ANALYSIS OF FREE OMEGA-3 AND OMEGA-6 FATTY ACIDS IN BEEF BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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Abstract – The traditional method of measuring fatty acid content in food is the analysis of fatty acid methyl esters using gas chromatography (GC). The high temperatures used in the GC method can affect the stability of the polyunsaturated fatty acids and the extraction technique requires the alteration of the sample. A new analytical methodology has been developed for the sensitive determination of free fatty acids (FFA) in beef using liquid chromatography/mass spectrometry (LC/MS). The LC/MS method enabled separation and identification of key omega-3 and omega-6 fatty acids without the need for derivatization into methyl esters. The limits of detection (S/N =3) and the limits of quantitation (S/N = 10) for the analysis were in the range of 0.21 - 1.10 mg/L and 0.67 - 3.70 mg/L, respectively. The method reported in this paper could potentially be used as a sensitive method for monitoring the levels of free fatty acids in beef.

Key Words – Liquid chromatography-mass spectrometry, Lipids, Omega 3, Omega 6, Steak

I. INTRODUCTION

Finishing diets of cattle can alter the fatty acid (FA) profile of beef in such a way as to be very desirable to the health conscious consumer [1]. For example, increasing the omega-3 fatty acid content of animal feed by utilizing more grass and forages in beef production or by feeding flax has been shown to increase Omega-3 levels and the subsequent nutritional value of the final meat product [2, 3]. Quantitative chemical techniques for the comprehensive determination of FA involves solvent extraction of the total lipids, hydrolysis, followed by derivitization of FA to their methyl esters for measurement by gas chromatography (GC). This technique is the current standard and works well as an analytical tool for these determinations but requires alteration of the sample. In addition GC analysis can also adversely affect temperature sensitive functional groups on specialized lipids. Novel techniques could be developed in order to improve the sensitivity of FFA analysis, as well as remove additional analytical steps which could lead to increased sample loss. Liquid chromatography-mass spectrometry (LC/MS) was chosen to separate and identify FFA in beef as LC/MS has high specificity and sensitivity [5] while skipping the need for derivitization which is often required in other techniques.

II. MATERIALS AND METHODS

Reference Standard Preparation: Reference standards were prepared in order to optimize the LC/MS procedure including perfecting the gradient conditions of the mobile phase. The standards were also used for identification purposes and for generating calibration curves in which to compare the extraction samples. The omega-3 and omega-6 standards (GLC 642 and GLC 643 purchased from Nu-Chek Prep) were made up to 5000 mg/L. This was done for the two standards separately by adding the entire contents of the factory standard to a 5-mL volumetric flask and topping it up with chloroform. This standard was then used to make 5 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L Omega-3, Omega-6, with a 25 mg/L internal standard. The internal standard used was heneicosanoic acid (C21:O). The varying parts per million stock standards were made up with 70:30 v/v methanol-chloroform with 3,5-di-tert-butyl-4-hydroxytoluene (BHT). BHT was used to help prevent oxidation.

Beef Preparation and Lipid Extraction: Beef steaks from the longissimus dorsi from various farms from the interior of British Columbia were kept frozen at - 80 ^{o}C before being utilized. A portion of a steak (approximately 2 cm^3) was ground up using a pestle and mortar with liquid nitrogen in its base. This process allowed for maximum mechanical breakdown of the meat. The lipid extraction method used was based on a modified

Bligh and Dyer method as per Lacaze et al. First, 0.20 ± 0.01g of the powder-like beef was weighed into a microcentrifuge tube. Methanol (400 µL) and chloroform (200 µL) were added into the microcentrifuge tube, along with 100 µL of the internal standard, and shaken using a Genie 2T mixer for 10 min. Next, chloroform (200 µL) was added and then mixed for one minute. Deionized water (200 µL) was then added and also mixed for one minute. The mixture was centrifuged for 5 min. The aqueous and organic layers were removed and put in a clean microcentrifuge tube, leaving the pellet for further extraction. Chloroform (400 µL) was put in with the pellet, mixed for 5 min, and then centrifuged for 5 min. The subsequent extract was added to the previously extracted aqueous and organic layers. Lastly, the microcentrifuge tube holding the aqueous and organic layers was centrifuged for 5 min. The bottom layer (chloroform layer) was transferred to a clean microcentrifuge tube. The extractions were "cleaned up" using a further solid phase extraction (SPE) method. This method was performed as described in Lacaze et al. SPE was done to separate the free fatty acids from the bound fatty acids. The samples were filtered under vacuum through bonded phase aminopropyl-silica cartridges (PrepSep-NH_2) using chloroform (3 mL) to condition them. The extracts were captured using 2-mL volumetric flasks. A 2:1 v/v chlorofrom-2-propanol (1 mL) wash was used before eluting the free fatty acids using 98:2 v/v diethyl ether-acetic acid (3 mL). The contents of the volumetric flask were evaporated to dryness via nitrogen blow-down (10 min). A 70:30 v/v methanol-chloroform mixture was added to the volumetric flask, mixed, and then transferred to a 2-mL vial for analysis.

LC/MS Procedure: The LC/MS analysis was done using an Agilent 1200 series LC coupled with an Agilent G6530 Quadrupole-Time of Flight (Q-TOF) MS. A Zorbax Eclipse Plus C_{18} column was used to separate the fatty acids. This column was 2.1 x 100 mm with a 1.8 um particle size. The mobile phases used to separate the free fatty acids consisted of: A - 5% acetonitrile, 95% water, with a 1% formic acid/ 1 mmol of ammonium formate buffer and B - 95% acetonitrile, 5% water, with a 1% formic acid/ 1 mmol of ammonium formate buffer. Separation took 35 min using gradient conditions of 60% B at 0-15 min, 60-80% B at 15-35 min, and then a 10 min post-time re-equilibration period. The flow rate was 0.7 mL/min, the injection volume was 5 µL and the column temperature remained consistent at approximately 50 °C. The MS operated in negative ion mode.

III. RESULTS AND DISCUSSION

Important omega-3's α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were identified and successfully separated in both the standards and meat samples using the method described. All omega 3's analyzed during this trial are listed in Table 1. Importantly, there was separation of omega 3 α-Linolenic and the omega 6 γ-Linolenic acid, allowing individual identification of the omega-3 18:3 chain.

Table 1. Omega 3's analysed using LCMS

Omega 3	Molecular Formula	Molecular Weight
18:3 (α-Linolenic Acid)	$C_{18}H_{30}O_2$	277.21304
20:3 (Eicosatrienoic Acid)	$C_{20}H_{34}O_2$	305.248604
22:3 (Docosatrienoic Acid)	$C_{22}H_{38}O_2$	333.279904
20:5 (Eicosapentaenoic Acid)	$C_{20}H_{30}O_2$	301.217304
22:5 (Docosapentaenoic Acid)	$C_{22}H_{34}O_2$	329.248604
22:6 (Docosahexaenoic Acid)	$C_{22}H_{32}O_2$	327.232954

Also, important omega-6's Linoleic Acid (LA) Arachidonic acid (AA) in omega-6 metabolism and elongation were separated and identified. All omega 6's analyzed during this trial are listed in table 2. This technique did have problems with the separation of two of the omega 3 and 6 fatty acids; 20:3 n-3 and n-6 were not separated, as well as 22:5 n-3 and n-6. This is important as the 22:5 n-6 is docospaentaenoic acid and is considered an important omega 6.

Table 2. Omega 6's analysed using LCMS

Omega 6	Molecular Formula	Molecular Weight
18:2 (Linoleic Acid)	$C_{18}H_{32}O_2$	279.232954
18:3 (γ- Linolenic Acid)	$C_{18}H_{30}O_2$	277.217304
19:2 (Nonadecadienoic Acid)	$C_{19}H_{34}O_2$	293.248604
20:2 (Eicosadienoic acid)	$C_{20}H_{36}O_2$	307.269254
20:3 (Dihomo-gamma-linolenic acid))	$C_{20}H_{34}O_2$	305.248604

20:4 (Arachidonic acid)	$C_{20}H_{32}O_2$	303.232954
22:2 (Docosadienoic acid)	$C_{22}H_{40}O_2$	335.29554
22:4 (Adrenic acid)	$C_{22}H_{36}O_2$	331.264254
22:5 (Docosapentaenoic acid)	$C_{22}H_{34}O_2$	329.248604

The chromatograms highlighting the separation of both the omega 3 and 6 fatty acid standards as well as the extract from a representative grass fed beef sample produced by the LC/MS are shown in both Figures 1 and 2 respectively. The retention times and the mass to charge (m/z) ratios of the various omega fatty acids are listed in Table 3. The relative standard deviation (% RSD) of the retention times for method repeatability (n = 3) were found to be lower than 2%. The low % RSD values indicate that this proposed LC/MS method is highly reproducible.

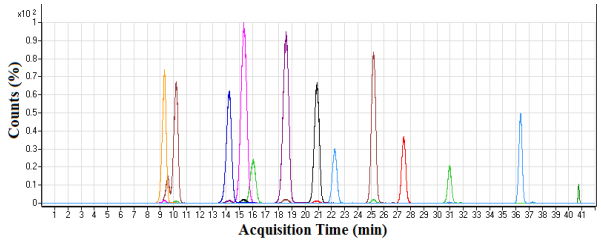

Figure 1. Chromatogram of Fatty Acid Standards

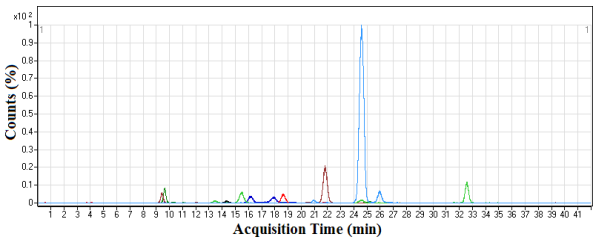

Figure 2. Chromatogram of Extract from Grass Fed Beef

Table 3. LC/MS Retention times of Omega FAs

Compound Name	Retention Time	M/Z Ratio
C18:2 (O6)	15.6	279.2
C18:3 (O3)	9.3	277.2
C18:3 (O6)	9.9	277.2
C19:2 (O6)	21.9	293.3
C20:2 (O6)	27.6	307.3
C20:3 (O3/O6)	20.5	305.3
C20:4 (O6)	15.0	303.2
C20:5 (O3)	9.1	301.2
C22:2 (O6)	36.0	335.3
C22:3 (O3)	30.6	333.3
C22:4 (O6)	24.9	331.3
C22:5 (O3/O6)	18.2	329.3
C22:6 (O3)	14.0	327.2

IV. CONCLUSION

This LC/MS method enables separation and identification of important free omega-3 and omega-6 fatty acids. The resulting information from the analysis can then be applied to quantification software to generate amounts of the free fatty acids and ratios. Sample preparation using lipid extraction and SPE is quite fast. The proposed method is sensitive and doesn't involve derivatization. The LC/MS method described in this paper can be used as a sensitive technique for monitoring variations in levels of free omega-3 and omega-6 fatty acids in beef samples and other tissue samples where you want to track and or quantify intracellular levels of free fatty acids. In the future a saponification step could potentially be added in place of SPE in order to release the bound omega 3 and 6 fatty acids in beef.

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