30% NCE CID-MS/MS acquisition as shown in Figure 2, which gave four specific fragments without any noise ion peaks. The precursor ion is commonly observed at 100% abundance, and the first loss is based on the dimethylamino group or C_2H_7N (−45.0578 Da). The following peaks are two related product ions and are based on the fatty acyl loss and an associated loss of C_2H_7N

(−45.0578 Da). The abundances were modeled similar to the observed abundances from the experimental reference MS/MS spectra.

Five representative spectra from measured reference compounds were used for development (DMED-9-PDAHSA, DMED-10-PAHMA, DMED-9-PAHPA, DMED-5-SAHPA, DMED-5-OAHSA). The remaining 34 spectra were used for validation purposes. SMILES codes, InChiKeys, and logP and logD values were updated, and the Visual Basic for EXCEL code was adapted to export NIST formatted MSP spectral files. These MSP files can be imported into the NIST MS Search software or the MS-Dial LC–MS/MS data processing software.[16]

RESULTS AND DISCUSSION

Underivatized FAHFAs commonly carry a negative charge in ESI-MS mode, causing low MS sensitivity. After labeling with DMED, FAHFAs were converted into DMED-FAHFAs to be detected in the more sensitive positive ESI-MS mode as shown in Figure 1. Yet, without a DMED-FAHFA mass spectral library, identifying novel, unknown DMED-FAHFA species would rely on tedious manual investigations. We therefore first investigated MS/MS fragmentations patterns of 35 DMED-FAHFA reference compounds by UHPLC–orbital ion trap high-resolution tandem mass spectrometry to develop and validate a generic in silico MS/MS DMED-FAHFA library. The theoretical MS/MS spectra were validated by experimental reference spectra and utilized to annotate DMED-FAHFAs from *Arabidopsis thaliana*.

Fragmentation Pattern Investigation.

The development of the DMED-FAHFA in silico library relied on heuristic information on MS/MS fragmentation patterns obtained by experimental spectra of the target compounds. Thus, studying the fragmentation pattern of DMED-FAHFAs was the first step. In order to obtain rich fragmentation information, high-resolution MS/MS spectra of DMED-5-PAHSA were acquired using CID collision energies at 30 %, 40%, and 50% NCE as representatives of DMED-FAHFA lipids. As shown in Figure 2, the 30% NCE spectrum yielded four fragment ions, while those at 40% and 50% NCE only gave three product ions. A detailed investigation of the DMED-5-PAHSA structure revealed reproducible fragmentation patterns. The peak at m/z 609.5935 represented the molecular ion $[M + H]^+$. The fragment ion peak at m/z 564.5358 was generated from the neutral loss of the dimethylamino group or C_2H_7N (−45.0578 Da) $[M + H - C_2H_7N]^+$ of the molecular ion. The product ion at m/z 353.3529 was annotated as the loss of an acyl group $[M + H - \text{acyl}]^+$, in this case, a palmitoyl group. Consequently, m/z 308.2591 represented the loss of both the palmitoyl group and dimethylamino group $[M + H - \text{acyl} - C_2H_7N]^+$. We further analyzed representative spectra in the MS/MS prediction software MassFrontier 8.0 as shown in Figure 3. To confirm the fragmentation rules learned from the 30% NCE CID-MS/MS spectrum of DMED-5-PAHSA, the 30% NCE high-resolution CID-MS/MS spectra of four more PAHSA positional regioisomers, including 9-PAHSA, 10-PAHSA, 12-PAHSA, and 13-PAHSA, and eight FAHFAs from eight different families, including 5-MAHMA, 5-MAHPA, 5-PAHMA, 5-PAHPA, 5-PDAHPA, 5-SAHMA, 5-SAHPA, and 9-PDAHSA, were investigated. Figure S1 demonstrates that no major differences of MS/MS fragmentation

patterns were observed in either positional regioisomers or different FAHFA families. This finding supported the notion that all DMED-FAHFAs might follow the same fragmentation rules under CID at 30% NCE. We therefore applied the obtained experimental fragmentation patterns and abundance values as heuristic information for the development of the complete DMED-FAHFA in silico library.

Validation of the In Silico Library.

The validation of the newly developed in silico DMED-FAHFA library was performed using two different data processing tools, specifically NIST MS Search 2.3 and MS-DIAL 4.00.[16] High-resolution MS/MS spectra of 35 DMED-FAHFAs reference standards were obtained by direct infusion and then submitted for annotation using NIST MS Search 2.3 with the new in silico DMED-FAHFA library. Supplement Figure S2 shows that all experimental MS/MS spectra were correctly annotated to their corresponding FAHFA families by the in silico DMED-FAHFA library with a reverse-dot score greater than 880.[17] DMED-FAHFAs cannot be annotated to specific regioisomers or stereoisomers, because they exhibit similar fragmentation patterns. Therefore, MS/MS spectra alone are not sufficient for unique assignment but require additional separation techniques.

As further validation, a mixture containing 26 DMED-FAHFAs was analyzed by UHPLC–Orbitrap MS, and the obtained RAW data was processed using MS-DIAL 4.00 with the in silico DMED-FAHFA library as the reference library. In the RAW data, retention time–MS^1 precursor ion peaks were clearly observed for all DMED-FAHFAs with limits of detection ranging from 3.6 to 208.3 fg (see Table S1). Yet, due to relatively slow MS^2 scan rates of the LTQ Orbitrap MS and lower responses of some DMED-FAHFA standards, only 21 of the standards triggered MS/MS data acquisitions. Using MS-DIAL 4.00, 15 DMED-FAHFAs were correctly annotated to their specific class with a reverse-dot score and total score greater than 636 and 795 (see Table S1). Six DMED-FAHFAs were missed by MS-DIAL that were either caused by relatively low intensities or due to coelution with other DMED-FAHFAs. Based on validation with authentic standards, we rationalized that MS/MS spectra with a reverse-dot score greater than 600 may serve to annotate DMED-FAHFA.

In order to investigate the rate of false-positive assignments using the DMED-FAHFA in silico library, we performed an additional test by matching 574 826 MS/MS spectra contained in the NIST17 mass spectral library against the in silico library. The precursor search width was set to 10 ppm, and the product ion width was set to 20 ppm. No false-positive hits were observed for the CID library at 30% and 50% NCE, leading to the conclusion that very low false-positive assignments can be expected during applications. A further analysis of the precursor values in the library revealed very unique precursor values, introduced by the derivatization process, leading to an extremely high specificity during untargeted search data processing.

A subsequent search of all the MS/MS spectra against the library itself revealed that all the 264 FAHFA classes were correctly identified. As mentioned before, stereoisomers and regioisomers could not be correctly assigned, because within a single DMED-FAHFA class, all in silico spectra show exactly the same fragmentation pattern.

In summary, the developed DMED-FAHFA in silico library is able to successfully annotate DMED-FAHFA from both direct infusion and LC–MS/MS data with high specificity. The library is compatible with multiple data processing tools and can lead to the detection of known but also novel FAHFA compounds.

Application of the DMED-FAHFA In Silico Library.

To further evaluate the performance of the in silico library, complex real samples, *Arabidopsis thaliana* shoots, were extracted and labeled with DMED. The samples were analyzed by UHPLC–LTQ Orbitrap MS/MS. The endogenous FAHFAs were annotated by matching experimental spectra to the in silico DMED-FAHFA library with MS-DIAL 4.00. A total of 19 DMED-FAHFAs from 16 FAHFA families were annotated using a 5 mDa MS^1 tolerance and a 10 mDa MS/MS tolerance. Every annotation was manually checked. We obtained reverse-dot-product scores of greater than 622 and total match scores of more than 656 according to the criteria obtained from authentic standards. Detailed information on the identified DMED-FAHFAs is summarized in Table 2. A total of 16 annotated FAHFA derivatives were previously reported. Chromatograms and MS/MS spectra of two representative DMED-FAHFAs are illustrated in Figure 4. Class information on the two compounds as DMED-PAHSA and DMED-SAHSA lipids was based on MS/MS spectra matching against the new in silico library, shown in Figure 4A,D. By comparing the retention time to the standards as shown in Figure 4B,C and Figure 4E,F, the ester positions of the two DMED-FAHFAs were annotated as specific regioisomers DMED-9-PAHSA and DMED-12-SAHSA. Due to the complexity of the *Arabidopsis thaliana* matrix, some interfering compounds coeluted with DMED-9-PAHSA and DMED-12-SAHSA within the Q^1 inclusion window, introduceing noise peaks in the experimental MS/MS spectra. DMED-9-PAHSA and DMED-12-SAHSA were annotated at the highest confidence level 1 of the metabolomics standards initiative (MSI),[18] because these annotations were based on the combined information on matching retention times, *m*/*z*, and MS/MS spectra of authentic standards. Interestingly, we have here annotated three novel DMED-FAHFAs that have never been detected endogenously in biological samples before, specifically POHHDA, ALAHLIA, and ALAHPA. Figure 5 shows the chromatograms, experimental spectra, and the in silico MS/MS spectral matches for these novel FAHFAs. The peak in Figure 5A was annotated as DMED-POHHDA, as all four signature product ions were perfectly matched to the in silico MS/MS spectra with a reverse-dot-product score of 622, indicating a high confidence of the annotation. For the annotations of DMED-ALAHPA and DMED-ALAHLIA in Figure 5B,C, all four signature fragment ions were found and matched those in the DMED-FAHFA in silico library. Additional fragment ions were interpreted as generated from coeluting compounds. These annotations are categorized as MSI level 3, because peaks were matched to *m*/*z* and in silico MS/MS spectra[18] but not confirmed by authentic standards, because these three novel FAHFA compounds are not commercially available yet.

Our previously reported work[11] employed UHPLC–triple quadrupole mass spectrometry to screen for DMED-FAHFA in *Arabidopsis*. The theory behind the strategy was that all fragment peaks observed in the multiple-reaction monitoring transitions of $[M + H]^+$ to $[M + H - \text{acyl} - C_2H_7N]^+$ could be potential DMED-FAHFAs. However, other compounds might

also show the same neutral loss without being DMED-FAHFAs, and hence, annotations based on a single (nominal-mass) MRM transition will likely have high false-positive rates. To test for such false-positive rates, we screened our UHPLC–LTQ Orbitrap MS/MS RAW data with a precursor and fragment ion tolerance at 0.6 Da for neutral losses of $[M + H - acyl - C_2H_7N]^+$ from $[M + H]^+$ precursor ions, to mimic the mass accuracy of a triple quadrupole MS. As given in Table S2, 24 potential DMED-FAHFAs were found based on the strategy; however, 11 peaks turned out to be false positives. Instead, using DMED-FAHFA annotations based on accurate masses and the new in silico library enabled accurate *m*/*z* matching of all four signature fragment ions, boosting overall compound annotation confidence. We propose the in silico DMED-FAHFA library as a reliable tool for FAHFA profiling in complex real samples.

Challenges and Outlook.

A total of 33 of the most common fatty acyl building blocks (from C14:0 to C24:6, Table 1) were used for building novel FAHFA compounds and their associated DMED derivatives. However, additional lower carbon counts than C14:0 and even very long chain fatty acyls chains (VLC-PUFA) have been observed in diverse matrices.[19] Here, the freely available and public EXCEL templates can be used to include additional species on demand.

The derivatized DMED-FAHFAs are chiral compounds;[20] hence, two enantiomers (*R*/*S*) are always chemically possible. Dependent on the number of double bonds, up to 128 stereoisomers (*E*/*Z*) could be theoretically observed. However, the library itself cannot be used to distinguish between stereoisomers and regioisomers. Chiral compound detection (tetrahedral *R*/*S* isomers) would require chiral column separations.[20] Double-bond isomers (*E*/*Z*) could be potentially separated, but the library does not contain any specific information to assign correct isomers. For further distinction of stereoisomers or regioisomers, additional orthogonal separation strategies such as multiple-stage mass spectrometry or ion mobility have to be used.[21] Specifically, the annotation of regioisomers can be resolved by retention time of reversed phase LC–MS/MS, which showed a strong correlation between retention times of DMED-FAHFA regioisomers and positions of the ester bonds.[11] A corresponding retention time model $\log_{10}k$ versus ester bond position was successfully built.[11]

False-positive identifications are always a concern. Here, confirmation with authentic reference compounds could increase the level of confidence. Cayman Chemical (https://www.caymanchem.com/) provides a wide range of biologically active FAHFA authentic reference standards, and different enantiomers have been synthesized.[20,22]

Interestingly, PAHSA-containing triacylglycerols (FAHFA-TG) have been recently described,[23,24] widening the array of known esterified lipids or estolides. The full mode of action, biology, and associated FAHFA pathways are not yet fully understood,[25–27] but the improved detection of FAHFAs in mammalian[28] and plant cells[4] opens a wide field of potential novel and exciting discoveries.

CONCLUSIONS

We have created an in silico library of 4290 high-resolution tandem mass spectra of DMED-FAHFA lipids covering 264 individual classes. The library is compatible with NIST MS Search and MS-DIAL and can be used to profile novel or yet unknown FAHFA lipids in plant or diverse other matrices. In comparison to the previously reported MRM method, the developed in silico library is helpful in reducing the false-positive rate of FAHFA identification and enabled high-throughput FAHFA annotation using high-resolution tandem mass spectrometry. All spectra, structures, and development tools are freely available at https://zenodo.org/record/3606905.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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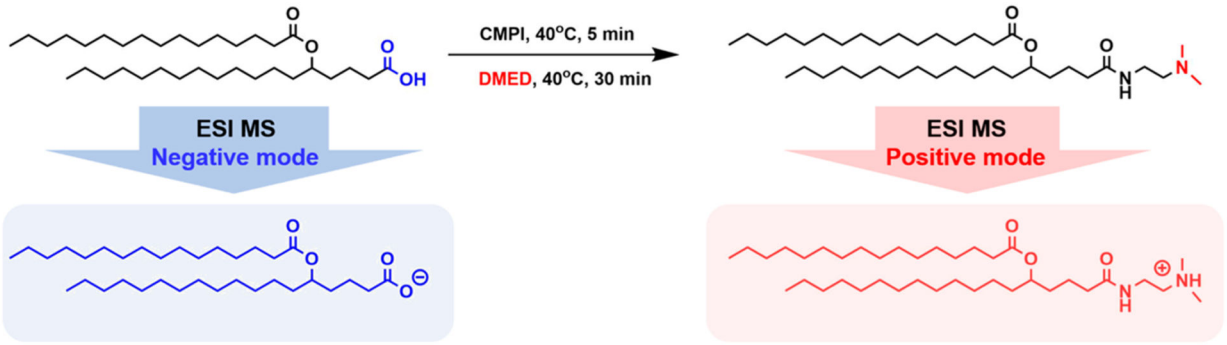

Figure 1.

DMED based FAHFA derivatization. A derivatization of FAHFAs is performed to increase detection limits and signal-to-noise ratios. The introduction of the [2-(dimethylamino)ethyl]amide group allows for easy protonation under acidic buffer conditions. The charged molecule greatly enhances the sensitivity in electrospray based LC–MS/MS measurements.

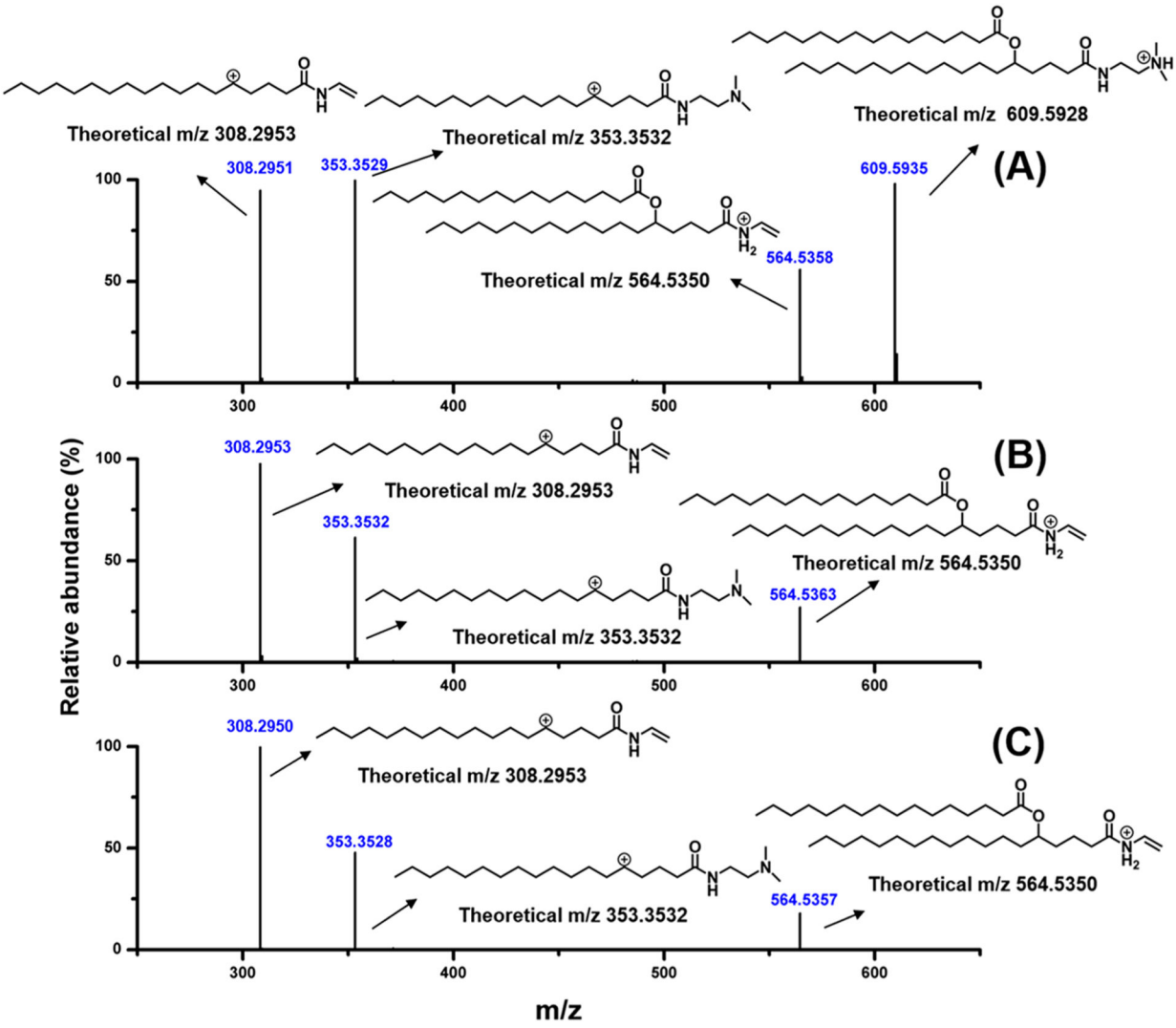

Figure 2.

MS/MS spectra of DMED-5-PAHSA at three CID energies of 30% (A), 40% (B), and 50% NCE (C). DMED-5-PAHSA (1 μg/mL) was analyzed via direct infusion on an LTQ-Orbitrap MS.

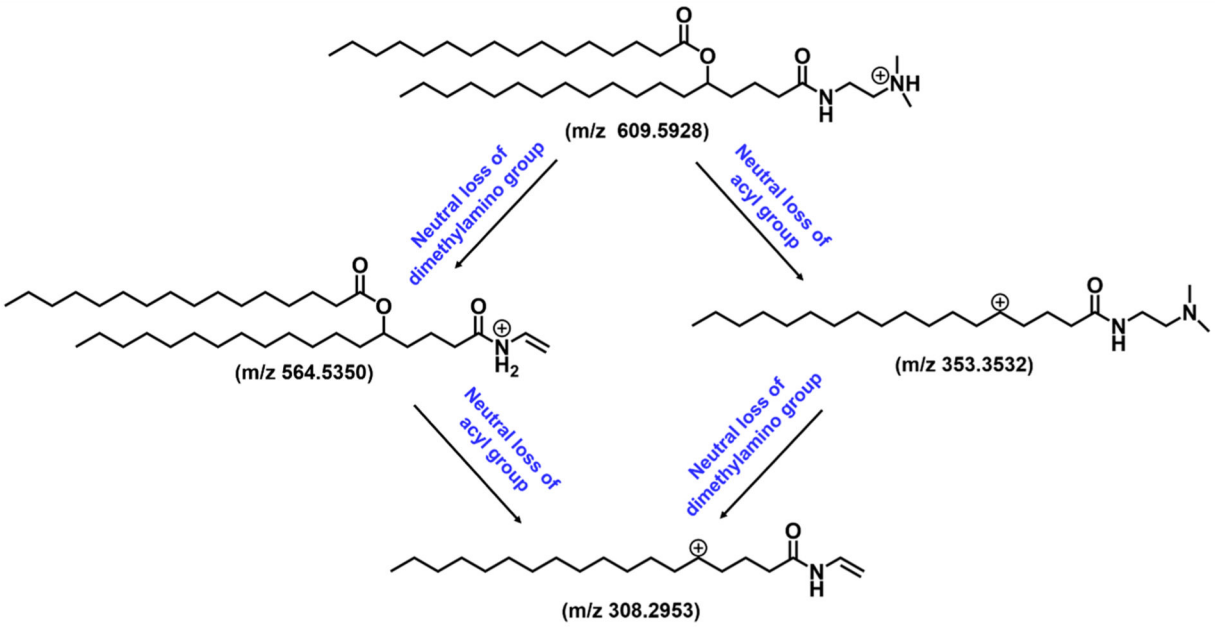

Figure 3.

Fragmentation reactions of DMED-FAHFAs, here DMED-5-PAHSA (*m*/*z* 609.5928). All four major product ion peaks are explained in the reaction, which was based on Mass Frontier 8.0 fragmentation prediction analysis.

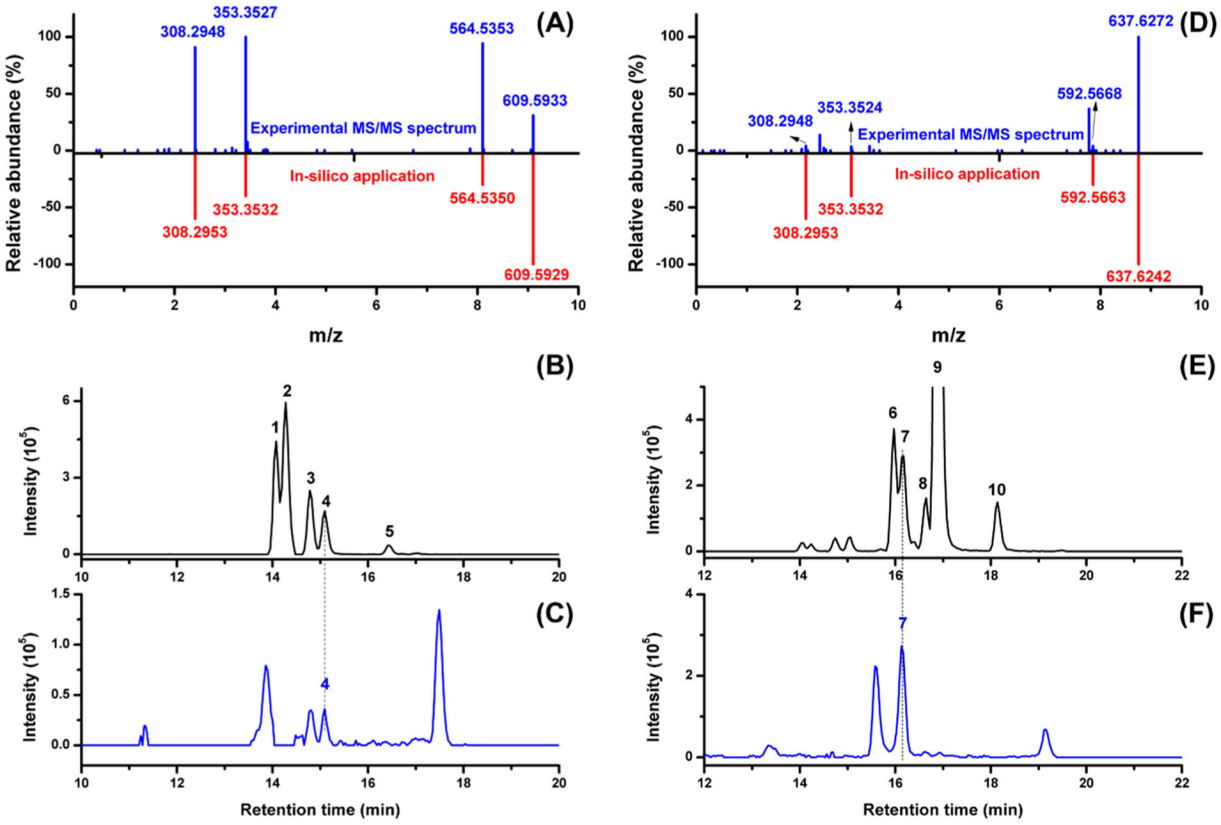

Figure 4.

MS/MS spectra of endogenous DMED-9-PAHSA annotated in *Arabidopsis thaliana* (A) and chromatograms of DMED-PAHSAs in standard solution (B) and *Arabidopsis thaliana* (C). MS/MS spectra of endogenous DMED-12-SAHSA annotated in *Arabidopsis thaliana* (D) and chromatograms of DMED-SAHSAs in standard solution (E) and *Arabidopsis thaliana* (F). Peaks: (1) DMED-13-PAHSA, (2) DMED-12-PAHSA, (3) DMED-10-PAHSA, (4) DMED-9-PAHSA, (5) DMED-5-PAHSA, (6) DMED-13-SAHSA, (7) DMED-12-SAHSA, (8) DMED-10-SAHSA, (9) DMED-9-SAHSA, (10) DMED-5-SAHSA.

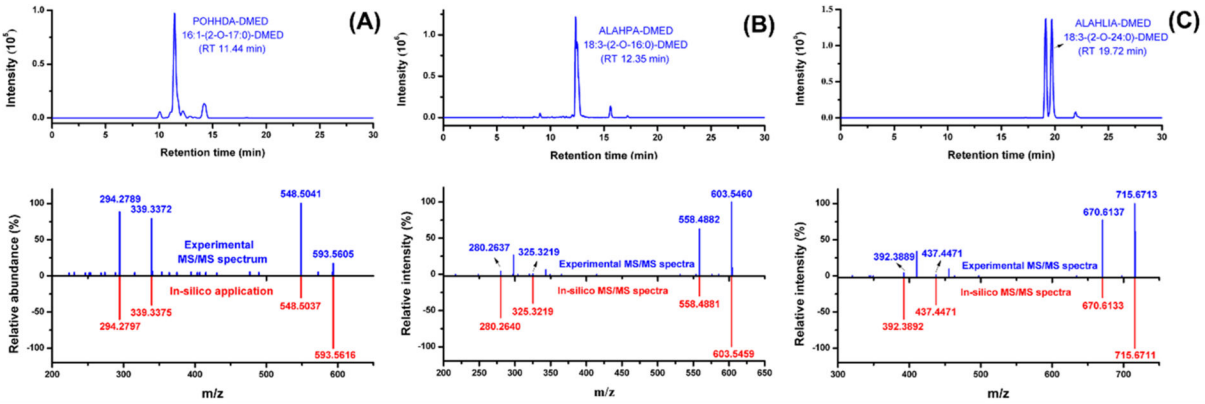

Figure 5.

Chromatogram and MS/MS spectra of novel endogenous DMED-FAHFAs annotated in *Arabidopsis thaliana*, including DMED-POHHDA (A), DMED-ALAHPA (B), and DMED-ALAHLIA (C).

Table 1.

Fatty Acyls Covered in the DMED-FAHFA Library[a]

ID	C:D	abbrev	Δ^x	full name
1	14:0	MA		myristic acid
2	14:1	MO	cis-Δ9	myristoleic acid
3	15:0	PDA		pentadecanoic acid
4	15:1	PDEA	cis-Δ10	pentadecenoic acid
5	16:0	PA		palmitic acid
6	16:1	PO	cis-Δ9	palmitoleic acid
7	17:0	HDA		heptadecanoic acid
8	17:1	HDEA	cis-Δ10	heptadecenoic acid
9	18:0	SA		stearic acid
10	18:1	OA	cis-Δ9	oleic acid
11	18:2	LA	all-cis-Δ9,12	linoleic acid
12	18:3	ALA	all-cis-Δ9,12,15	a-linolenic acid
13	18:4	SDA	all-cis-Δ6,9,12,15	stearidonic acid
14	20:0	AA		arachidic acid
15	20:1	EA	cis-Δ11	eicosenoic acid
16	20:2	EDA	all-cis-Δ11,14	eicosadienoic acid
17	20:3	ERA	all-cis-Δ11,14,17	eicosatrienoic acid
18	20:4	ARA	all-cis-Δ5,8,11,14	arachidonic acid
19	20:5	EPA	all-cis-Δ5,8,11,14,17	eicosapentaenoic acid
20	22:0	BA		behenic acid
21	22:1	ERU	cis-Δ13	erucic acid
22	22:2	DDA	all-cis-Δ13,16	docosadienoic acid
23	22:3	DTRA	all-cis-Δ13,16,19	docosatrienoic acid
24	22:4	DTA	all-cis-Δ7,10,13,16	docosatetraenoic acid
25	22:5	DPA	all-cis-Δ7,10,13,16,19	docosapentaenoic acid
26	22:6	DHA	all-cis-Δ4,7,10,13,16,19	docosahexaenoic acid
27	24:0	LIA		lignoceric acid
28	24:1	NA	cis-Δ15	nervonic acid
29	24:2	TDA	all-cis-Δ15,18	tetracosadienoic acid
30	24:3	TTRA	all-cis-Δ9,15,18	tetracosatrienoic acid
31	24:4	TTA	all-cis-Δ9,12,15,18	tetracosatetraenoic acid
32	24:5	TPA	all-cis-Δ9,12,15,18,21	tetracosapentaenoic acid
33	24:6	THA	all-cis-Δ6,9,12,15,18,21	tetracosahexaenoic acid

[a]The current implementation covers one adduct or ion form $[M + H]^+$ at CID energy of 30% NCE and 33 of the most important building block fatty acyls.

Table 2.

Annotated FAHFAs Found in *Arabidopsis thaliana* Using the Novel DMED-FAHFA Library

number	name	abbreviation	retention time (min)	*m/z*	reverse-dot score	total score	MSI level
1	18:3-(O-24:0)-DMED	DMED-ALAHLIA	19.7	715.6726	745	819	3
2	18:3-(O-16:0)-DMED	DMED-ALAHPA	12.3	603.5464	813	922	3
3	22:6-(O-18:0)-DMED	DMED-DHAHSA	13.2	681.5942	842	729	3
4	17:0-(O-18:0)-DMED	DMED-HDAHSA	15.7	623.6090	825	900	3
5	18:2-(O-16:0)-DMED	DMED-LAHPA	14.2	605.5623	819	923	3
6	18:1-(O-16:0)-DMED	DMED-OAHPA	15.6	607.5763	832	926	3
7	18:1-(10-O-18:0)-DMED	DMED-10-OAHSA	14.8	635.6025	830	899	1
8	16:0-(O-15:0)-DMED	DMED-PAHPDA	14.7	567.5465	819	908	3
9	16:0-(O-18:0)-DMED	DMED-PAHSA	14.2	609.5873	771	860	3
10	16:0-(9-O-18:0)-DMED	DMED-9-PAHSA	15.1	609.5933	835	896	1
11	16:0-(10-O-18:0)-DMED	DMED-10-PAHSA	14.8	609.5935	809	881	1
12	15:0-(O-18:0)-DMED	DMED-PDAHSA	14.1	595.5779	840	908	3
13	16:1-(O-17:0)-DMED	DMED-POHHDA	11.4	593.5566	622	736	3
14	16:1-(O-18:0)-DMED	DMED-POHSA	12.1	607.5777	786	879	3
15	18:0-(O-16:0)-DMED	DMED-SAHPA	17.5	609.5936	832	928	3
16	18:0-(12-O-18:0)-DMED	DMED-12-SAHSA	16.2	637.6244	863	922	1
17	18:0-(O-18:0)-DMED	DMED-SAHSA	15.6	637.6249	625	846	3
18	14:0-(O-15:0)-DMED	DMED-MAHPDA	12.3	539.5151	802	880	3
19	16:0-(O-16:0)-DMED	DMED-PAHPA	15.6	581.5623	811	656	3