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LC-MS/MS quantitative analysis of Polyunsaturated Omega 3, 6,7 and 9 Fatty Acids in Serum for Research Use

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ABSTRACT

Purpose: An LC-MS/MS analytical method was developed and verified for the quantitation of Polyunsaturated Omega 3, 6, 7 and 9 fatty acids in serum for research use. Simple sample preparation techniques including protein crash and liquid-liquid extraction were evaluated. A Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer in negative Electrospray mode with a Thermo Scientific™ Dionex™ Vanquish™ Horizon HPLC system was used.

Methods: 200 µL of serum were used for the analysis of the Omega fatty acids. Various columns were evaluated and a Thermo Scientific™ Accucore™ Vanquish™ C18, 100 x 2.1 mm, 1.5 µm with 5mM Ammonium Acetate in water and acetonitrile mobile phases achieved baseline chromatographic separation in approximately 10 minutes run time. Quantitative analysis was performed using scheduled reactive monitoring (SRM) transition pairs for each steroid and internal standard in positive and negative mode and accuracy of the analytical method was verified using pooled reference samples.

Results: Good linearity and reproducibility were obtained across the dynamic range of the fatty acids with a coefficient of determination R^2>0.95 or better for all compounds in the various matrices. The limits of detection and quantitation were determined to the ng/ml levels with very good reproducibility observed for all compounds.

INTRODUCTION

Polyunsaturated Fatty acids are a class of diverse chemical compounds that are carboxylic acids with a long aliphatic chain that are unsaturated that are found in ones diet and have a good and bad physiological impact. There are many different kinds which are active depending on their saturated state and the length of their aliphatic chain and can have a significant influence on the body.

In this case, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of Polyunsaturated Omega 3, 6, 7 and 9 fatty acids in serum for research. The fatty acids analyzed include Omega-3- Alpha-Linolenic Acid, Stearidonic Acid, Eicosapentaenoic Acid, Heneicosapentaenoic Acid, Docosapentaenoic Acid, Docosahexaenoic Acid; the Omega-6- Linoleic Acid, Gamma-Linolenic Acid, Eicosadienoic Acid, Dihomo-Gamma-Linolenic Acid, Arachidonic Acid, Docosadienoic Acid, Adrenic Acid, Docosapentaenoic Acid; the Omega-7- Palmitoleic Acid, Vaccenic Acid and the Omega-9- Oleic Acid, Elaidic Acid, Gondoic Acid, Mead Acid, Erucic Acid, Nervonic Acid. The sample preparation choices were kept simple and included protein crash and a two step liquid-liquid extraction. The methodologies were developed on a TSQ Quantiva triple quadrupole mass spectrometer in positive and negative Electrospray ionization modes with a Vanquish Horizon HPLC system with a 10 minute analytical gradient.

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MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from Cayman Chemical Company, Ann Arbor, MI

Omega-3			
Alpha-Linolenic Acid (ALA):	50 mg	Alpha-linolenic Acid-D14:	100 μg
Stearidonic Acid (SDA):	1 mg		
Eicosapentaenoic Acid (EPA):	50 mg	Eicosapentaenoic Acid-D5:	50 μg
Heneicosapentaenoic Acid (HPA):	1 mg	Heneicosapentaenoic Acid-D6:	25 μg
Docosapentaenoic Acid (DPA):	1 mg		
Docosahexaenoic Acid (DHA):	50 mg	Docosahexaenoic Acid-D5:	50 μg
Omega-6			
Linoleic Acid (LA):	50 mg	Linoleic Acid:	500 μg
Gamma-Linolenic Acid (GLA):	50 mg		
Eicosadienoic Acid: (EDA)	50 mg		
Dihomo-Gamma-Linolenic Acid (DGLA):	10 mg	Dihomo-Gamma-Linolenic Acid-D6:	100 μg
Arachidonic Acid (AA):	50 mg	Arachidonic Acid-D8:	1 μg
Docosadienoic Acid (DDA):	1 mg		
Adrenic Acid:	10 mg		
Docosapentaenoic Acid (DPA-6):	1 mg		
Omega-7			
Palmitoleic Acid (PMA):	100 mg	Palmitoleic Acid-D14:	100 μg
Vaccenic Acid:	50 mg		
Omega-9			
Oleic Acid:	500 mg	Oleic Acid-D17:	1 μg
Elaidic Acid:	50 mg		
Gondoic Acid:	100 mg		
Mead Acid:	1 mg	Mead Acid-D6:	100 μg
Erucic Acid:	50 mg		
Nervonic Acid:	100 mg		

Reagents

The following Fisher Scientific™ acids, reagents and solvents were used

HPLC grade Water	Hexane
Methanol	Acetonitrile
Ammonium Acetate	Sodium Hydroxide
Hydrochloric Acid	

Sample Preparation- Protein Crash

- 200 μL of Serum/BSA mixture calibrators, controls and serum sample were added to 1.5 ml eppendorf tubes and 20 μL of fatty Acid ISTD at 1000 ng/mL were added to each tube and vortexed briefly
- 400 μL of Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and 200 μL of water was added to each vial and capped.
- All in-house calibrators were prepared in drug-free serum and bovine serum albumin mixture (Golden West Biological, Inc., Temecula, CA)

Sample Preparation- Liquid-Liquid Extraction

- 200 μL of Serum/BSA mixture calibrators, controls and serum samples were added to a test tube and 20 μL of fatty acid at 1000 ng/mL were added to each and vortexed briefly
- 400 μL of Acetonitrile:6N Hydrochloric Acid (90:10, v:v) was added to each tube and vortexed briefly and then heated at 100ºC for 45 min
- 400 μL of Methanol:10N Sodium Hydroxide (90:10, v:v) was added to each tube and vortexed briefly and then heated at 100ºC for 45 min
- The sample was then re-acidified with 200 μL of 6N Hydrochloric Acid
- 4 mL of Hexane were added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature
- The extract was reconstituted in 200 μL of water and methanol (100 μL of each)
- The supernatant was transferred to an MS vial and capped.

The calibration curves ranged from 0.1 ng/mL to 5000 ng/mL and various pooled samples were used as control material.

Data Analysis

The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Endura Tune™ 2.1 SW, and Thermo Scientific™ Tracefinder™ 4.1 SW

Method

HPLC Conditions-

Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore Vanquish C18, 100 x 2.1 mm, 1.5 μm
Column Temperature:	50 °C
Injection Volume:	20 μL
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	5mM Ammonium Acetate in Water
Mobile Phase B:	Acetonitrile
Flow Rate:	0.6 ml/min
Gradient:	0 min- 40%A:60%B
	6.5 min- 40%A:60%B
	6.6 min- 2%A:98%B
	9.0 min- 2%A:98%B
	9.1 min- 40%A:60%B
Run time:	10.0 mins

MS and Ion Source Conditions-

Quantiva triple quadrupole mass spectrometer

Ion mode:	Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	350 °C
Ion Transfer Tube Temperature:	300 °C
Sheath Gas:	30
Aux Gas:	15
Sweep Gas:	0
Spray Voltage:	Negative Ion (V):3000V
Q1/Q2 Resolution:	0.7 (FWHM)
Cycle time (sec):	0.5
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

Table 1. Scan Parameters- SRM Table

Compound	RT (Min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	RF Lens (V)
SDA-18:4 (n-3)	1.61	Negative	2.75.3	231.2/177.1	10/14	88
EPA-20:5 (n-3)	2.21	Negative	301.2	257.2/203.2	14/10	99
ALA-18:3 (n-3)	2.33	Negative	277.3	275.2/259.2/233.2	18/17/15	94
GLA-18:3 (n-6)	2.47	Negative	277.3	275.2/259.2/233.2	17/14/14	94
PMA-16: (n-7)	2.81	Negative	253.3	252.8	10	99
DHA-22:6 (n-3)	2.92	Negative	327.3	283.2/229.2	10/14	92
HPA-21:5 (n-3)	3.10	Negative	315.3	271.2/217.2	14/14	95
AA-20:4 (n-6)	3.46	Negative	303.3	259.2/205.2	13/15	93
LA-18:2 (n-6)	3.71	Negative	279.3	278.8	10	96
DPA-22:5 (n-3)	4.35	Negative	329.3	285.2/231.2	14/15	98
DPA-6-22:5 (n-6)	4.65	Negative	329.3	285.2/231.2	13/15	93
DGLA-20:3 (n-6)	5.11	Negative	305.3	287.2/261.2	19/16	97
Mead-20:3 (n-9)	5.89	Negative	305.3	287.2/261.2	18/15	99
Oleic-18:1 (n-9)	6.34	Negative	281.3	280.8	10	94
Vaccenic-18:1 (n-7)	6.57	Negative	281.3	280.8	10	95
Elaidic-18:1 (n-9)	7.20	Negative	281.3	280.8	10	98
Adrenic- 224:4 (n-6)	7.13	Negative	331.2	287.3/233.2	15/10	100
EDA-20:2 (n-6)	7.31	Negative	307.3	306.8	10	99
Gondoic-20:1 (n-9)	7.65	Negative	309.3	308.8	10	95
DDA-22:2 (n-6)	7.73	Negative	335.3	334.8	10	100
Erucic-22:1 (n-9)	8.17	Negative	337.3	336.8	10	107
Nervonic	8.78	Negative	365.3	364.8	10	107

RESULTS

Table 2. Sensitivity.

Compound	Protein Crash LOD/LOQ (ng/ml)	LLE-Underiatized LOD/LOQ (ng/ml)
SDA-18:4 (n-3)	1/ 2.5	0.25/0.5
EPA-20:5 (n-3)	10/25-Inter	0.25/0.5
ALA-18:3 (n-3)	25/50-Inter	1/ 2.5
GLA-18:3 (n-6)	10/25--Inter	2.5/5
PMA-16: (n-7)	10/25-Inter	1/ 2.5
DHA-22:6 (n-3)	5/10-Inter	0.1/0.25
HPA-21:5 (n-3)	1/ 2.5	0.1/0.25
AA-20:4 (n-6)	25/50-Inter	0.5/1
LA-18:2 (n-6)	Interference	5/10
DPA-22:5 (n-3)	25/50-Inter	1/ 2.5
DPA-6-22:5 (n-6)	1/ 2.5	0.25/0.5
DGLA-20:3 (n-6)	25/50-Inter	5/10
Mead-20:3 (n-9)	5/10	1/ 2.5
Oleic-18:1 (n-9)	10/25-Inter	5/10
Vaccenic-18:1 (n-7)	Interference	10/25
Elaidic-18:1 (n-9)	Interference	Interference
Adrenic- 224:4 (n-6)	2500/5000	2.5/5
EDA-20:2 (n-6)	2.5/5-Inter	2.5/5-Inter
Gondoic-20:1 (n-9)	5/10-Inter	1/ 2.5-Inter
DDA-22:2 (n-6)	2.5/5-Inter	1/ 2.5-Inter
Erucic-22:1 (n-9)	Interferences	25/50-Inter
Nervonic	10/25-Inter	2.5/5

Linearity/Sensitivity

The linear range of the fatty acids in serum/BSA matrix was from 1 ng/ml to 5000 ng/ml with the LLE extraction only. The linearity was determined in triplicate over 3 days and the results are shown with LOD and LOQ being determined as 3:1 and 10:1 of signal to noise respectively where possible and the mean coefficient of determination (R2) > 0.98 for each matrix and the %CV for each calibration point were all <15% only for the LLE extraction. Too many interferences in the PPX extraction prevented the determination of the linear range as expected since there are a lot of lipid compounds present.

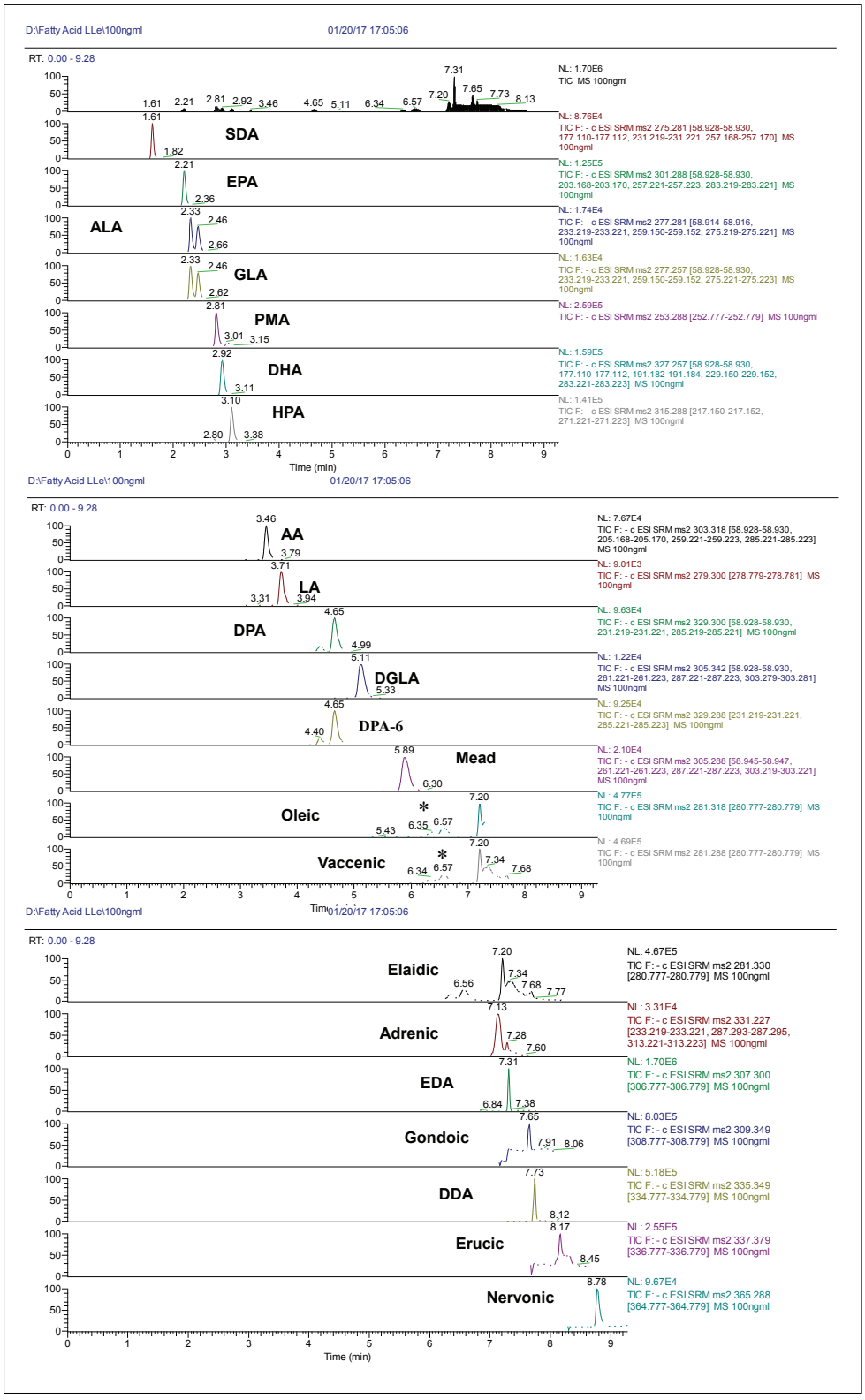
Accuracy

The accuracy was determined by the analysis of pooled sample control material as the percentage deviation from the targeted mean and the results were <15% for all levels in each matrix. The serum pooled control material concentrations were 25 ng/nl and 250 ng/ml. Therefore, the analytical method with the LLE extraction can achieve research laboratory required accuracy for the analysis of the fatty acids in serum.

Precision/Specificity

The intra–assay precision (%CV) of the fatty acids in serum were determined by extracting and quantifying three replicates of the pooled sample control material for the LLE extraction only. The inter-assay precision was determined over 3 consecutive days and was found to have a %CV <15% for each fatty acid within their respective linear range for the three levels of pooled serum sample control material respectively. Therefore, the analytical method can achieve the required precision for the analysis of the omega fatty acids in serum. Due to the similarity between the various fatty acids tested and other similar compounds, there were interferences present as well as ion suppression which made obtaining consistent results and may require a larger diameter LC column.

Figure 1: Chromatograms



CONCLUSIONS

- Baseline separation of the omega fatty acids with good LOD/LOQ was achieved in serum for most of the compounds but interferences were present.
- Simple LLE sample preparation achieved desirable LOD/LOQ to the relevant levels with further work to be carried out to fine tune these techniques to obtain more sensitive results and to remove the interferences and achieve better separation and removal of interferences while maintaining ease of use and low cost
- Good linearity of calibration curves with acceptable accuracy, precision and reproducibility in negative mode was achieved <15% for %CV for the majority of the omega fatty acids within their linear range and the sample preparation techniques and analytical methodologies will be further extended, verified and optimized to obtain results than can be achieved by GC/MS.

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