The University of South Bohemia in České Budějovice

Faculty of Science

Analysis of hydroxy fatty acids using GC-EI-MS and HPLC-ESI-MS

Master thesis

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Annotation:

The analysis of three different Hydroxyeicosatetraenoic acid in human blood plasma shall be determined using gas chromatography with electron ionization and high pressure liquid chromatogtaphy with electron spray ionization. The results shall be compared in terms of sensitivity, linearity and simplicity. Additionally the extraction of the lipids via protein precipitation shall be optimized.

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 26.04.2021

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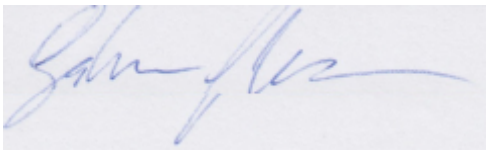

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1. Abstract

The goal of this thesis is to develop a method for the analysis of free hydoxyeicosatetraenoic acids (HETE) found in blood plasma via GC-EI-MS or HPLC-ESI-MS. HETEs are of importance for several biological functions such as inflammatory responses and were also found to be related to the development of cancer cells. Thus, the ability to quantify them reliably is of importance. Gas chromatography (GC) has several desirable properties for the analysis of biological fluids, including minimal matrix effects, great separation efficiency and resolution, while also being a comparatively low-cost method. High performance liquid chromatography (HPLC) on the other hand does not require derivatization prior to analysis and higher sensitivities may be achieved.

The lipids were extracted via protein precipitation. Analysis was carried out using GC as well as HPLC separation coupled to mass spectrometric detection employing electron ionization (EI) and electrospray ionization (ESI) with a triple-quadrupole (QqQ) mass analyzer. Deuterated HETEs were used as internal standards (ITSD) for calibration.

In GC analysis the need for derivatization resulted in side products that complicated the spectrum and chromatogram, interfering with the quantification and method development. The inability to remove excess reagents due to the volatility of the derivatized analyte puts additional strain on the liner and GC system. Additionally, the hard electron ionization appears to induce heavy fragmentation, possibly reducing the sensitivity of the method and increasing the limit of quantification. HPLC provided excellent separation of the analytes and the run time per sample was reduced to half of the GC method. The lack of derivatization also removed the need of evaporating excess reagents and eliminated a possible source of error. Furthermore, the gentler ionization generated mostly the molecular ion, which simplified the setup of a sensitive multiple reaction monitoring (MRM) method due to the higher intensities of fragments formed from the single precursor.

HPLC-ESI-QqQ analysis was found to be the superior method for the analysis of HETEs. The simpler sample preparation procedure and shorter run time speed up the analysis considerably. Furthermore, the elimination of the unreliable derivatization procedure, makes the analysis simpler and eliminates the risk of wasting the expensive isotope-labeled lipids, which are necessary to account for matrix effects observed in HPLC.

2. Introduction

Arachidonic acid is an important polyunsaturated fatty acid needed for the synthesis of several lipids controlling physiological pathways. One subgroup of lipids produced from arachidonic acid by pathways controlled via several lipoxygenases are hydoxyeicosatetraenoic acids (HETEs). Common oxidation sites in human peripheral blood polymorphonuclear leukocytes were found to be the 5-, 12- and 15 position [1]. Major expression sites for 5-lipoxygenase are leukocytes [2], platelets for 12-lipoxygenase [3] and certain myeloid cells for 15-lipoxygenase [4].

Assigning a specific function to one of these eicosanoids is difficult since the biosynthesis of these lipids often occurs in tandem with the formation of different substances, making identification of the effect of a single compound challenging.

While it was previously assumed that the majority of eicosanoids was composed of free fatty acids, more recent studies suggest that HETEs can be incorporated into phospholipids of different kind by several cell types [5; 6]. In case of lung epithelial cells 5-HETE and 12-HETE are mainly incorporated into the more common phosphatidylcholine while 15-HETE is found in the less frequent phosphatidylinositol [7].

The HETEs and their derivatives show a wide range of different biological functions. For example, the 5-HETE derivative 5-oxo-eicosatetraenoic acid (5-oxo-ETE) is proven to play a role in control of neutrophile activity [8]. Functions of 12-HETE are assumed to include inhibition of platelet aggregation by PGH2 [9] and it may have effects on the senescence of epithelial cells through increasing the expression of NADPH oxidase [10]. While the physiological effects of 15-HETE are not completely understood, high levels can be found in eosinophiles [11] and tracheal epithelial cells [12]. Reticulocytes also express 15-lipoxygenase at certain stages, which may indicate an effect on cell development [13]. Furthermore 15-HETE is a precursor of certain lipoxins in some cells, thus playing a role in inflammatory events [5]. Recent experiments also suggest the ability of 15-HETE to increase the expression of cyclooxygenase-2 in the presence of IL-1β which consequently causes an increase in the rate of production of prostaglandins [14].

Furthermore, 5- and 12-HETE have been found to be connected to cancer cells. They were observed to produce 5-HETE [15; 16] and oxidation products such as 5-oxo-ETE which may support proliferation in some cell lines [17]. Tumor cells have also been observed to produce 12-HETE [18]. Further experiments showed that 12-HETE was vital for the survivability via

prevention of apoptosis [19] and improved expression of certain integrins [20]. Additionally to enhancing the longevity of cancer cells 12-HETE also promotes metastasis via rearrangements in the cytoskeleton of some cell lines [21] and is simplifying access to the blood stream by facilitating release of the lysosomal cysteine protease Cathepsin B [22]. The significance of 15-HETE in this context is largely unknown. Due to the physiological effects of these HETEs and their metabolites, they are of increasing interest to researchers. Regarding their analysis it is important to note that plasma shows a lower concentration for all analytes compared to serum according to some literature [23]. Thus, plasma may resemble physiological conditions more closely.

Prior to analysis it is necessary to extract the lipids of interest. Several methods such as protein precipitation, liquid-liquid extraction and solid phase extraction (SPE) are commonly used for sample preparation and yield good results as demonstrated by Ostermann et al. [24]. SPE was found to be the most suitable method for the preparation of biological samples, since it has the potential to reduce matrix effects if chosen correctly [25]. However, a major drawback of certain SPE columns for the analysis of polyunsaturated fatty acid is the risk of oxidation in the presence silica gel [26]. This problem can be avoided by using liquid-liquid extraction or protein precipitation, which also yield good results. The latter is especially viable due to its simplicity [24]. Matrix effects can complicate HPLC-ESI analysis as they tend to cause ion suppression which results in a lower signal intensity. This is due to the large number of molecules found in such samples, which may elute together with the substances of interest. While they may not appear in the chromatogram through use of methods such as MS/MS analysis, they can still compete with the analyte during ionization and suppress the signal [27; 28]. Another problem one might run into is the possibility of oxidation of the fatty acids during sample extraction. Antioxidants such as butylated hydroxytoluene (BHT) are proven to prevent oxidation of polyunsaturated fatty acids in dried blood spots [29] and have also been used during sample extraction previously [30]. Another aspect to consider is the possibility of increasing or decreasing HETE concentration, which may be observed when blood plasma or serum samples are stored over an extended period of time or thawed and frozen several times. Taking this behavior into account only once thawed samples should be used for analysis [31].

To properly profile various lipids different methods are often necessary. GC-MS is commonly used for the analysis of steroids and free fatty acids. However, gas chromatography requires the analyte to be volatile which is often not the case for lipids. Thus, it is necessary to modify certain functional groups via derivatization. A common

procedure employed to increase the volatility is the formation of trimethyl silyl esters (TMS). Well established substances used are *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide and N,O-bis -(trimethylsilyl)trifluoroacetamide (BSTFA) [32–34]. Another option is the modification with groups undergoing dissociative electron capture such as pentafluorobenzyl (PFB). These compounds can greatly increase the sensitivity by improving the ionizability when using appropriate ionization sources [35]. This type of modification can be used in HPLC methods such as atmospheric pressure chemical ionization (APCI) but also in electron ionization (EI) which is commonly used for GC analysis in combination with TMS derivatization [36].

HPLC-MS is generally used to detect neutral lipids and phospholipids. The biggest advantage of this method over GC-MS is that no derivatization is necessary. To separate the lipids reversed phase chromatography or hydrophilic interaction chromatography is often used. Further separation can be achieved by addition of modifiers such as formic acid to the mobile phases, as this keeps the fatty acids protonated, enhancing the retention to the stationary phase.

A further increase in sensitivity of mass spectrometric methods can be achieved by use of MS/(MS) techniques, which reduce random noise through certain conditions that need to be met for a signal to be recorded. This can be done by using methods such as selected ion monitoring (SIM), where only a few selected m/z values of the complete spectrum are used for the measurement and multiple reaction monitoring (MRM) in which selected precursor ions are further fragmented via collision induced dissociation. The products of these collisions are dependent on characteristic properties of a compound such as mass and chemical structure.

The group of Yasukazu et al. [33] achieved derivatization of cholesterol, hydroxy cholesterols and linoleic acid using hydroxyhexadecanoic acid as an internal standard for GC analysis. After extraction, the solvent was evaporated and the analyte derivatized directly using pure BSTFA. After incubation at 60°C an aliquot of the residual derivatization solvent was directly used for injection. The omission of excess reagent evaporation eliminates a source of errors and may speed up the sample preparation process slightly. HPLC-ESI-MS of fatty acids was carried out in negative mode using water containing 2 mM ammonium acetate and methanol in acetonitrile (5:95) as the mobile phases. The HPLC as well as the GC method developed were successfully employed for quantification of biomarkers corresponding to certain diseases.

Derivatization of HETEs with PFB esters and trimethylsilyl ethers (TMS) for GC-EI-MS was tested by Wheelan et al. [36] and compared to derivatization with methyl esters. The observed results were similar to data collected using methyl ethers. This eliminated the need for the use of toxic or carcinogenic alkylating agents. Furthermore, the fragmentation was found to be directed by the position of the TMS ether. It was determined to occur in the presence of a double bond in the beta position relative to the derivatized alcohol, making assessment of the chemical structure possible.

Comparatively, after extraction Knott et al. [37] and Leis et al. [38] used BSTFA as well as PFB for the analysis of prostaglandins via GC-NICI-MS. After evaporation under nitrogen the samples were derivatized with PFB in ACN, and BSTFA in pyridine, among others. Knott et al. claim that 18 samples can be run per day using this method.

Meng Wang et al. [39] extracted and successfully analyzed several lipids, including hydroxy-unsaturated fatty acids via protein precipitation and a HPLC-IMS-QTof setup using methanol and acetonitrile (1:1) and water, both containing 0.1% formic acid (FA), as the mobile phases. The developed method was used for metabolomic profiling of acromegaly patients and quantification was possible without derivatization.

3. Goals of the thesis

The goal of this thesis is to compare gas chromatography (GC) and high-performance liquid chromatography (HPLC) for the quantification of free, unesterified HETEs in human blood plasma as well as the development and optimization of appropriate sample preparation and quantification methods. Different derivatization approaches, which are necessary for GC analysis shall be compared using 16-Hydroxyhexadecanoic acid as a model substance. Furthermore, the extraction of HETEs from human plasma shall be optimized to achieve the best recovery across all analytes of interest. To ensure a sensitive detection of the analyte it is required to identify characteristic fragments. For quantification, the goal was to develop MS/MS methods to reduce noise and lower the limit of quantification via optimization of collision energies and instrument parameters.

4. Materials and Methods

4.1. Chemicals

Table 1: *List of chemicals used, purity, manufacturer, and catalogue number*

Compound	Purity	Manufacturer	Catalogue number
Acetonitrile	≥99.9%	VWR Chemical	20060.320
N,O-Bis(trimethylsilyl)trifluoro-Acetamide with 1% trimethylchlorosilane (BSTFA)	99%	Sigma Aldrich	15238-10X1ML
2,6 – Di-tert-butyl-4-methylphenol (BHT)	≥99 %	Sigma Aldrich	B1378-1KG
n-Hexane	≥95 %	VWR Chemical	139386-2.5L
16-Hydroxyhexadecanoic acid (HHDA)	98%	Sigma Aldrich	177490-1G
Methanol	≥99.8%	VWR Chemical	20847.320
N,N-Diisopropylmethylamine	≥98 %	Sigma Aldrich	38431-10ML
2,3,4,5,6-Pentafluorobenzyl Bromide (PFB)	≥98.5%	Sigma Aldrich	90257-5G
Pyridine	≥99.8%	Sigma Aldrich	270970-25ML
5(S)-HETE	≥98%	Cayman Chemical	34230
12(S)-HETE	≥98%	Cayman Chemical	34570
15(S)-HETE	≥98%	Cayman Chemical	34720
5(S)-dHETE	≥99%	Cayman Chemical	334230
12(S)-dHETE	≥99%	Cayman Chemical	334570
15(S)-dHETE	≥99%	Cayman Chemical	334720

4.2. Materials

All solvents and reactants used are specified in Table 1. The mass detectors and chromatographic equipment used for analysis were purchased from Agilent Technologies and are specified in Table 2. The deuterated internal standards of 5-, 12- and 15-HETE and their non-deuterated counterparts necessary for analysis and calibration were manufactured by Cayman Chemicals and Sanova Pharma GesmbH and kindly provided by prof C. Gerner from the institute of analytical chemistry at the university of vienna. Aliquots of the Lipids dissolved in 25 µl acetonitrile at a concentration of 25 $\mu g \cdot ml^{-1}$ were diluted to a volume of 625 µl resulting in a final concentration of 1 $mg \cdot l^{-1}$ prior to use.

Table 2: *Designation of the instruments used.*

Setup	Instrument	Type	Series
GC-QqQ	QqQ	Agilent G7000D	Agilent 7000D GC/TQ
	Sampler	Agilent G2614A	Agilent 7638B
	GC-Oven	Agilent G3440A	Agilent 7980A
HPLC-QTof	QTof	Agilent 6510 Q-Tof	Agilent 6510 LC/MS
	Column compartment	Agilent G1316A	Agilent 1260 Infinity
	Sampler	Agilent G1329B	Agilent 1260 Infinity
	Pump	Agilent G1311B	Agilent 1260 Infinity
HPLC-QqQ	QqQ	Agilent G6420A	Agilent 1260 Infinity
	Column compartment	Agilent G1316A	Agilent 1260 Infinity
	Sampler	Agilent G1367E	Agilent 1260 Infinity
	Pump	Agilent G1311B	Agilent 1260 Infinity

4.3 Eicosanoid extraction and synthesis of derivatives

Prior to analysis all plasma samples were stored at -80°C and only thawed just before use. Extraction of the free eicosanoids was achieved via protein precipitation using a mixture of methanol and acetonitrile (1:1) as described by Fan et al. [40]. The precipitation agent was spiked with a mixture of all three internal standards to a final concentration of 5 $\mu g \cdot l^{-1}$ and BHT at a concentration of 25 $mg \cdot l^{-1}$ to avoid oxidation of the unsaturated fatty acids. 800 µl of the precipitation reagent were added to 200 µl of a pooled plasma sample obtained by mixing of plasma from 5 different individuals in a clean 1.5 ml glass HPLC vial. After vortexing for 30 seconds the samples were centrifuged at 4700 rpm at 4°C for 20 minutes. From the supernatant 800 µl could reliably be transferred to a new vial without disturbing the pellet and were then evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was taken up in 50 µl of ACN/MeOH and vortexed again for 30 seconds, increasing the concentration of plasma components by a factor of 3.2 compared to the original sample. Finally, the mixture was transferred into a fresh HPLC vial containing a glass insert due to the low volume of the sample and injected.

For the analysis of the compounds via gas chromatography a volatile sample is necessary. To achieve higher volatility, one needs to derivatize polar functional groups such as hydroxy groups and carboxylic acids. To optimize the derivatization process different procedures using pentafluorobenzyl bromide (PFB) and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were compared using 16-hydoxyhexadecanoic acid as a model substance. For the sake of simplicity all samples are diluted to 400 µl during method development to remove the need for inserts.

4.3.1 Derivatization with PFB in ACN

Using a modified version of protocol proposed by Leis et al. [38] the residue was taken up in 50 µl of a 7 w% solution of PFB in ACN after evaporation. Next 10 µl of the catalyst N-methyl diisopropylamine (DIPMA) were added and the mixture was incubated for 10 minutes at room temperature. Prior to injection the mixture was filled up to 400 µl with hexane.

4.3.2 Derivatization with BSTFA and pyridine

Derivatization with BSTFA and pyridine was tested using a modified procedure given by Leis et al. [38]. The solvent was evaporated and 50 µl of a mixture of BSTFA and pyridine (2:1) were added. The sample was then incubated at 60°C for 20 minutes. After cooling to room temperature, the sample was filled up to 400 µl with hexane

4.3.3. Derivatization with pure BSTFA

Lastly, a method based on the one provided by Yasukazo [33] et al. was used. 50 µl of BSTFA were added to the analyte after evaporation of the solvent and incubated at 60°C for 1 hour. The derivatized samples were left to cool to room temperature and filled up to 400 µl with hexane before injection.

4.3.4. Removal of excess reagents

Additionally, the effect of evaporation of the excess solvent was investigated. After the end of the reaction the excess solvent or reagent was evaporated to dryness under a gentle stream of nitrogen at 50°C. The residue was then redissolved in 400 µl of hexane and vortexed for 30 seconds before injection.

4.4. Preparation of standards

All working standards used during method optimization for GC-MS were prepared by serial dilution of a 1 $g \cdot l^{-1}$ stock solution obtained by dissolving 100 mg of 16-hydroxyhexadecanoic acid in 100 ml methanol. The dilution steps were carried out in glass HPLC vials. For derivatization 200 µl of each standard were transferred into a new vial and the solvent was removed by evaporation at 40°C under a nitrogen stream. Due to several problems during the development of the protocol and low signal intensities for derivatized HETEs in preliminary SCAN experiments no further measurements were conducted using the lipids and exceedingly expensive deuterated internal standard.

In contrast to the GC method, derivatization is optional in HPLC analysis and only increases the sensitivity of the method. Omitting it eliminates the risk of side product formation and reduces the time needed for preparation considerably. 100 µl of each of the non-deuterated

eicosanoid stocks were combined, filled up to 500 µl with MeOH/ACN (1:1) and two interdilutions with concentrations of 200 µg• l^{-1} (ID A) and 40 µg• l^{-1} (ID B) were prepared by dilution with MeOH/ACN (1:1) from which the remaining working standards were prepared. To correct for ion suppression caused by matrix effects equal volumes of the deuterated lipids were combined, resulting in a mixture with a concentration of 333.33 µg•l^{-1} for each labeled acid. To each calibration standard 48 µl of internal standard were added, resulting in a concentration of 32 µg• l^{-1} for each deuterated HETE. A summary of the preparation procedure is given in Table 3:

Table 3: *Preparation of calibration standards for HPLC analysis*

Concentration (µg • l^{-1})	Volume (µl)		Interdilution	V ITSD (µl)	V ACN (µl)
40	100	From	ID A	48	352
30	75	From	ID A	48	377
20	50	From	ID A	48	402
10	25	From	ID A	48	427
5	62,5	From	ID B	48	389,5
2	25	From	ID B	48	427

4.5. GC analysis

The GC method used was a slightly modified version of the variant used by Yakuzaku et al. [33]. An Agilent phenyl methyl siloxane column (Agilent 19091S-433, HP-5MS 5% Phenyl Methyl Siloxane, 30m x 250 µm x 0.25 µm) was chosen. A sample volume of 1 µl was injected at a temperature of 250°C and a flow of 1.2 ml⋅min^{-1} in pulsed split-less mode. Separation was achieved using a linear temperature gradient starting at 60°C and heating to 280°C with a slope of 10°C per minute and a transfer line temperature of 280°C. The final temperature was held for 5 minutes. Mass spectrometric analysis was carried out using an Agilent G7000D-QqQ instrument. Ionization was achieved using an electron ionization (EI) source operated at 70 eV. Latter MS/MS experiments were done using nitrogen as the collision gas.

Using the model substance as specified previously, method optimization was carried out using a single quadrupole instrument. Three standards containing 50, 30 and 10 mg ⋅l^{-1} of

the model substance were prepared in methanol, the solvent was evaporated to dryness and the residue derivatized according to the previously mentioned methods. The sample was injected and measured in SCAN mode for acquisition of a full spectrum of the substance. It was identified using concentration dependent peak heights and the molecular ion peak of the derivatized sample. The three major product ions formed were used for setting up a SIM method.

Attempts were made to develop an MRM method employing the GC-EI-QqQ setup. The known precursors were fragmented further using nitrogen as the collision gas. Energy optimization was done using the MassHunter Optimizer software with a 375 $\mu g \cdot l^{-1}$ standard used for injection

4.6. HPLC analysis

Due to the preparation of samples for GC-MS analysis being complicated through by-product formation related to the derivatization process, which was interfering with the quantification, variation between repeated measurements, limited sample stability and the possibility of the derivatization failing completely, the choice was made to switch to an MRM based HPLC-ESI method in negative mode without any derivatization. Due to the softer ionization method the formed number of fragment ions is lower, while their intensity is higher. For development of an appropriate MRM method the fragmentation pattern was recorded using a QTof mass detector. Based on the data a method for a QqQ instrument was written and the collision energies optimized.

Separation of the HETEs was carried out using a modification of the method reported by Meng Wang et al. [39]. The mobile phases were 0.1% formic acid in water (A), 0.1% formic acid in methanol (B) and 0.1% formic acid in acetonitrile (C) for analysis and pure acetonitrile to flush the column in between runs. For the preparation of HPLC mobile phases and aqueous samples Milli-Q water was used. It was purified using a Millipore Elix instrument. To ensure the run time was long enough for the system to return to the initial conditions before the next injection the pressure was recorded, and the run-time adjusted to give it enough time to stabilize. The detailed gradient used is given in Table 4.

Table 4: *Elution gradient for the separation of HETEs*

Time / min	mobile phase A	mobile phase B	mobile phase C
0.00	70.0%	15.0%	15.0%
2.50	40.0%	30.0%	30.0%
9.00	10.0%	45.0%	45.0%
10.00	0.0%	50.0%	50.0%
10.10	70.0%	15.0%	15.0%
14.00	70.0%	15.0%	15.0%

For chromatography, a C18 column (Poroshell 120 EC-C18, 2.7 µm, 3 * 75 mm) was used. 10 µl were injected at a flow rate of 0.6 $ml \cdot min^{-1}$ and the column compartment was kept at a temperature of 45°C for the duration of the run. The source gas temperature was set to 350°C with a flow of 10 liters per minute, a nebulizer pressure of 45 psi and a fragmentor voltage of 100 V. For further method development two different types of mass detectors were employed. To set up an MRM method a HPLC-ESI-QTof instrument was used for the identification of product fragments due to its higher resolution. Two standard mixtures, one containing the normal variants and the other the deuterated HETEs were prepared and measured in SCAN mode. From the full spectra the molecular ion peak (M^-) and specific as well as unspecific product ions were determined. Further experiments and the analysis of plasma samples were carried out in MS/MS mode on a HPLC-ESI-QqQ instrument using the precursor and product ions identified via the QTof mass detector. When plasma extracts were analyzed pure acetonitrile was used to flush the column in between each measurement and at the end of each sequence to clean the column of any acid residues and prevent carry over or damages that might be caused during down times.

4.6.1. Peak identification

Since the three eicosanoids are constitutional isomers and have the same molecular ion peaks and functional group losses during analysis it is necessary to identify the individual substances. By preparing standards for each of the lipids and injecting them individually the order of the peaks was determined. Using the given gradient 15-HETE eluted first, followed by 12- and 5-HETE. The internal standards eluted slightly earlier than the normal lipids. It is important to note that the deuterated fatty acids are not baseline separated from their un-deuterated counterparts. This is intended and required for the assessment of matrix effects at

a certain retention time, since the analyte and the internal standard will be affected equally. To observe their peaks individually and compare them in a physiological sample one must extract the chromatogram for each substance via their characteristic parent and product ions.

4.6.2 Identification of fragments

Using HPLC-ESI-QTof the characteristic fragmentation patterns were recorded at a collision energy of 10 eV with nitrogen as the collision gas. Characteristic product ions that were observed among all analytes were the loss of water and the loss of the carboxylic acid group, producing peaks at m/z values of 301.27 and 257.34, respectively. While these losses can be found in all HETEs with a single hydroxylation and are thus not very specific, they are suitable for optimization of parameters during the extraction.

These fragments had similar abundances in 15- and 5-HETE but were less prominent in 12-HETE resulting in a lower peak height.

The collision energies for both fragmentations were further optimized using HPLC-ESI-QqQ with nitrogen as a collision gas. The optimal conditions were found to be 5 eV for the observation of the water loss and 7 eV for the loss of the carboxylic acid.

To further increase the sensitivity and avoid unwanted peaks in the real plasma sample, resulting from other HETEs present, more specific fragments were chosen. The fragments appear to be formed via bond dissociation between the hydroxyl group and the unsaturation between the alpha and beta carbon, followed by a proton transfer from the alcohol to the cleaved fragment. The limit of quantification was improved considerably using these fragments.

5. Results

5.1. Results of the Derivatization

Derivatization with PFB and BSTFA was employed for GC analysis, while HPLC analysis was carried out without derivatization. The success of the methods was evaluated via the presence of a molecular ion peak or characteristic fragments and analysis of mass spectra using database searches. BSTFA was found to be superior compared to PFB modification. The results and possible reasons are explained in detail in the following paragraphs.

5.1.1. Derivatization of HHDA with PFB

All methods employing PFB as a derivatization agent were unsuccessful for this kind of fatty acid. The reason for this outcome was most likely the formation of an unexpected product. The mass spectrum of the analyte was found to match the one of the HHDA lactone. This theory was further supported by the presence of the alcohol of the derivatization agent in the chromatogram, despite using water free environment. The presence of a base that was needed for the derivatization of the carboxylic acid might have further catalyzed the nucleophilic attack of the terminal hydroxy group to the newly formed ester. Due to this reaction mechanism the derivatization with PFB was unsuitable for the analyte of interest. At high concentrations, another peak appeard in the chromatogram, which is likely to correspond to the desired derivatization product due to the observation of a characteristic fragment at an m/z value of 181.

5.1.2. Derivatization using BSTFA based protocols

The derivatization with both BSTFA protocols was successful as evident by the albeit small molecular ion peak. Further verification was achieved by injecting standards of various concentrations and observing the change in peak height. The SCAN spectrum is provided in Figure 1. For the development of a SIM method three ions with good intensity were selected for further analysis. Generally heavier fragments are more specific for a compound than smaller fragments, thus the ions with m/z values of 311.3, 385.3 and 401.3 were chosen.

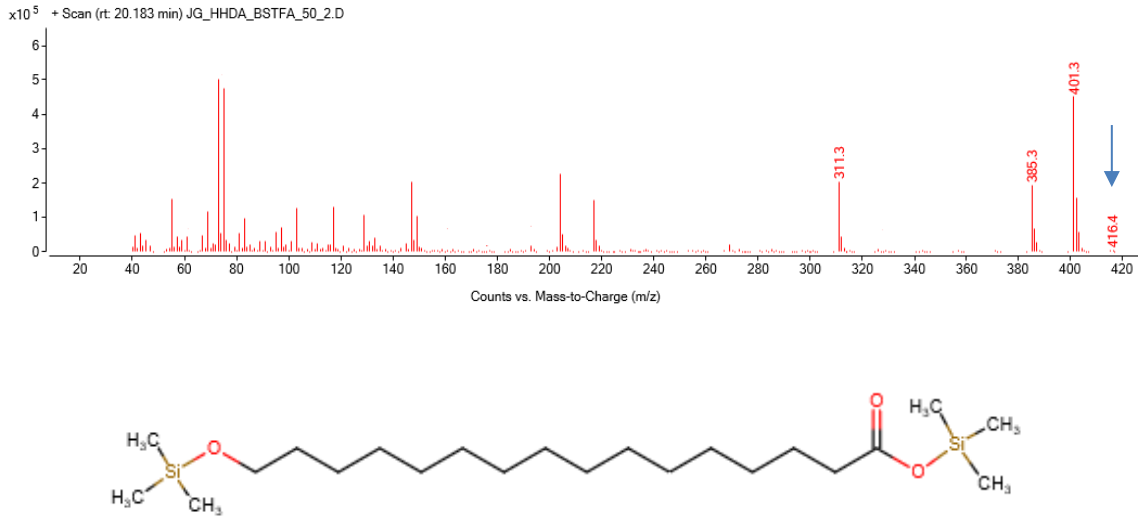

Figure 1: *Chemical structure of the BSTFA derivative and SCAN spectrum of a 50 mg ·l^{-1} standard of derivatized HHDA. The molecular ion peak is marked with an arrow.* The compound was found to elute at 20.19 minutes

5.1.3 Derivatization with BSTFA and pyridine

In total 5 calibration standards were used to check the linearity of the method before switching to the QqQ instrument. They were ranging from a concentration of 375 to 1.25 µg·l^{-1}. After finishing the derivatization procedure, the samples were filled up to 400 µl and 1 µl was injected for analysis. While the trend appeared linear when measuring higher concentrations, this could no longer be observed when measuring low concentrated standards. Table 5 displays the peak areas and their according standard deviations.

Table 5: *Peak areas measured for standards of different concentrations.*

c (µg·l^{-1})	Mean	Std. dev.	N
375	18515.67	1133.42	4
125	6545.86	452.72	4
25	3194.16	618.72	4
3.75	2127.13	390.24	4
1.25	1698.99	268.12	4

Additionally, it could be observed that the signal intensity was decreasing over time, when doing repeated measurements. This issue is addressed in detail later. While this was not an immediate problem it had to be considered when conducting many runs, such as a calibration series and several samples.

The individual and average peak areas of four standard series were plotted against the concentration, resulting in a calibration curve with an R-squared value of 0.9963. The respective graphs are shown in Figure 2 and a representation as a bar plot, including error bars is shown in Figure 3. The non-linear behavior is not immediately evident from the graph due to the largest standard dominating the calibration, however it is easily noticeable in the bar plot. While the change in peak area from 375 to 125 $\mu g \cdot l^{-1}$ corresponds to a decrease of about two thirds, the expected trend does not continue when comparing the peak areas observed for lower concentrations. The signal for 25 $\mu g \cdot l^{-1}$ is about half of the value observed for 125 $\mu g \cdot l^{-1}$ and the measurements stray even further from the expected value at 3.75 and 1.25 $\mu g \cdot l^{-1}$

5.1.4 The loss of linearity

The most likely explanation for these results is the limited sensitivity of the old instrument used or the presence of a base signal which additionally increases all peaks by the same amount, which has a greater effect at lower concentrations. This could be caused by derivatization side products with a similar retention time to the fatty acid, thus increasing the observed signal. Using an MRM method may resolve this issue, since only specific mass losses are detected with this approach. However, such an experiment is not possible using a single quadrupole instrument. It was necessary to employ the MS/MS capable QqQ mass analyzer for further measurements. The MRM approach and its problems shall be discussed in the following sections.

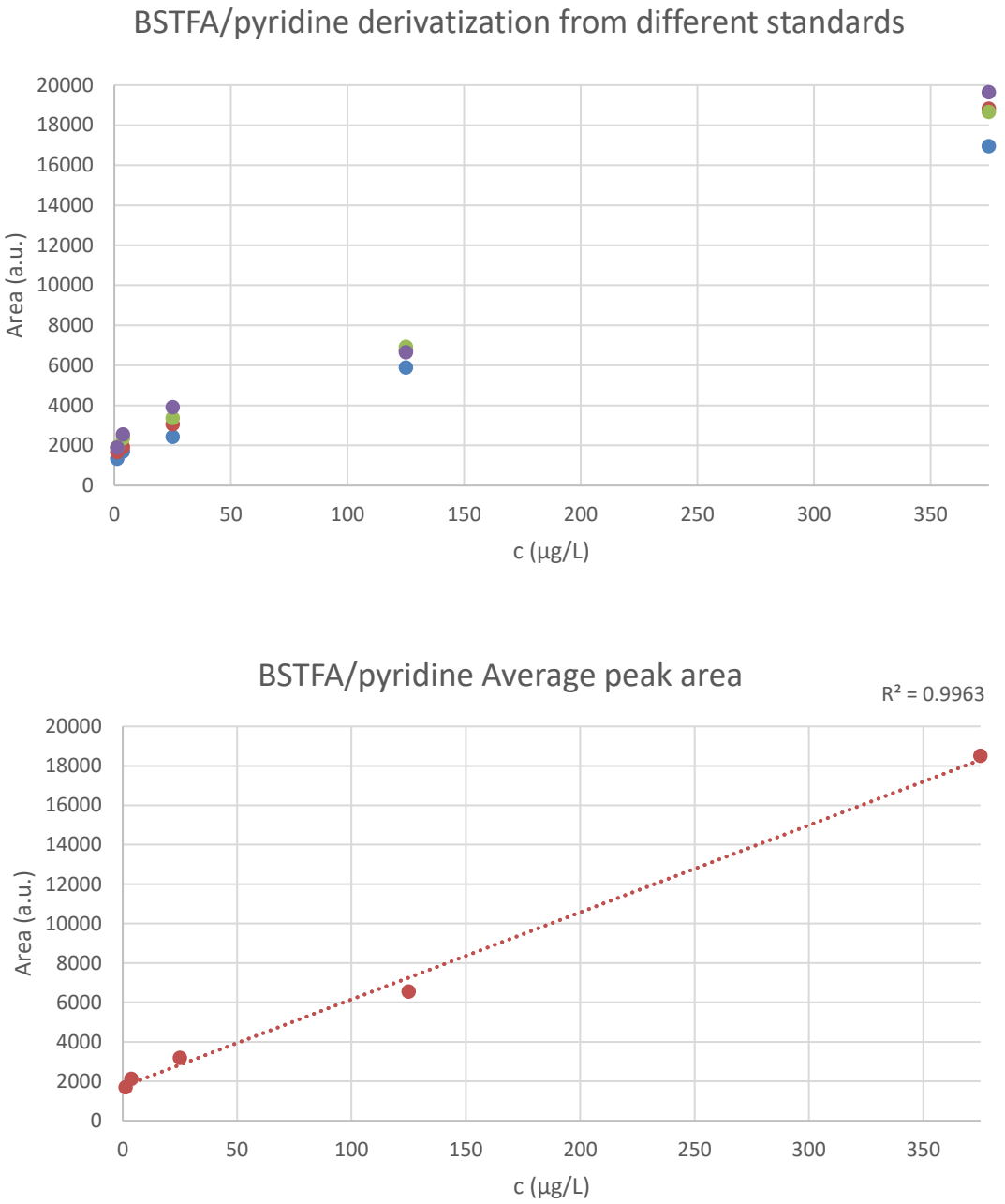

Figure 2: *Comparison of the individual (top) and average (bottom) peak areas of 4 different standard series of identical concentration derivatized with BSTFA and pyridine plotted against the concentration. The dotted line shows the linear regression.*

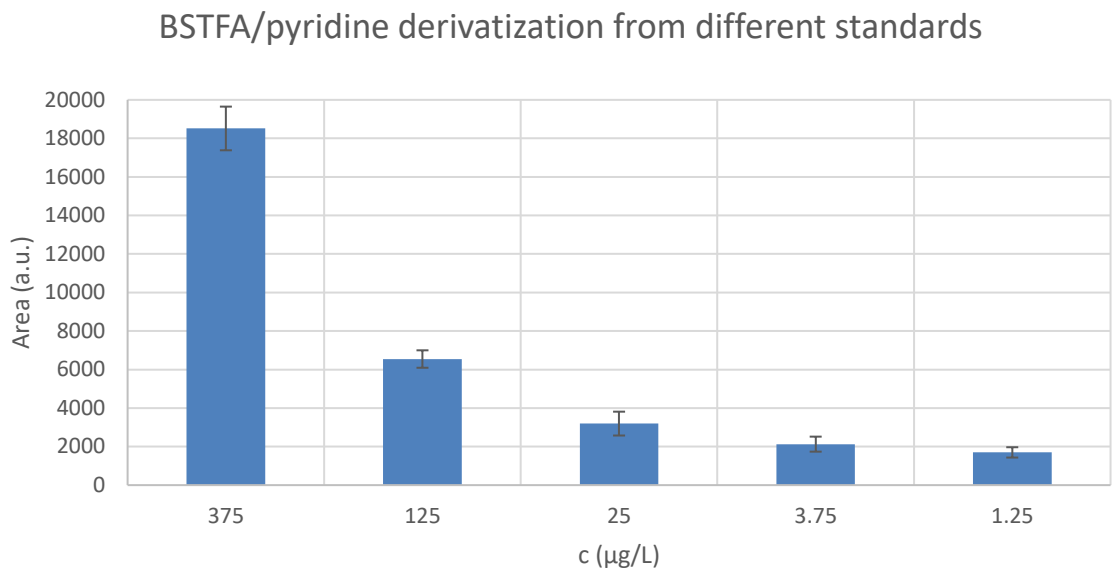

Figure 3: *Bar plot of the average peak areas and corresponding standard deviations at different concentrations after derivatization with BSTFA and pyridine.*

5.1.5. Derivatization of HHDA with pure BSTFA

Like in the standards derivatized with pyridine 5 calibration standards were prepared. The samples were filled up to 400 µl and 1 µl was used for injection. Similarly to the previous method a non-linear trend was observed in a standard series of the same concentrations. The observed peak areas and their respective standard deviations are listed in Table 6.

Table 6: *Peak areas measured for standards of different concentrations.*

c ($\mu g \cdot l^{-1}$)	Mean	Std. dev.	N
375	17631.27	1227.54	4
125	6799.36	557.12	4
25	2570.43	419.12	4
3.75	1576.66	166.80	4
1.25	1288.25	105.24	4

As in the procedure with pyridine the signal intensity decayed slightly over multiple measurements, however the spread between the measured intensities was smaller overall. Just as observed previously the linearity of the method was lost at lower concentrations, as indicated by the bar plot in Figure 5. The individual, as well as the average peak areas of four standard series plotted against the concentration are displayed in Figure 4.

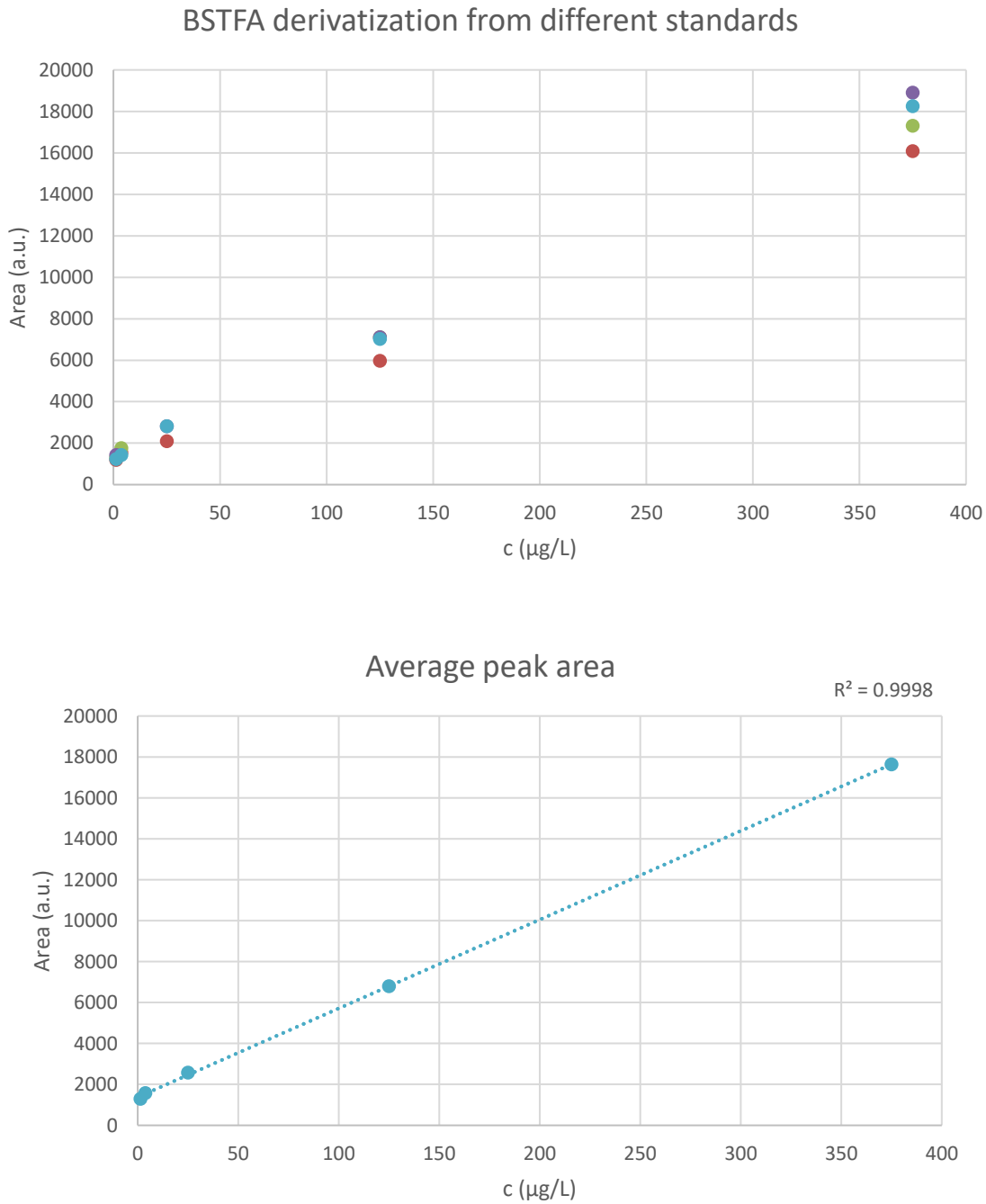

Figure 4: *Comparison of the individual (top) and average (bottom) peak areas of 4 different standard series of identical concentration derivatized with BSTFA plotted against the concentration. The dotted line shows the linear regression.*

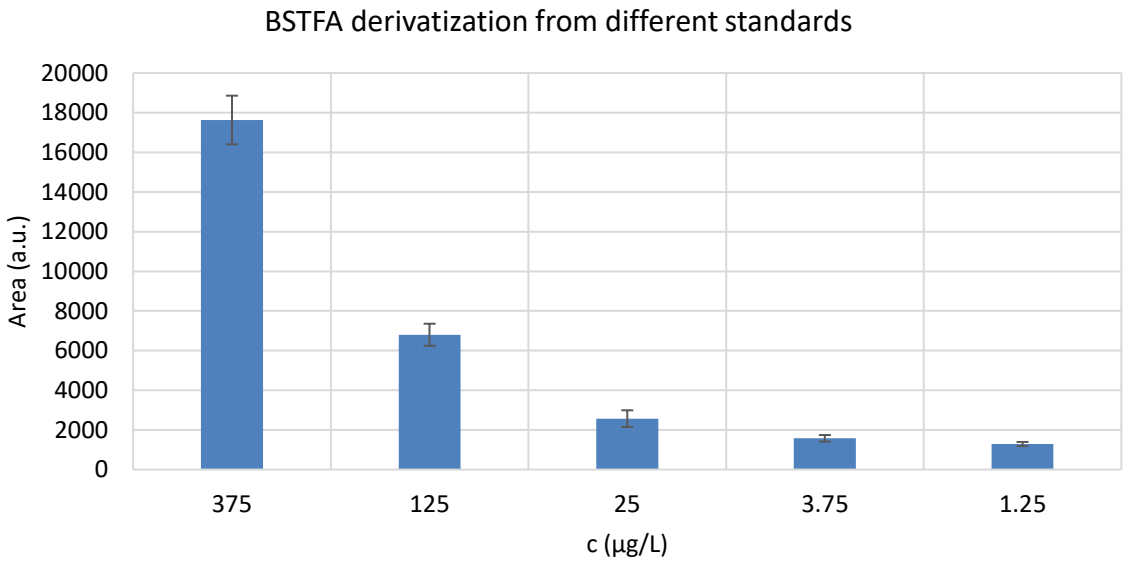

Figure 5: *Bar plot of the average peak areas and corresponding standard deviations at different concentrations after derivatization in pure BSTFA.*

5.2. Effects of evaporation of excess reagent

While evaporation was found to be part of some protocols [37; 38] it was omitted from the methods employed for the derivatization of the used substance. This was due to a significant signal reduction of the analyte peak when compared to a non-evaporated sample. Figure 6 shows a comparison between the injection of a 375 $\mu g \cdot l^{-1}$ standard with and without evaporation measured in SIM mode. The evaporation was conducted at 50°C under a stream of nitrogen. Even at lower temperature the loss was considerable which made evaporation of the excess reagent unviable for the analysis of the low concentrated eicosanoids in plasma samples. This issue may be caused by a reaction with moisture during solvent removal or the increased volatility after derivatization. However, the exact cause is unknown.

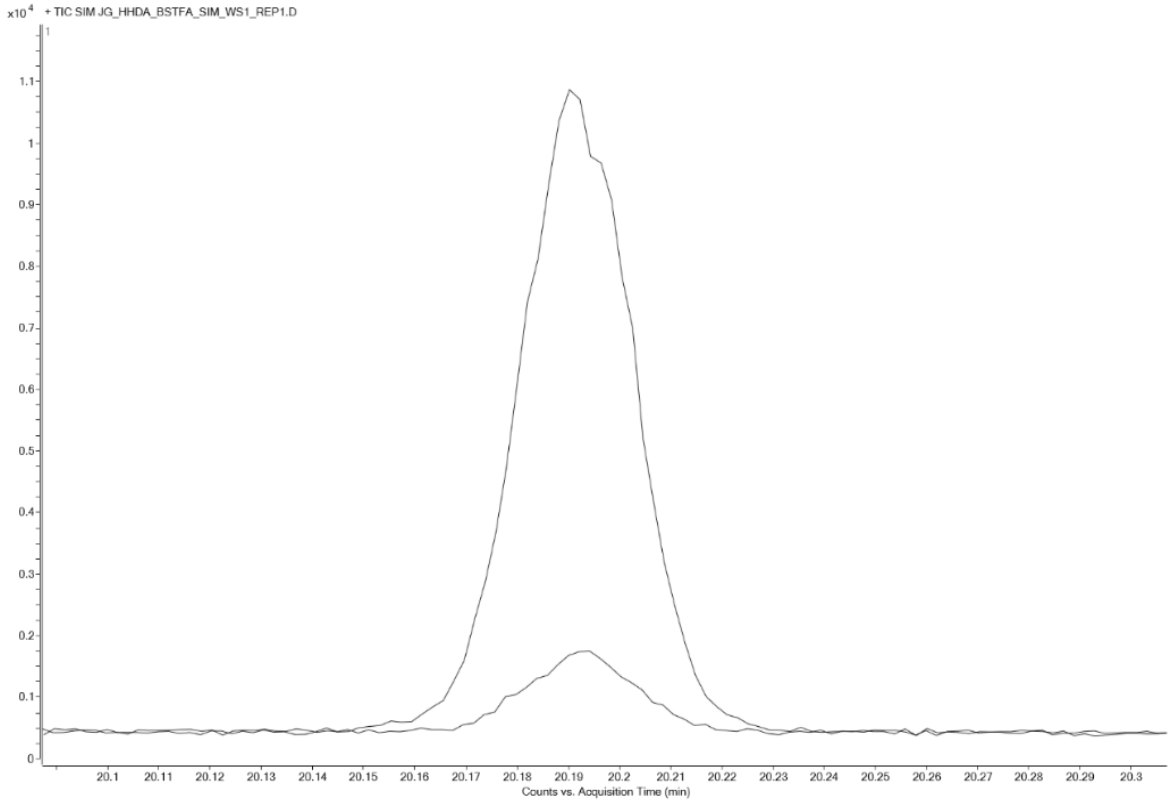

Figure 6: *Comparison of signal intensity of standards of the same concentration with (lower line) and without (upper line) evaporation of excess reagents. The x-axis shows the retention time*

After optimization using the single quadrupole instrument and choosing the best derivatization option the choice was made to switch to the newer triple quadrupole instrument. With the higher sensitivity it was possible to achieve calibration curves that showed linearity even at concentrations ranging from 5 $\mu g \cdot l^{-1}$ to 0.5 $\mu g \cdot l^{-1}$ as depicted in the calibration curve shown in Figure 7. The corresponding data is given in Table 7. Standards of lower concentration started to exhibit non-linear behavior once again (not shown). Due to the number of standards measured for calibration only two repetitions were made because of problems encountered with sample stability over several hours and the long run time. The longevity of the analyte is addressed in detail in the next section.

Table 7: *Peak areas and standard deviations for concentrations in the low µg and ng range.*

c (µg · l^{-1})	Mean	N
5	85611,25	2
3.75	70688,32	2
2.5	52836,61	2
1.25	36691,05	2
0.5	28119,98	2

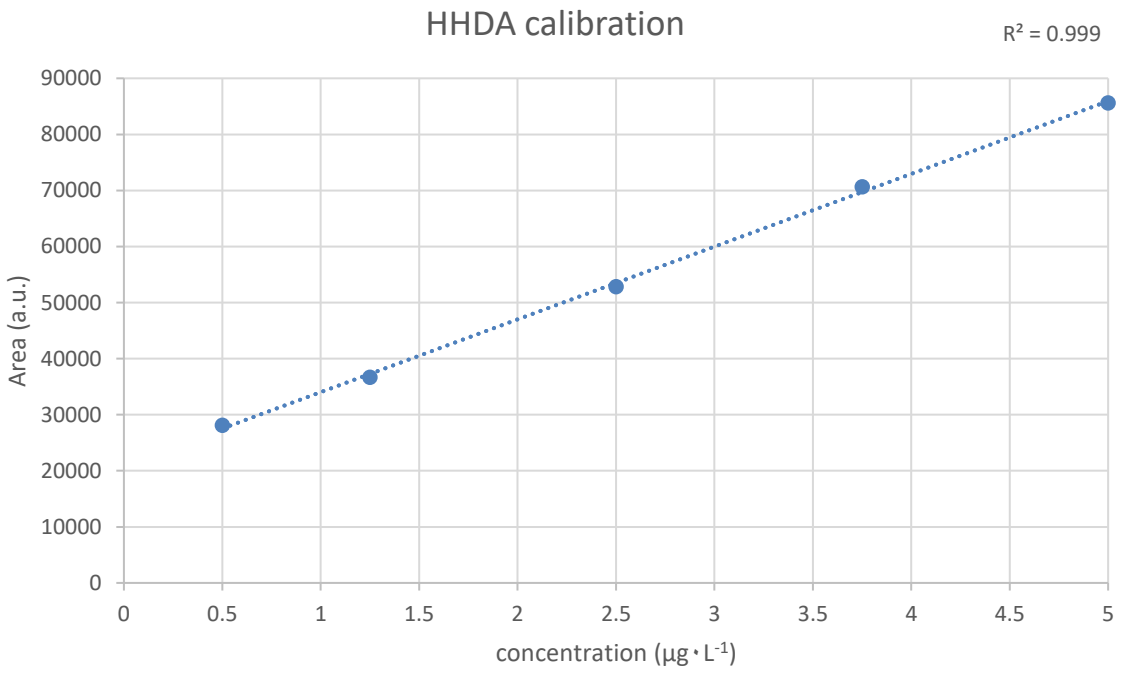

Figure 7: *Calibration curve for concentrations from 5 µg · l^{-1} to 0.5 µg · l^{-1} using the triple quadrupole Mass analyzer in SIM mode. The dashed line indicates the linear regression.*

5.3. Results of GC analysis

Similarly to the analysis on the single quadrupole instrument, a peak appeared to be coinciding with the analyte, which could also be observed in a blank sample. This caused an increase in peak area. In Figure 7 this is indicated by the calibration curve not intercepting the y-axis at the origin, but at around 20000 a.u. Most likely this is the result of side reactions during the derivatization process, forming side products with similar retention time to the analyte. While this was not a big problem when operating the MS in SIM mode due to the significantly larger peak area, it prevented proper analysis of the substance when using an MRM method, since some of the side products appeared to still be present. Development was further complicated by the low abundance of ions. The fragments used for SIM analysis were chosen as precursors for an MRM method and the fragmentation energies were optimized using the MassHunter software. However, the observed MRM signals were already approaching the limit of detection in the higher standards. Additionally, an unidentified compound seemed to have a similar retention time as well as identical fragments, thus interfering severely with the analysis.

5.3.1 Stability of the analyte

Since evaporation of the excess reagent significantly reduced the signal intensity, the longtime stability of the derivatized analyte without removal of the excess derivatization agents was recorded. To compare the procedures one standard with the same concentration was prepared for each method and measured 100 times. Figure 8 shows the peak area plotted against time. While the mixture containing pyridine showed a higher initial peak area it suffered from a much faster onset of signal decay after approximately 4 hours while the sample in pure BSTFA showed a stable signal for about 8 hours. Since a single measurement took approximately 35 minutes, including the time the GC oven required to cool back down, this severely limited the number of samples that can be run before the analyte starts to decay. Due to the longer lifetime, pure BSTFA is better suited for the analysis of the compounds of interest.

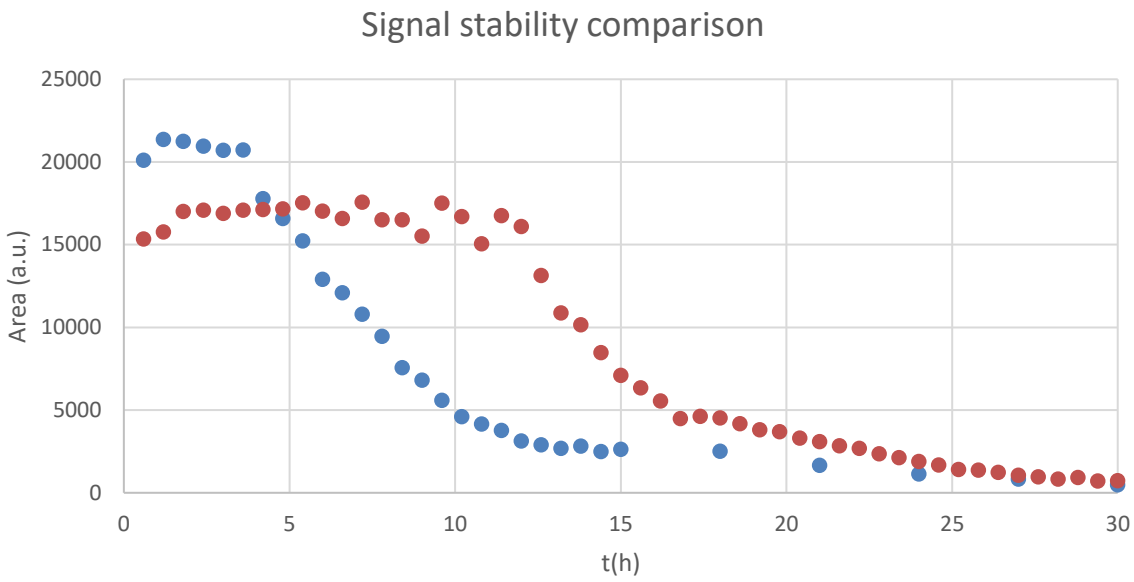

***Figure 8**: Comparison of the signal intensity of the analyte between the two BSTFA methods plotted against time. The results for pure BSTFA are plotted in red and for BSTFA and pyridine in blue.*

5.3.2. Analysis of HETEs

Figure 9 shows the overlayed chromatograms of individually injected standards for each of the lipids. The upper chromatogram further highlights the lack of base line separation for 5-, and 12-HETE as well as 15-HETE and an unknown peak present in all runs. The lower chromatogram shows the height of the analyte peaks compared to what appears to be residual matrix components from previous injections or derivatization side products.

Despite the sensitivity of the method being theoretically sufficient for the analysis of the eicosanoids in a plasma sample there were several issues making quantification difficult. Besides the insufficient separation of the analytes an unexpectedly small signal intensity after derivatization was observed when measuring in SCAN mode. The standards used had a concentration of about 3 $mg \cdot l^{-1}$ which is over 1000 times higher than what can be expected in a plasma sample.

Additionally, the analytes were eluting in the onset of the column bleeding, which caused a background signal, making identification of fragments that were specific to the sample and had good signal intensity even more difficult. Consequently, the setup of a SIM method was complicated due to the uncertain origin of the fragment ions.

The development of an MRM method, which would be ideal for quantification, would suffer even more from the low intensity as mentioned previously and was not pursued further.

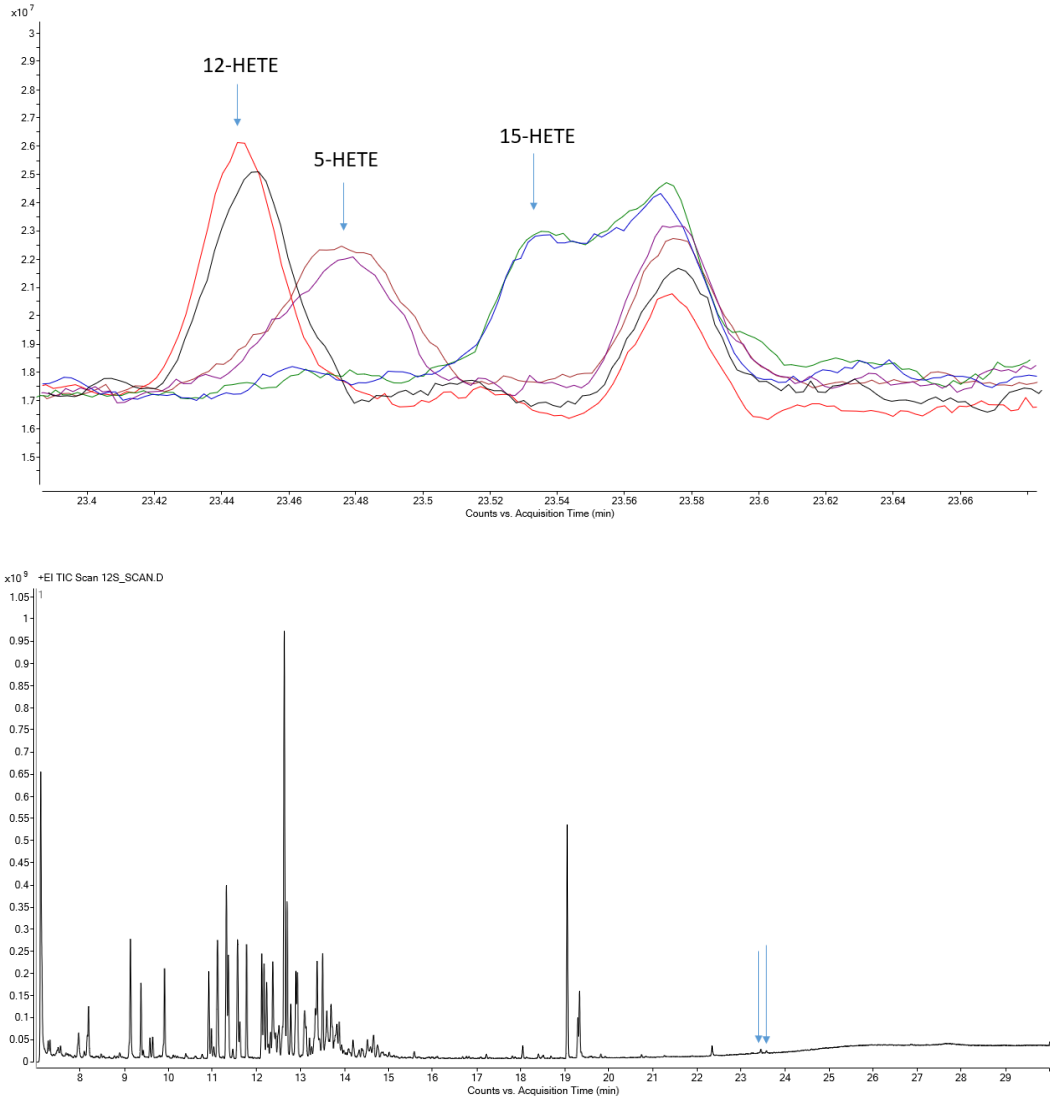

Figure 9: *Chromatograms recorded for HETEs. The overlayed chromatograms were observed for the individual lipids derivatized using pure BSTFA (upper image). The whole chromatogram (lower image) also shows other signals of considerable intensity which may be matrix components from previous injection or derivatization sideproducts.. The analytes are marked by arrows in both spectra.*

5.4. Results of HPLC analysis

Using a HPLC-ESI-QTof setup characteristic fragmentation patterns for the individual HETEs and their deuterated counterparts (not shown) were determined. The SCAN spectra of the acids are depicted in Figure 10. The molecular ion peaks [M^-] of the HETEs were found to be at an m/z value of 319.22 for the regular eicosanoids and 327.27 for the internal standards. It seems that the formation of the major fragments, that do not correspond to the loss of water or the carboxylic acid group was dictated by the position of the hydroxyl group. Using ChemDraw it was determined that the precursors of the observed fragments were likely formed by dissociation of the carbon-carbon bonds between the alcohol group and a double bond between its alpha and beta carbons. Figure 11 shows the formation of the precursors as well as the most likely candidates for the observed ions.

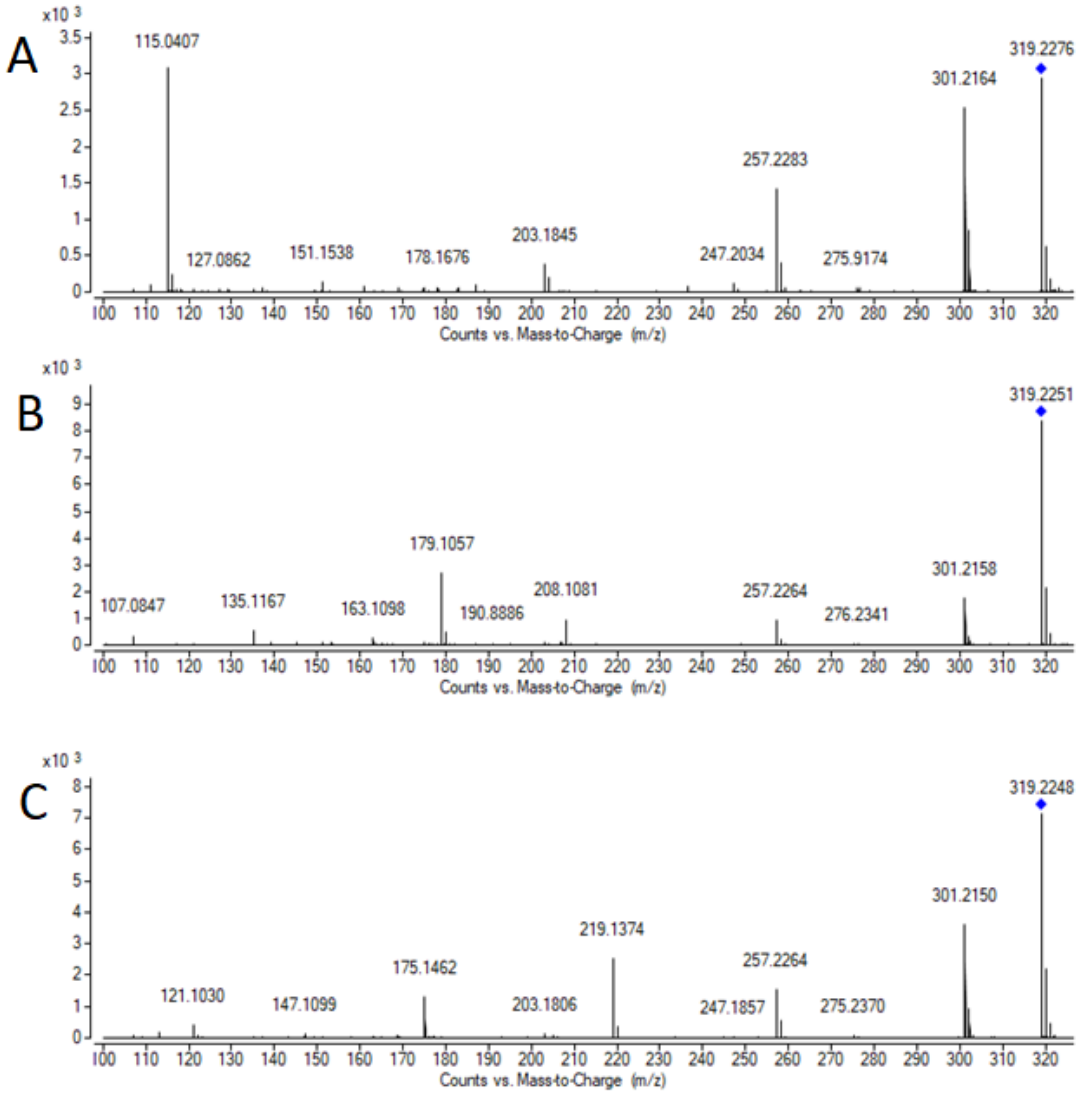

Figure 10: *MS/MS spectra acquired for 5-HETE (A), 12-HETE (B) and 15-HETE (C). The precursor ion is indicated by the diamond.*

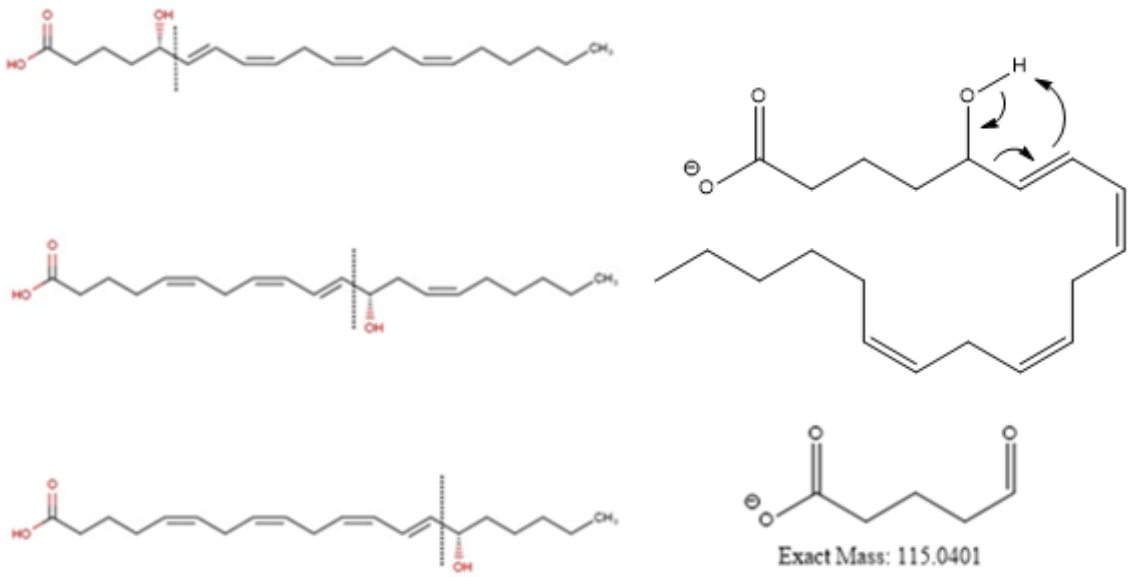

Figure 11: *Suggestion on the observed ions and the fragmentation mechanism in ESI ionization, using 5-HETE as an example. The dashed lines indicate the position of fragmentation. All masses were calculated using ChemDraw.*

Table 8 lists the fragments used for the quantification of plasma samples as well as the according retention times. Their viability is further proven by the fact that similar results were observed by different groups such as *Mazaleuskaya et al.* [41].

Table 8: *Lipids, their corresponding retention times and fragments used in MS/MS analysis*

Compound	Retention time	parent ions (m/z)	product ions (m/z)	collision energy (eV)
5-HETE	9.8 min	319.22	115.0407	7
5-dHETE	9.7 min	327.27	116.0443	7
12-HETE	9.5 min	319.22	179.1057	7
12-dHETE	9.4 min	327.27	184.1379	7
15-HETE	9.2 min	319.22	219.1374	7
15-dHETE	9.1 min	327.27	226.1374	7
all HETEs*	-	319.22	301.21	5
			257.22	7
all dHETEs*	-	327.27	309.26	5
			265.27	7

*these fragments are non-specific and correspond to the respective losses of the carboxylic acid group (-44) and water (-18). Both losses can be observed in all of the analytes as shown by the mass spectra displayed in Figure 10 and also other HETEs

5.4.1. Effect of sample preparation

After extracting the lipids from blood plasma, the substances of interest were identified in the chromatogram via their retention time. When comparing the effects of cooling, anti-oxidative properties of BHT and sonication it appeared that different sample preparation procedures did affect the result. Cooling the sample to -20°C for 2 hours has shown a negative effect on the amount of extracted lipid when compared to the uncooled sample.

Using uncooled samples, the effects of BHT and sonication were investigated further. Compared to the untreated sample the addition of BHT reduced the peak area of 5-HETE but increased the signal for 12- and 15-HETE. Sonication additionally improved the extraction of 15-HETE, while reducing the peak area of 12-HETE.

A further improvement was achieved by combining the methods employing addition of BHT and sonication. Despite the signal intensity for 5-HETE being slightly lower compared to the other methods an overall improvement was observed for the other eicosanoids.

5.4.2. Standards compared to plasma extracts

Figure 12 shows chromatograms of normal as well as deuterated lipid standards at different concentrations. The first peak for each set of doublets corresponds to the internal standard while the second belongs to the non-deuterated variant. The signal produced by the deuterated fatty acids appears to be of similar intensity for 15- and 12-HETE while it is lower for 5-HETE. As previously mentioned, the concentration of the internal standard was kept constant for all measurements while the amount of the normal lipid changed.

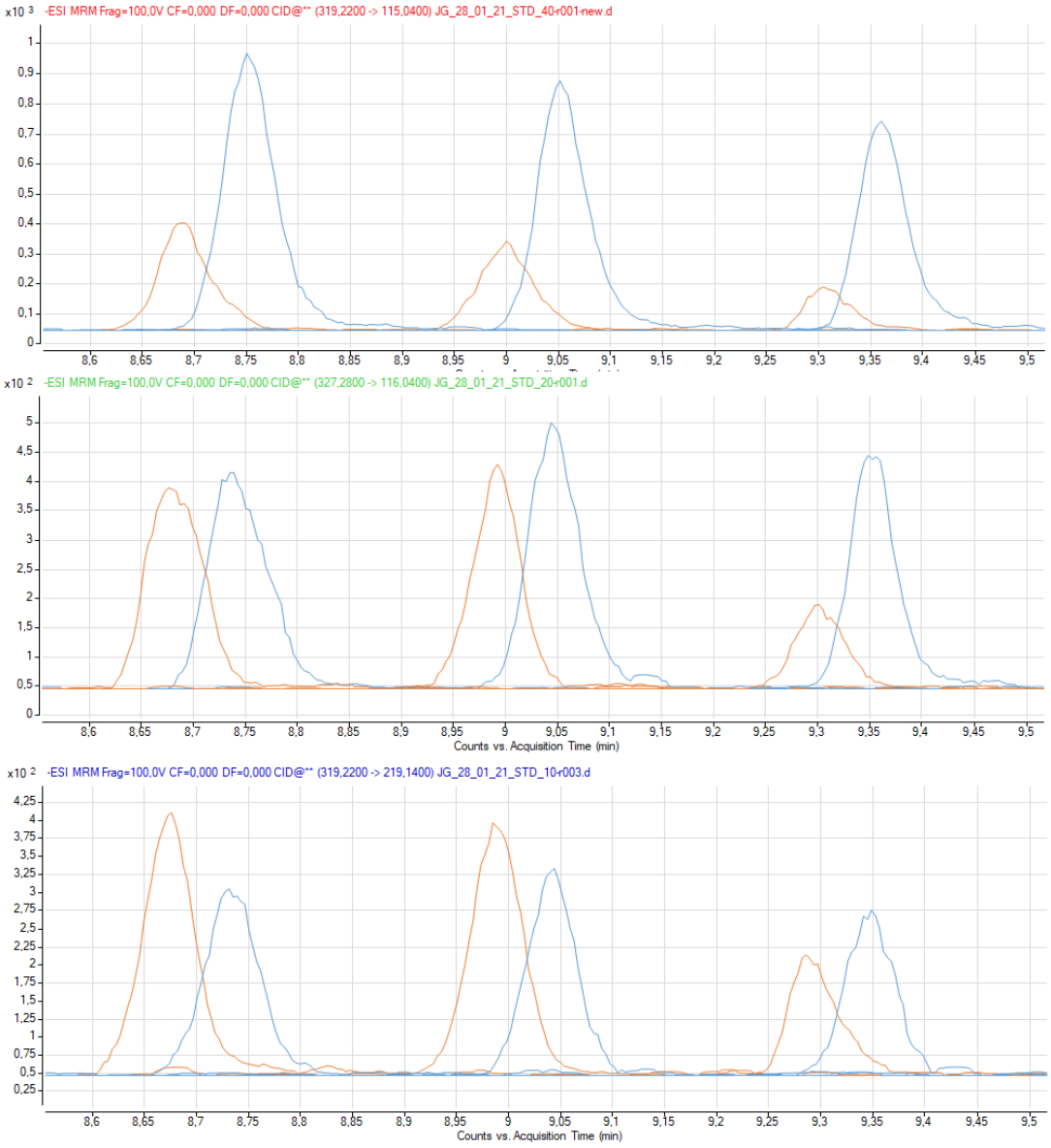

Figure 12: *Measurement of calibration standards. The blue lines indicated unlabeled lipids and the orange lines show the internal standards. The concentrations are 40 (top), 20 (middle) and 10 (bottom) µg ·l^{-1} for non-deuterated HETEs and 32 µg · l^{-1} for dHETEs.*

The chromatograms shown in Figure 13 depict a measurement in a plasma sample. The internal standards and lipids are easily identifiable by extracting the characteristic MRM fragmentations. Some fragment ions other than the quantifier can be observed near some analytes as indicated by the presence of multiple peaks. However, this does not interfere with quantification, since only the fragmentation specific to the compound is used. A likely explanation is the presence of additional HETEs. A compound such as 5-,12-diHETE may have similar retention times and produce fragments also observed in other HETEs due to the identical position of hydroxy groups. The higher intensities of the internal standards compared to the calibration solutions are the result of switching from a protocol using 100 µl plasma and 400 µl precipitation agent to one using double the amounts. After taking up the residue in 50 µl of ACN/MeOH this leads to double concentrations for lipids and internal standards. This needs to be accounted for when calculating the final concentration.

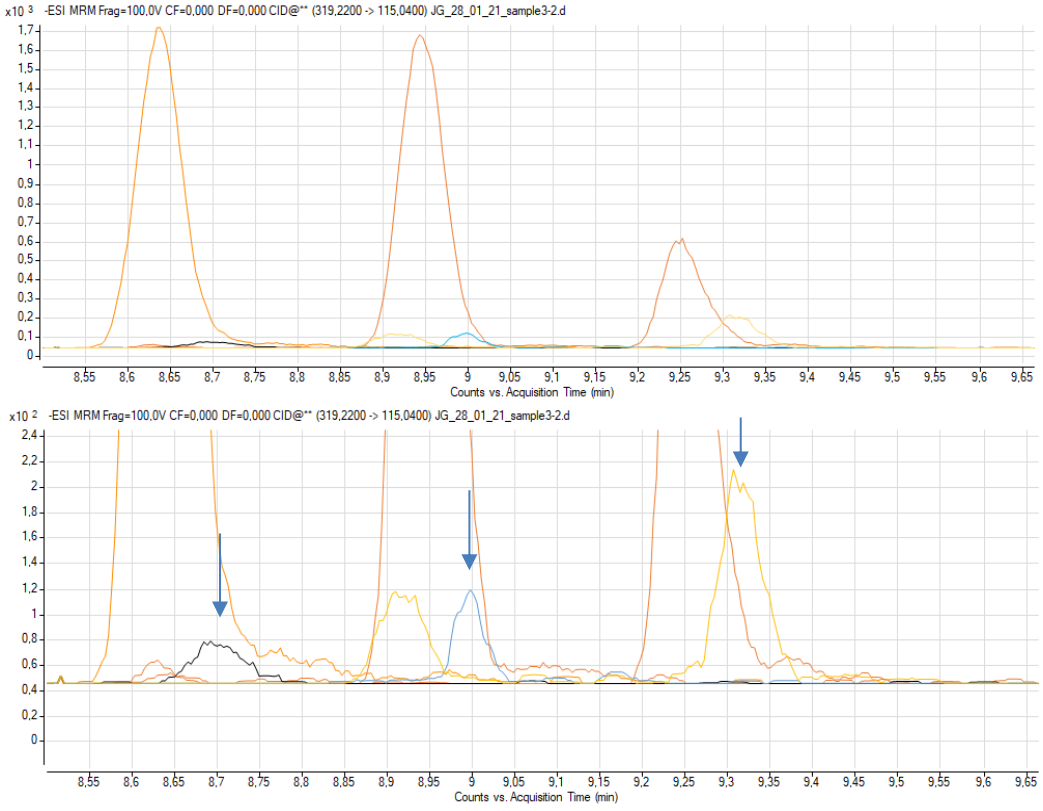

Figure 13. *Measurements of plasma samples. Some of the chosen fragments were not only observed in one lipid, as shown in the lower chromatogram. This does not interfere with analysis since the signal observed is not the same as the quantifier. The yellow, blue (319 → 179) and black (319 → 219) lines each indicate a specific fragmentation. Orange lines show the according signals of internal standards. The correct peak for quantification was identified according to retention time and ion mass. All analytes are indicated by blue arrows.*

5.4.3. Assessment of matrix effects

To check for the presence of matrix effects the area of the internal standards measured in a plasma sample was compared to the area observed in a blank sample containing only the internal standards dissolved in MeOH/ACN like in the calibration standards. Matrix effects are caused by the analyte coeluting with other molecules, competing during ionization. However due to the MRM analysis these molecules are not visible in the chromatogram. The matrix effect (MEF) was calculated according to the following formula [42]:

$$MEF = \frac{A_{IS} - A_S}{A_{IS}} * 100$$

where A_{IS} is the average area of the internal standard in the blank sample and A_S the average area in presence of a biological matrix. The data used for calculation of the matrix effects is compiled in Table 9

Table 9: *Areas of the eicosanoids with and without biological matrix and the according matrix effect.*

Compound	A_S / a.u.	A_{IS} / a.u.	MEF / %
15-HETE	6092,63	6921,00	11,97
12-HETE	5739,50	5853,90	1,95
5HETE	2162,13	5786,05	62,63

The chromatograms show slight matrix effects for 15-HETE, basically no matrix effect for 12-HETE and a huge suppression for 5-HETE as depicted in Figure 14. However, as previously mentioned this is irrelevant if the signal intensity is high enough for quantification. Since the internal standard and the analyte are affected in the same way, their area ratio will stay constant, regardless of signal suppression.

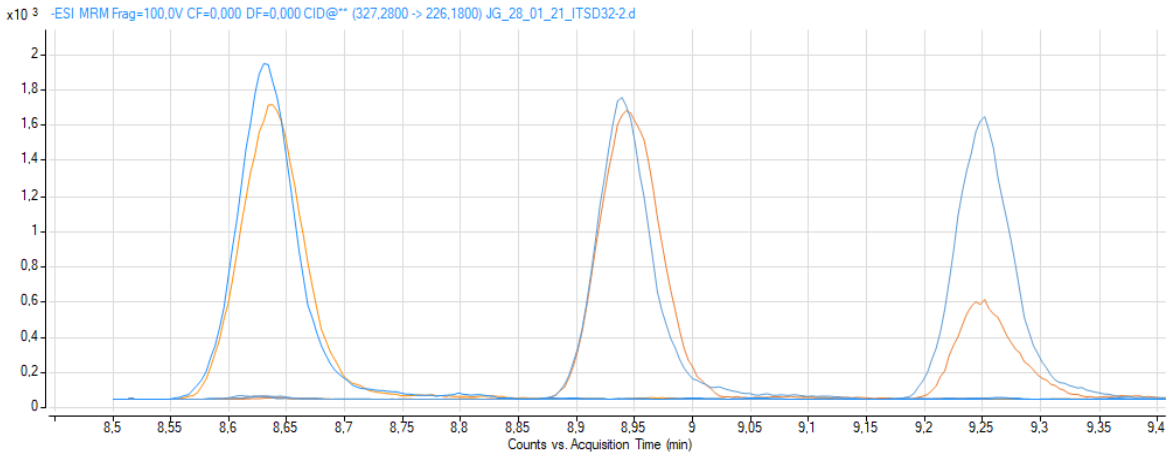

Figure 14: *Effect of biological Matrix. Chromatograms in the presence of matrix components are depicted in orange and blue in the absence of matrix.*

5.4.4. Standard calibration and quantification

In the standards 15-HETE produced the biggest signal, followed by 12-HETE and 5-HETE. Calibration curves were prepared by plotting the ratio of the area of the analyte and the internal standard against the concentration. Each of the standards was measured 3 times and the area ratio recorded. The overall standard deviation of the area ratios was the highest for 5-HETE. The detailed data is presented in Table 10.

Table 10: *Ratios of peak areas of standards divided by internal standard and standard deviation.*

c (µg/l)	15-HETE		12-HETE		5-HETE		N
	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	
40	2,4192	0,1773	2,4812	0,0618	6,2511	0,5341	3
30	1,7807	0,0692	1,7767	0,1002	4,2399	0,5251	3
20	1,2141	0,0706	1,2439	0,0299	2,6699	0,1454	3
10	0,4166	0,0489	0,5524	0,0483	1,3976	0,2332	3
5	0,3506	0,0131	0,4101	0,0151	0,7975	0,0900	3
2	0,1158	0,0182	0,1352	0,0167	0,3056	0,0576	3
1	0,0682	0,0068	0,0608	0,0101	0,1694	0,0460	3

As evident from Figures 15-17, showing the calibration curves for the different eicosanoids and the corresponding coefficients of determination, the method used for analysis provides the linearity necessary for measuring real plasma samples.

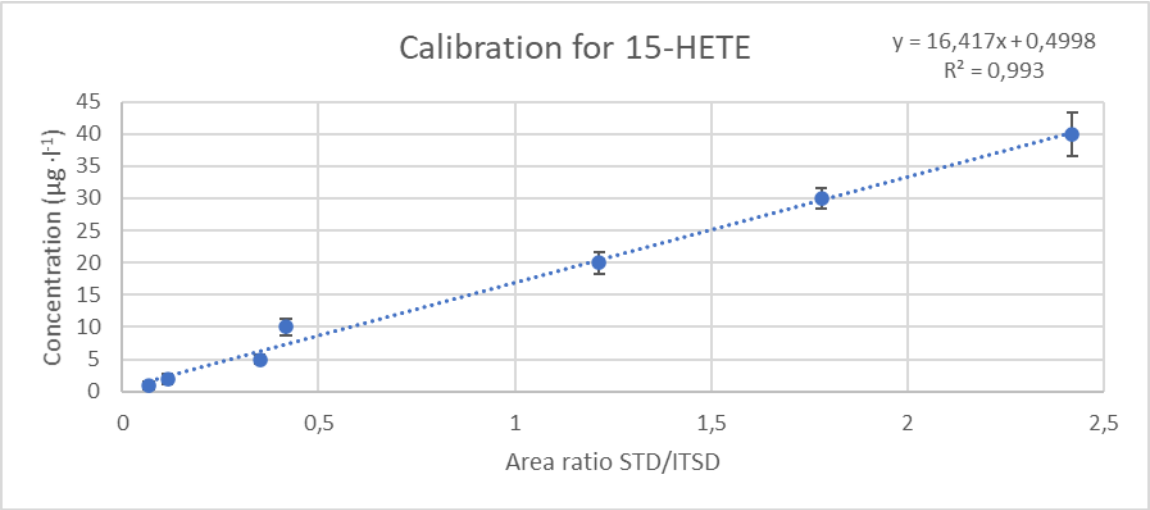

Figure 15: *Calibration curve for 15-HETE. The average concentration of three measurements was plotted against the ratio of the peak areas of the analyte and the internal standard, including error bars.*

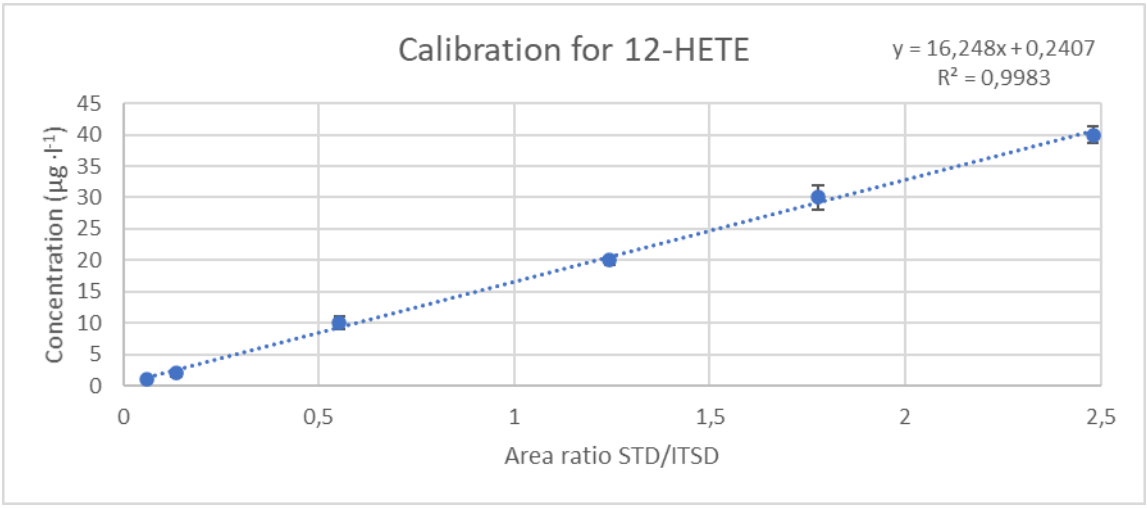

Figure 16: *Calibration curve for 12-HETE. The average concentration of three measurements was plotted against the ratio of the peak areas of the analyte and the internal standard, including error bars.*

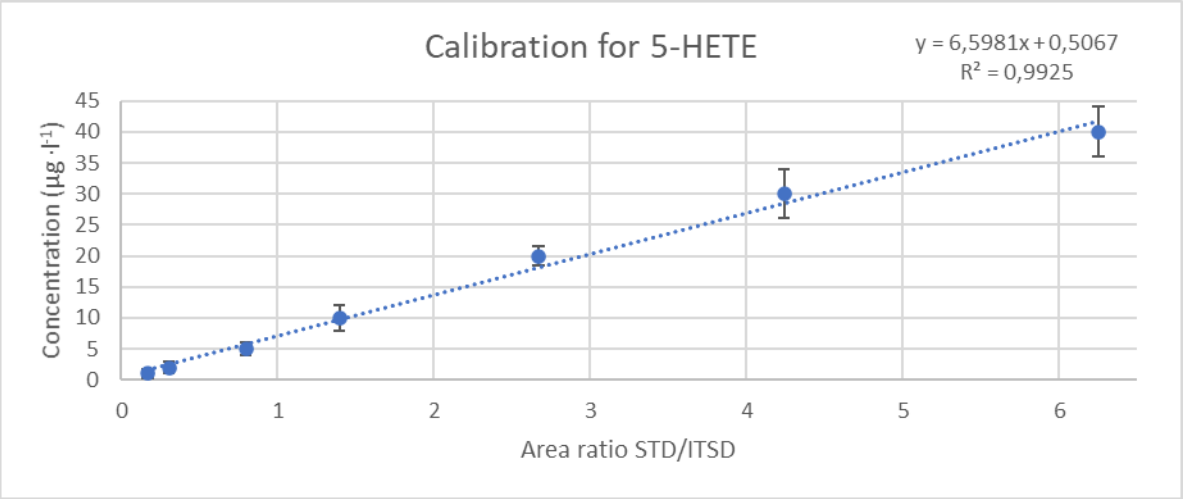

Figure 17: *Calibration curve for 5-HETE. The average concentration of three measurements was plotted against the ratio of the peak areas of the analyte and the internal standard, including error bars.*

Using the obtained calibration curves, the concentration of free HETEs in blood plasma samples was calculated and listed in Table 11. These values are within the range described by Psychogios et al. [43] for the most part. The value for 15-HETE is very similar to the one reported, while the concentration of 12-HETE is lower, but within the standard deviation. The mean concentration of 5-HETE exceeds the literature value.

The measurements were conducted using a pooled plasma sample consisting of plasma from five different persons of unknown age, gender, and health condition. The sample was separated into 5 portions of 200 µl each of which were treated as described previously. The resulting plasma extracts were measured twice, with a washing step between each measurement to remove leftover matrix.

Table 11: *Concentration (nM) of free HETEs in blood plasma*

Analyte	Mean / nM	Standard deviation	N
15-HETE	1.98	1.16	9
12-HETE	1.53	0.58	10
5-HETE	3.98	1.39	10

The high standard deviation observed may be the result of the low concentrations of the analytes in plasma at about 1 $\mu g \cdot l^{-1}$. Derivatization combined with a different ion source may remedy this issue.

6. Discussion

Since derivatization is a crucial part of the analysis of eicosanoids in GC-MS it is critical that the process is reliable. Between the two tested derivatization agents BSTFA was vastly superior over PFB. No PFB-esters could be formed by using the methods described in literature by Knott et al. [37] and Leis et al. [38] with HHDA as the analyte. It is possible that this may be related to the chemical structure of the compound chosen for method development. Despite working in water free environment SCAN experiments showed peaks in the spectrum that can be assigned to the alcohol of the derivatization agent, the reaction product of the excess reagent with the base catalyst and the lactone of the original compound. It might be the case that the desired product is formed, but immediately reacts further into a cyclic ester via a nucleophilic substitution, releasing the pentafluorobenzyl alcohol. High concentrations of the fatty acids additionally show a peak appearing past the 20-minute mark. Since a characteristic fragment for PFB esters is present it is possible, that it is the analyte. This derivatization procedure was abandoned due to the undesired side reaction.

For the derivatization with BSTFA two different methods were compared. Yasukazo et al. employed pure BSTFA [33] and Leis et al. additionally used pyridine to catalyze the reaction [38]. Derivatization in the presence of pyridine was found to be unsuitable in the experiments conducted with HHDA and was thus not further used on the lipid standards. While the catalysis with pyridine lead to better peak areas it was also related to higher standard deviations. Furthermore, the presence of the base seems to negatively affect the stability of the derivatized analyte, as evident from the repeated measurement of a single standard. With a signal lifetime of about 3.5 – 4 hours and an average run time of 35 minutes this significantly limits the number of measurements that can be carried out. Thus, the use of pure BSTFA is recommended if one wishes to run a larger number of samples or calibration standards at the same time. A drawback observed was the formation of by-products that in this case coincided with an analyte peak and caused contamination of the liner, especially when using plasma extracts.

Evaporation of the excess reagent at 50°C under nitrogen is not recommended due to the large reduction in signal intensity. The volatility of the analyte may have been too high to remove the excess of BSTFA without loss even at low temperatures.

Another problem observed with GC analysis was the harsh ionization, forming many fragments with lower intensity. This caused low peak areas when measuring in MRM mode, increasing the limit of quantification considerably.

Furthermore, it happened that the derivatization would fail. While this may be a coincidence, it was the reason to switch to an HPLC method due to the very limited amount and high price of the HETE standards used. However, even when the derivatization was successful the analytes showed poor signal intensity and separation. One lipid additionally coincided with a peak that appeared to be a reaction side product, making the identification of characteristic fragments for development of a SIM method difficult.

A major advantage of the HPLC method is that no derivatization is required. This eliminates the formation of unwanted side products and removes a source of contamination and possible errors. With the softer ionization method, the fragmentation is less intense, resulting in lower fragment ion formation and higher intensities for individual fragments. The ions used for quantification were also observed in other studies [41], suggesting good reproducibility and suitability as a quantifier. This made the development of an MRM method considerably easier compared to analysis via GC. Additionally, the compounds could be reliably separated, and carryover of matrix components could be avoided by flushing with pure acetonitrile between runs.

While the analysis of the eicosanoids worked well using the specific fragments the measured value was found to be at the lower end of the calibration curve in case of 15- and 12-HETE. If necessary, a further signal increase may be achievable by different ionization methods such as APCI or APPI in combination with derivatization using electron capturing groups such as PFB as previously mentioned. Meckelmann et al. tested derivatization using N-(4-aminomethylphenyl)pyridinium chloride in combination with positive mode ESI, however generally no significant improvement over negative mode ESI, as it was also used in the present analysis, was observed [44]. Another possible enhancement over the method used may be the use of higher injection volumes. However, this caused a significant increase in peak width in HPLC which may be related to volume overload or the high amount of organic solvent in the sample compared to the mobile phase.

Compared to the plasma levels reported by Psychogios et al. [43] the concentrations measured in the current experiments are similar to the literature value in case of 15-HETE, less than half for 12-HETE and about four times higher in case of 5-HETE. When comparing the results to the findings stated by Meckelmann et al. [44] the measured amount of 12-HETE is closer to the literature value, while the concentration of 15-HETE is roughly two times higher. The difference in the concentration of 5-HETE is even greater than compared to the measurements of Psychogios et al. The most likely explanation for the observed data is the unknown medical history of the persons the samples were taken from, since the concentration may be affected by factors such as health, diet, and certain drugs. Furthermore, pre-analytical handling of the samples is unknown. Aspects such as the storage conditions and the number of freeze-thaw cycles may affect the concentration of the analytes, either increasing or decreasing them as described by Dorow et al. [31].

The observed matrix effect was found to be especially high for 5-HETE at over 60% while only being around 10% for 15-HETE and almost non-existent for 12-HETE. One might even be able to omit the addition of the expensive deuterated lipids for analytes not suffering from significant matrix effects. If one wishes to remove as much of the biological matrix as possible, the ideal sample preparation method was found to be a carefully chosen SPE procedure as demonstrated by Ostermann et al. [25] However, their data also shows good recovery when using the much simpler protein precipitation approach and the matrix effects did not cause major problems with the method used.

7. Conclusion

GC analysis was found to be less reliable for the analysis of HETEs due to formation of side products and inferior separation power compared to a HPLC method. Further, derivatization with BSTFA alone may not be ideal for the analysis of HETEs, despite working very well for HHDA. While additional modification with PFB was omitted due to undesired side reactions with the model substance it may be worth conducting further experiments with eicosanoids. This is supported by the fact that derivatization with PFB has successfully been used for HETEs before by groups such as Mazaleuskaya et al. [41]

The observed contamination of the GC liner or column may be reducible by use of SPE to remove a part of the biological matrix before analysis. The evaporation of excess reagents prior to injection, to remove compounds that could potentially damage the column, was

found to have a critical influence on the signal intensity of the analyte. The peak area was observed to decrease dramatically after solvent removal and is thus not recommended.

In comparison to GC, HPLC analysis was found to be considerably easier and less prone to errors. Especially the lack of derivatization and need of removal of excess solvents further simplified the analysis and eliminated problems such as peaks coinciding with the analyte. Matrix effects were found to be an important factor to consider when using protein precipitation. Internal standards are crucial for the analysis of 15- and 5-HETE which showed signal suppression of about 10 and 60% respectively. 12-HETE only suffered from little suppression.

Regarding sample preparation the addition of 25 $mg \cdot l^{-1}$ BHT to the precipitation agent and sonication when resuspending the dried residue have been found to be beneficial. Pre-analytical factors to consider are the storage conditions and the number of freeze-thaw cycles the samples go through. As previously mentioned, these factors can affect eicosanoid concentrations which may be relevant for the experiments conducted. Ideally only once thawed samples that had been stored at -80°C prior to sample preparation should be used.

8. References

1 Borgeat P, Samuelsson B. Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc Natl Acad Sci U S A* 1979;**76**:2148–52.

2 Woods JW, Evans JF, Ethier D *et al.* 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. *J Exp Med* 1993;**178**:1935–46.

3 Mats H, Bengt S. Prostagland Endoperoxides. Novel Transformations of Arachidonic Acid in Human Platelets. *Proc. Nat. Acad. Sci. USA* 1974;**71**:3400–04.

4 Levy D. Bruce, Romano Mario, Chapman A. Harold, Reilly J. John, Drazen Jeffry, Serhan N. Charles. Human Alveolar Macrophages Have 15-Lipoxygenase and Generate 15(S) -Hydroxy-5,8,1 1 -cis-l 3-trans-eicosatetraenoic Acid and Lipoxins. *J Clin Invest* 1993;**92**:1572–79.

5 Brezinski ME, Serhan CN. Selective incorporation of (15S)-hydroxyeicosatetraenoic acid in phosphatidylinositol of human neutrophils: agonist-induced deacylation and transformation of stored hydroxyeicosanoids. *Proc Natl Acad Sci U S A* 1990;**87**:6248–52.

6 Joulain C, Meskini N, Anker G *et al.* Esterification of 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid into the phospholipids of human peripheral blood mononuclear cells: inhibition of the proliferative response. *J Cell Physiol* 1995;**164**:154–63.

7 Alpert SE, Walenga RW. Human tracheal epithelial cells selectively incorporate 15-hydroxyeicosatetraenoic acid into phosphatidylinositol. *Am J Respir Cell Mol Biol* 1993;**8**:273–81.

8 Powell WS, Gravel S, MacLeod RJ *et al.* Stimulation of human neutrophils by 5-oxo-6,8,11,14-eicosatetraenoic acid by a mechanism independent of the leukotriene B4 receptor. *Journal of Biological Chemistry* 1993;**268**:9280–86.

9 Croset M, Lagarde M. Stereospecific inhibition of PGH2-induced platelet aggregation by lipoxygenase products of icosaenoic acids. *Biochemical and Biophysical Research Communications* 1983;**112**:878–83.

10 Chung EJ, Reedy JL, Kwon S *et al.* 12-Lipoxygenase is a Critical Mediator of Type II Pneumocyte Senescence, Macrophage Polarization and Pulmonary Fibrosis after Irradiation. *Radiat Res* 2019;**192**:367–79.

11 Holtzman MJ, Pentland A, Baenziger NL *et al.* Heterogeneity of cellular expression of arachidonate 15-lipoxygenase: implications for biological activity. *Biochim Biophys Acta* 1989;**1003**:204–08.

12 Hunter JA, Finkbeiner WE, Nadel JA *et al.* Predominant generation of 15-lipoxygenase metabolites of arachidonic acid by epithelial cells from human trachea. *Proc Natl Acad Sci U S A.* 1985;**82**:4633–37.

13 Nadel JA, Conrad DJ, Ueki IF *et al.* Immunocytochemical localization of arachidonate 15-lipoxygenase in erythrocytes, leukocytes, and airway cells. *J Clin Invest* 1991;**87**:1139–45.

14 Kim J, Choe J. A paracrine effect of 15 (S)-hydroxyeicosatetraenoic acid revealed in prostaglandin production by human follicular dendritic cell-like cells. *Prostaglandins Other Lipid Mediat* 2020;**151**:106487.

15 Ghosh J, Myers CE. Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc Natl Acad Sci U S A* 1998;**95**:13182–87.

16 Gupta S, Srivastava M, Ahmad N *et al.* Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. *Cancer* 2001;**91**:737–43.

17 O'Flaherty JT, Rogers LC, Paumi CM *et al.* 5-Oxo-ETE analogs and the proliferation of cancer cells. *Biochim Biophys Acta* 2005;**1736**:228–36.

18 Chen YQ, Duniec ZM, Liu B *et al.* Endogenous 12(S)-HETE production by tumor cells and its role in metastasis. *Cancer Research* 1994;**54**:1574–79.

19 Graham. PP, Kandouz M, Meram A *et al.* Mechanisms Controlling Cell Cycle Arrest and Induction of Apoptosis after 12-Lipoxygenase Inhibition in Prostate Cancer Cells. *Cancer Research* 2002;**62**:2721–27.

20 Graham PP, Tang K, Cai YL *et al.* Overexpression of Platelet-type 12-Lipoxygenase Promotes Tumor Cell Survival by Enhancing v3 and v5 Integrin Expression. *Cancer Research* 2003;**63**:4258–67.

21 Timar J, Tang D, Bazaz R *et al.* PKC mediates 12(S)-HETE-induced cytoskeletal rearrangement in B16a melanoma cells. *Cell Motil Cytoskeleton* 1993;**26**:49–65.

22 Honn KV, Timár J, Rozhin J *et al.* A Lipoxygenase Metabolite, 12-(S)-HETE, Stimulates Protein Kinase C-Mediated Release of Cathepsin B from Malignant Cells. *Experimental Cell Research* 1994;**214**:120–30.

23 Walenga RW, Sunderji S, Stuart MJ. Formation of Hydroxyeicosatetraenoic Acids (HETE) in Blood From Adults Versus Neonates: Reduced Production of 12-HETE in Cord Blood. *Pediatric Research* 1988;**24**:563–67.

24 Ostermann AI, Koch E, Rund KM *et al.* Targeting esterified oxylipins by LC-MS - Effect of sample preparation on oxylipin pattern. *Prostaglandins Other Lipid Mediat* 2020;**146**:106384.

25 Ostermann AI, Willenberg I, Schebb NH. Comparison of sample preparation methods for the quantitative analysis of eicosanoids and other oxylipins in plasma by means of LC-MS/MS. *Anal Bioanal Chem* 2015;**407**:1403–14.

26 Guey-Shuang Wu, A. Stein, Robert, Mead JF. Autoxidation of fatty acid monolayers adsorbed on silica gel: II. Rates and products. *Lipids* 1977;**12**:971–78.

27 Matuszewski BK, Constanzer ML, Chavez-Eng CM. Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per milliliter concentrations. *Anal Chem* 1998;**70**:882–89.

28 Fu I, Woolf E, Matuszewski B. Effect of the sample matrix on the determination of indinavir in human urine by HPLC with turbo ion spray tandem mass spectrometric detection. *Journal of Pharmaceutical and Biomedical Analysis* 1998;**18**:347–57.

29 Metherel AH, Hogg RC, Buzikievich LM *et al.* Butylated hydroxytoluene can protect polyunsaturated fatty acids in dried blood spots from degradation for up to 8 weeks at room temperature. *Lipids in Health and Disease* 2013;**12**:22.

30 Stone W. L. ,Farnsworth C. C. ,Dratz E. A. A Reinvestigation of the Fatty Acid Content of Bovine, Rat and Frog Retinal Rod Outer Segments. *Exp. Eye Res.* 1979;**28**:387–97.

31 Dorow J, Becker S, Kortz L *et al.* Preanalytical Investigation of Polyunsaturated Fatty Acids and Eicosanoids in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry. *Biopreserv Biobank* 2016;**14**:107–13.

32 Wu Z, Shon JC, Liu K-H. Mass Spectrometry-based Lipidomics and Its Application to Biomedical Research. *J Lifestyle Med* 2014;**4**:17–33.

33 Yasukazo Y, Shintaro K, Shigekazu T *et al.* Simultaneous measurement of F2-isoprostane, hydroxyoctadecadienoic acid, hydroxyeicosatetraenoic acid, and hydroxycholesterols from physiological samples. *Analythical Biochemistry 379* 2008;**379**:105–15.

34 Wenclawiak BW, Jensen TE, Richert JFO. GC/MS-FID analysis of BSTFA derivatized polar components of diesel particulate matter (NBS SRM-1650) extract. *Fresenius J Anal Chem* 1993;**346**:808–12.

35 Singh G, Gutierrez A, Xu K *et al.* Liquid chromatography/electron capture atmospheric pressure chemical ionization/mass spectrometry: analysis of pentafluorobenzyl derivatives of biomolecules and drugs in the attomole range. *Anal Chem* 2000;**72**:3007–13.

36 Wheelan P, Zirrolli JA, Murphy RC. Analysis of hydroxy fatty acids as pentafluorobenzyl ester, trimethylsilyl ether derivatives by electron ionization gas chromatography/mass spectrometry. *J Am Soc Mass Spectrom* 1995;**6**:40–51.

37 Knott I. et al. Routine Prostaglandin Assay by GC-MS in Multiwell Tissue Culture Plates: Application to Human Synoviocytes and Chondrocytes. *Analythical Biochemistry* 1993;**210**:360–65.

38 Leis HJ, Hohenester E, Gleispach H *et al.* Measurement of prostaglandins, thromboxanes and hydroxy fatty acids by stable isotope dilution gas chromatography/mass spectrometry. *Biomed Environ Mass Spectrom* 1987;**14**:617–21.

39 Wang M, Guo S, He M *et al.* High-Performance Liquid Chromatography-Mass Spectrometry-Based Lipid Metabolite Profiling of Acromegaly. *J Clin Endocrinol Metab* 2020;**105**.

40 Fan Y, Li Y, Chen Y *et al.* Comprehensive Metabolomic Characterization of Coronary Artery Diseases. *J Am Coll Cardiol* 2016;**68**:1281–93.

41 Mazaleuskaya LL, Salamatipour A, Sarantopoulou D *et al.* Analysis of HETEs in human whole blood by chiral UHPLC-ECAPCI/HRMS. *J Lipid Res* 2018;**59**:564–75.

42 Zhou W, Yang S, Wang PG. Matrix effects and application of matrix effect factor. *Bioanalysis* 2017;**9**:1839–44.

43 Psychogios Nikolas et al. The Human Serum Metabolome. *Plos One* 2011:p 12.

44 Meckelmann SW, Hellhake S, Steuck M *et al.* Comparison of derivatization/ionization techniques for liquid chromatography tandem mass spectrometry analysis of oxylipins. *Prostaglandins Other Lipid Mediat* 2017;**130**:8–15.

9. List of Abbreviations

Abbreviation	Meaning
ACN	Acetonitrile
BHT	Butylhydroxytoluol
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
EI	Electron Ionization
ESI	Electron Spray Ionization
ETE	Eicosatetraenoic acid
GC	Gas Chromatography
HETE	Hydroxyeicosatetraenoic acid
HHDA	Hydroxyhexadecanoic acid
HPLC	High Performance Liquid Chromatography
ITSD	Internal Standard
MEF	Matrix Effect
MeOH	Methanol
MRM	Multiple reaction Monitoring
MS	Mass Spectrometry
PFB	Pentafluorobenzyl bromide
QqQ	Tripple Quadrupole
QToF	Quadrupole Time of Flight
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
TMS	Trimethylsilyl